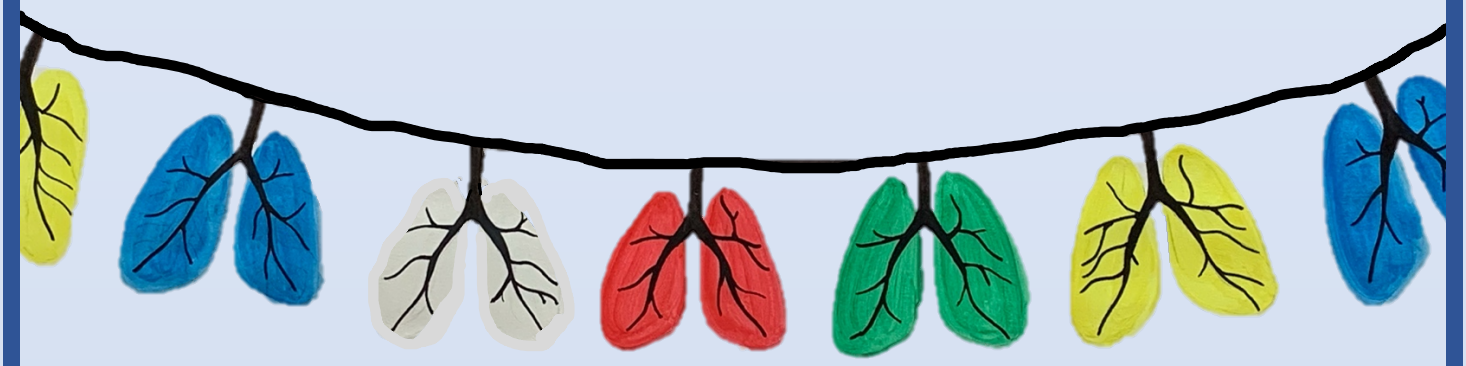
Characterization of pneumonia among children under five years of age hospitalized in Thimphu, Bhutan

Sophie Jullien



Characterization of pneumonia among children under five years of age hospitalized in Thimphu, Bhutan

Doctoral thesis

Sophie Jullien

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Caracterización de la neumonía en niños menores de cinco años hospitalizados en Thimphu, Bután

Doctoral thesis report presented by **Sophie Jullien**

to apply for the doctoral degree from the University of Barcelona

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El Dr Quique Bassat, investigador del Instituto de Salud Global de Barcelona y del Centro de Investigação em Saúde de Manhiça,

hace constar

que la tesis titulada

Characterization of pneumonia among children under five years of age hospitalized in Thimphu, Bhutan

presentada por Sophie Jullien ha sido realizada bajo su dirección, y cumple todos los requisitos que dicta la normativa vigente para la presentación de tesis doctorales como un compendio de artículos en la Facultad de Medicina de la Universitat de Barcelona,

y considera,

que la memoria resultante es apta para optar al grado de Doctor en Medicina con mención Internacional por la Universidad de Barcelona.

Y para que quede constancia, firma el presente documento

Quique Bassat

Barcelona, 9 de diciembre de 2020

C'est le temps que tu as passé pour ta rose

qui fait ta rose si importante

Le Petit Prince (Antoine de Saint-Exupéry)

Acknowledgments

The accomplishment of this doctoral thesis has been a long journey. I am not sure when it began but does it matter? Just like happiness, the start or the destination does not matter, but the journey itself. Here, I do not intend to detail how wonderful and how not so wonderful this journey has been, but I would like to show my sincere gratitude to all the people who made this thesis possible. The order of acknowledgments below is not relevant and was not created by a classification and regression tree analysis or any other statistical model.

First, I would like to give a brief introduction of the RIBhuC project. Working with the Cochrane Infectious Diseases Group taught me the importance of being rigorous, systematic, and critical in research. After critically appraising articles included in the systematic reviews I was working on, I was keen on conducting field research myself. I needed to better understand the challenges involved in the process, and what better way is there than doing it oneself? As I was on my way back to clinical work and about to reach Bhutan, if you add Quique to the ingredient list, then everything was ready to start this new journey! I must admit that starting a research project in a setting where research is pretty uncommon, is, in one word, challenging. On the other hand, it has been such an amazing and enriching learning experience, as this allowed me to deal directly and to learn from each and every step required in conducting this modest study.

Kadinchey la to all the children and families who consented to participate to the RIBhuC study. I wish the findings of this thesis could help Bhutanese children to celebrate their fifth birthday.

Name same kadinchey la to all the staff at JDWNRH who have been involved in the study and without whom this study would not have been possible: in particular to the sisters and brothers (nurses) for sample collection, to all my colleagues in the paediatrics department for their contribution to the study, to the ward staff, and to the radiology and microbiology departments for their support. *Kadinchey* to the residents for their willingness in learning and contributing to this research, particularly to Dinesh and Tashi. I am grateful to Ragunath Sharma, who coordinated all the steps that involved the laboratory at JDWNRH. I would also like to show my gratitude to the two lovely ladies at the medical records section. I wish I could remember their names. Despite their

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A huge evidence-based thank you to Dee for proof-editing the whole document. I would like to take this opportunity to show my gratitude to Paul Garner. He made a meaningful impact on my professional path and continue to be a great mentor and reference for inspiration and excellence. I genuinely wish him a full recovery.

This is an acknowledgment for a doctoral thesis and not a list of loved ones throughout my life. However, the emotional and personal support throughout this journey has been at least as important as the professional support in order to complete this thesis.

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Table of contents

List of abbreviations

AIDS	Acquired immunodeficiency syndrome
Angpt-2	Angiopoietin-2
ARI	Acute respiratory infections
ALRI	Acute lower respiratory infections
AUROC	Area under the receiver operating characteristics
CI	Confidence interval
CRP	C-reactive protein
EDTA	Ethylenediaminetetraacetic acid
EPI	Expanded Programme on Immunization
ESR	Erythrocyte sedimentation rate
GAPPD	Global Action Plan for Pneumonia and Diarrhoea
HIV	Human immunodeficiency virus
IL-6	Interleukin-6
IL-8	Interleukin-8
IMCI	Integrated Management of Childhood Illness
JDWNRH	Jigme Dorji Wangchuck National Referral Hospital
LMIC	Low- and middle-income country
LODS	Lambaréné Organ Dysfunction Score
LRTI	Lower respiratory tract infections
MDGs	Millennium Development Goals
NPW	Nasopharyngeal washing
PCR	Polymerase chain reaction
PCT	Procalcitonin
PCV	Pneumococcal conjugate vaccine
PICU	Paediatric intensive care unit
PNC	Pneumococcal nasopharyngeal carriage
RIBhuC	Respiratory Infections in Bhutanese Children
RISC	Respiratory Index of Severity in Children
RSV	Respiratory syncytial virus

RT-PCR	Real-time polymerase chain reaction
SARI	Severe acute respiratory infections
SDGs	Sustainable Development Goals
sFlt1	Soluble fms-like tyrosine kinase 1
sTNFR1	Soluble tumour necrosis factor receptor 1
sTREM-1	Soluble triggering receptor expressed on myeloid cells-1
UNICEF	United Nations International Children's Emergency Fund
WBC	White blood cells
WHO	World Health Organization

Articles included in the thesis

This thesis is presented as a compendium of articles. The thesis consists of 11 objectives and six articles:

Article 1, related to objective 1

Jullien S. The challenges of combining clinical work with research in Bhutan: a changing status quo. J Trop Pediatr. 2019;65(3):207-209.

2019 impact factor 0.940, Infectious Diseases Q3

Article 2, related to objective 2

Jullien S, Pradhan D, Bassat Q. Pneumonia in Bhutanese children: what we know, and what we need to know. Pneumonia (Nathan). 2020;12:1.

2019 impact factor pending

Article 3, related to objectives 3 to 5

Jullien S, Pradhan D, Tshering T, Sharma R, Dema K, Garcia-Garcia S, et al. Pneumonia in children admitted to the national referral hospital in Bhutan: a prospective cohort study. Int J Infect Dis. 2020;95:74-83.

2019 impact factor 3.202, Infectious Diseases Q1

Article 4, related to objectives 6 and 7

Jullien S, Sharma R, Lhamu Mynak M, Henares D, Muñoz-Almagro C, Bassat Q. Pneumococcal nasopharyngeal carriage among Bhutanese children hospitalized with clinical pneumonia: serotypes and viral co-infection. BMC Infectious Diseases. 2020;20(1):940.

2019 impact factor 2.688, Infectious Diseases Q1

Article 5, related to objectives 8 and 9

Jullien S, Richard-Greenblatt M, Casellas A, Tshering K, Ribó JL, Sharma R, et al. Association of clinical signs, host biomarkers, and aetiology with radiological findings in Bhutanese children hospitalized with pneumonia.

Submitted to PLoS ONE.

2019 impact factor 2.740, Multidisciplinary Q1

Article 6, related to objectives 10 and 11

Jullien S, Richard-Greenblatt M, Ngai M, Lhadon T, Sharma R, Dema K, et al. Performance of host-response biomarkers versus standard clinical and laboratory parameters to risk-stratify children with clinical pneumonia in Bhutan

Submitted to Scientific Reports.

2019 impact factor 3.998, Multidisciplinary Q1

Summary

Background and rationale

Pneumonia is the leading infectious cause of death in children under five years of age. One child dies of pneumonia every 39 seconds. In 2018, pneumonia was responsible for 800,000 child deaths, which is more than the deaths due to malaria, tuberculosis, human immunodeficiency virus, and measles combined. Although pneumonia affects children worldwide, the main burden remains disproportionately concentrated in low- and middle-income countries in Southeast Asia and sub-Saharan Africa. Global efforts have been made in the last decades, which have led to considerable progress in reducing morbidity and mortality associated with this disease. However, unless global efforts accelerate to specifically and more proactively tackle this disease, 3.2 million children will die unnecessarily from pneumonia between 2020 and 2030.

The challenges to prevent and address this disease are multiple. First, characterizing the aetiology of pneumonia remains challenging and this has not yet been properly addressed. Viruses are the main cause of pneumonia in children under five years of age, with the respiratory syncytial virus repeatedly identified as the most common pathogen in this age group. *Streptococcus pneumoniae* and *Haemophilus influenzae* type b are the principal bacterial causes of childhood pneumonia. Nowadays, the cases of pneumonia caused by these bacteria can be prevented with highly-effective conjugate vaccines, and their local incidence has dramatically decreased in settings where vaccines against them have been adequately implemented. The wide range of pathogens causing pneumonia implies that different treatments are required and that no single effective vaccine is sufficient to prevent pneumonia. Furthermore, the identification of respiratory viruses in the nasopharynx of children with clinical pneumonia requires careful interpretation. The distinction between nasopharyngeal carriage and causative agent is difficult, and respiratory virus detection does not imply causation nor exclude a bacterial infection. There is growing evidence that shows an overlap of viral and bacterial aetiology in respiratory infections, and the probable important interaction between them in the pathogenesis of pneumonia. Besides, pneumonia aetiology studies conducted in different settings have reported contradictory findings, which limit the generalizability of

findings to other countries and regions and contribute to the difficulties in understanding the key etiological pathogens causing childhood pneumonia.

Second, there is no standardised case definition of pneumonia and there is no simple clinical criteria or diagnostic tool that allows accurate detection and thus diagnosis of the disease. The overlap of symptoms and signs between pneumonia and many other common diseases in children, such as malaria, severe anaemia, sepsis, and congenital heart diseases, leads to difficulties in differentiating these diseases and translates the low specificity of clinical characteristics for the detection of pneumonia. Clinical diagnostic criteria for pneumonia, such as those proposed by the World Health Organization (WHO), are highly sensitive to identify all children that would benefit from antibiotic treatment, but poorly specific. Indeed, an additional challenge is to distinguish between viral or bacterial aetiology. Clinical criteria are unable to discern between children with bacterial pneumonia who require antibiotics from those who present with self-limited pneumonia. This leads to overtreatment of clinical pneumonia cases with antibiotics, with potential implications for the emergence of antimicrobial resistance. Radiological criteria were developed to increase specificity in identifying bacterial pneumonia and are still used as a reference standard, both in clinical practice and for research purposes. However, the chest radiograph is an imperfect tool to discriminate between bacterial and viral pneumonia for guiding antibiotic treatment. The difficulty in identifying the causative pathogen, together with the complexity around causality and the role of co-infections, support the need for a novel and different approach to reducing the high mortality associated with pneumonia.

Early identification of children with severe pneumonia encourages a prompt and more aggressive treatment, leading to better prognosis and reduced mortality. Therefore, early recognition of children at risk of progression to severe disease is needed from their first contact with the healthcare system to help decision-making for early referral, prioritization of care, and the need of hospitalization and intensive care. Several prognostic scores have been developed with this purpose, based on clinical characteristics and simple laboratory testing, but they lack sensitivity and specificity and have not been adequately validated for childhood pneumonia.

Host-response biomarkers have been increasingly investigated in different clinical areas for pneumonia and other infectious diseases for diagnosis, aetiology, risk stratification and triage decisions, severity of illness, and guidance for the initiation and discontinuation of antibiotic therapy. Specific biomarkers of host response including inflammatory, immune, and endothelial activation pathways are of particular interest, as these mediators are involved in the pathogenesis of life-threatening infections, irrespective of their aetiology. In addition, biomarkers can be quantified in the blood and present the benefit of accurate and reproducible measures. A biomarker as a point-of-care tool fulfilling the WHO ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable to end-users) criteria to risk-stratify children with pneumonia in both community settings and at the hospital level could have a significant impact. C-reactive protein and procalcitonin are commonly used in the diagnosis of infectious diseases in high-income countries. The measurement of procalcitonin in children with pneumonia for guiding antibiotic treatment reduces antibiotic use in high-income countries without negative influence on clinical outcomes. However, these inflammatory biomarkers are rarely available as a point-of-care tool in resource-constrained settings. Also, there is insufficient evidence on their performance in the epidemiological context of low- and middle-income countries with different demographic factors and co-existing diseases. Nevertheless, there are positive findings from other biomarkers investigated in low- and middle-income countries. For example, in Uganda, elevated angiopoietin-2/angiopoietin-1 ratio at presentation in children with pneumonia was associated with disease severity and an increased risk of fatal outcome. Although further investigation is needed to validate their diagnostic and prognostic value, these emerging biomarkers seem promising to contribute to reducing the morbimortality associated with childhood pneumonia.

In low- and middle-income countries with limited healthcare resources, diagnosis and management of pneumonia in children under five years of age is particularly challenging. In addition, epidemiology and causative pathogens of childhood pneumonia differ widely across the world. The characterization of pneumonia and its associated challenges in a country like Bhutan can shed light on the most effective strategies for ensuring child survival. In Bhutan, where pneumonia was estimated to be responsible for 15% of under-

five deaths and 27% of post-neonatal deaths, improving care for this disease will have a major impact on child health.

The Kingdom of Bhutan is a small landlocked country in the Eastern Himalayas, with a population of around 800,000 people. In this predominantly mountainous country, elevation rises from around 100 m in the Indian border (South) to the high Himalayan peaks above 7500 m (North). The capital, Thimphu, stands at 2334 m. The climate varies with altitude, from tropical in the South to alpine with very cold winters in the North. Bhutan is classified as a lower-middle-income country. Essential health services in both modern and traditional medicines are free for Bhutanese citizens based on a primary healthcare approach, reaching the most remote areas despite the geographical difficulties.

Hypotheses

- There is a dearth of epidemiological, clinical, and aetiological data that hampers a comprehensive characterization of childhood pneumonia in Bhutan.
- Childhood pneumonia remains a major public health problem in Bhutan.
- Bacteria account for around 25% of the pneumonia cases in hospitalized children, with *Streptococcus pneumoniae* as the main bacterial pathogen. Viruses are frequently detected in the nasopharynx of children hospitalized with pneumonia; the most common are respiratory syncytial virus and rhinovirus.
- Most pneumococcal serotypes that circulate among children hospitalized with pneumonia are preventable by pneumococcal conjugate vaccines.
- Clinical characteristics are poor at predicting radiological outcomes.
- Host-response biomarkers are helpful for discerning bacterial from viral pneumonia and for identifying children at risk of poor prognosis.

Objectives

The general objective of this thesis was to describe the epidemiology, aetiology, clinical presentation, and radiological findings of pneumonia among Bhutanese children to better characterize childhood pneumonia in Bhutan and to contribute to the understanding of this disease in the local context. This thesis also aimed to assess the diagnostic and prognostic performance of host-response biomarkers alone, combined, or

in addition to clinical scoring scales to risk-stratify children hospitalized with pneumonia and predict their outcome.

Methods and summary of articles

The first article acknowledges the need for local research in Bhutan and comments on the specific challenges experienced when trying to conduct it.

The second article is a systematic review that summarizes current knowledge around childhood pneumonia in Bhutan and identifies knowledge gaps in this area. The findings of this review were used as the starting point to guide further research and to establish the objectives of the Respiratory Infections in Bhutanese Children (RIBhuC) study.

We reported the findings of the RIBhuC study in **articles 3 to 6** of this thesis. In brief, the RIBhuC study took place between 1 July 2017 and 30 June 2018. We prospectively enrolled all children between 2 and 59 months admitted to the Jigme Dorji Wangchuck National Referral Hospital (JDWNRH) in Thimphu with WHO-defined clinical pneumonia, provided parents or caregivers consented to study participation. On admission, we performed a comprehensive physical examination, including anthropometric and vital signs measurements. We recorded demographic and clinical data from medical files and through family interviews. We performed an antero-posterior chest radiograph within 24 hours of admission and classified children according to radiological findings following WHO radiological criteria. We collected blood samples upon enrolment or as soon as possible after enrolment for haematology, biochemistry, and bacterial culture, and two drops of blood on filter paper for the identification of *Streptococcus pneumoniae* by real-time polymerase chain reaction (RT-PCR). In addition, we measured plasma levels of eleven host-response biomarkers, including six markers of immune and endothelial activation: interleukin-6 (IL-6), interleukin-8 (IL-8), soluble triggering receptor expressed on myeloid cells-1 (sTREM-1), soluble tumour necrosis factor receptor 1 (sTNFR1), angiopoietin-2 (Angpt-2), and soluble fms-like tyrosine kinase 1 (sFlt1). Finally, we collected respiratory samples through nasopharyngeal washing for the molecular identification of seventeen respiratory viruses and four atypical bacteria and the detection and capsular typing of *Streptococcus pneumoniae*.

The third article describes the aetiological profile and the demographic and clinical characteristics of this cohort of children admitted with WHO-defined clinical pneumonia.

The fourth article reports data on the prevalence of pneumococcal nasopharyngeal carriers and on the pneumococcal serotypes circulating among Bhutanese children with clinical pneumonia before the introduction of the pneumococcal conjugate vaccine in the country. We identified and compared respiratory viruses among children with and without pneumococcal nasopharyngeal colonization to contribute to the understanding of the interplay between pneumococcal nasopharyngeal colonization and viral co-infections.

The fifth article describes the radiological findings of the RIBhuC cohort and the differences in radiological outcomes by demographic characteristics, aetiology, clinical features, and host-response biomarker levels. We also evaluated the utility of host-response biomarkers in discerning between bacterial and viral pneumonia, taking radiological endpoint pneumonia as a proxy for bacterial aetiology**.**

The sixth and last article of this thesis assessed the performance of a wide range of clinical characteristics, laboratory testing, clinical scoring scales, and host-response biomarkers to risk-stratify children with clinical pneumonia in Bhutan and predict their outcome.

Main results

The first article of this thesis illustrates how essential reliable local data are to inform evidence-based policies and practices. The main challenges identified that prevent the expansion of local research in Bhutan were, among others: the lack of awareness that research is needed; the lack of motivation, methodological and analytical skills, and critical appraisal; funding; and priority for clinical work, which represents a huge workload.

The systematic review (**second article**) included 44 records that we summarized qualitatively. We found that pneumonia is still accountable for a high burden and mortality rate in Bhutanese children. The national surveillance system in place focuses mainly on influenza virus identification, although it has recently introduced other viral aetiologies to monitor. We found very limited or no data regarding the epidemiological, bacterial aetiology, clinical, radiological, and prognostic characteristics of childhood pneumonia in Bhutan.

The RIBhuC study included 189 children with WHO-defined clinical pneumonia hospitalized at JDWNRH, which corresponds to 11.9% of all paediatric admissions during the study period. We reported the demographic, clinical, and microbiological characteristics of this cohort in **the third article**. Six children died, corresponding to a case fatality rate of 3.2%. These six children were referred from healthcare centres far from Thimphu and were in a critical condition when they reached the hospital. Children were hospitalized for a median of four days. Over half (53.4%) of the cohort were infants (<12 months of age). Most children (75.7%) were adequately immunized according to age; the remaining children were partially immunized as per the national schedule. One in ten children were wasted. Counterintuitively, the lowest number of pneumonia-related admissions was during the winter (21 December to 20 March), which is the coldest season in Bhutan. Most children (79.4%) presented with severe pneumonia, as per the WHO criteria. Upon admission half of the children were breathing fast according to age, three-quarters were hypoxemic (defined as SpO2 <90%), and 54.3% presented with lower chest retractions. Of the 149 readable chest radiographs, 53.0% were normal, 26.2% were classified as primary endpoint pneumonia, and 20.8% presented other infiltrates. Non-contaminated bacterial growth was detected in 8/148 (5,4%) blood cultures, with only two cases of *Streptococcus pneumoniae* isolated. *Bordetella pertussis* was detected by molecular methods in three children, of which a five-month old child who had a fatal outcome. Viral detection in upper respiratory secretions was common, with at least one virus detected in 103/115 (89.6%) children, of which 34.0% were mixed infections. Respiratory syncytial virus (52/115; 45.2%), rhinovirus (42/115; 36.5%), and human parainfluenza virus (19/115; 16.5%) were the three most-commonly isolated viruses.

The fourth article shows that 76/121 (62.8%) children were pneumococcal nasopharyngeal carriers. Thirty different serotypes (or groups of serotypes when it was not possible to differentiate them) were identified among these 76 children and half of the children had at least two and up to five different serotypes. We considered the serotypes 1, 3, 4, 5, 7F, 14, 18C, and 19A as highly invasive, according to findings from other studies and refer to the remaining serotypes as “less-highly invasive”. Most children presented less-highly invasive serotypes; the most common were 7B/C or 40 (43.4%), followed by 6A/B (15.8%), and 23F (7.9%). Around one-third of the children

presented with highly invasive serotypes; the most common were 14 (11.8%), 3 (6.6%), and 1 (6.6%). Over half of the children (57.9%) presented at least one serotype included in the 13-valent pneumococcal conjugate vaccine, and half of the children presented at least one serotype included in any of the two commercially available 10-valent pneumococcal conjugate vaccines. Concerning the association of pneumococcal nasopharyngeal carriers with viral co-infection, we detected respiratory viruses in a similar proportion among children with (62/70; 88.6%) and without pneumococcal colonization (36/40; 90.0%). Rhinovirus was less common among pneumococcal carriers (20/70; 28.6% versus 19/40; 47.5%; p=0.046) but we found no further significant differences regarding the detection of other viruses.

In **the fifth article**, we assessed the association of clinical signs, biomarkers, and aetiology with radiological findings among the 149 children with readable chest radiographs (26.2% with endpoint pneumonia). We found no significant differences in the identification of respiratory viruses by radiological outcomes. A higher proportion of children with endpoint pneumonia reported symptoms (64.1% versus 38.5%, p=0.007) or presented fever (42.1% versus 21.3%, p=0.045) for at least five days prior to admission and fulfilled clinical criteria for WHO severe pneumonia (92.3% versus 75.5%, p=0.033). No single clinical sign was more suggestive of presenting endpoint pneumonia or non-endpoint pneumonia. However, erythrocyte sedimentation rate and plasma levels of C-reactive protein and procalcitonin were higher among endpoint pneumonia and remained significantly higher after adjusting for observed confounders. Procalcitonin presented the best overall discriminatory ability to identify radiological pneumonia as a proxy for bacterial aetiology with 72% sensitivity and 66.2% specificity. None of the immune and endothelial activation markers demonstrated good discriminatory ability between radiological endpoint and non-endpoint pneumonia.

The sixth article assessed the performance of inflammatory, immune, and endothelial activation markers alone or in addition to clinical signs or scoring scales to risk-stratify the 118 children who had biomarker quantification. Overall, 23/118 (19.5%) children progressed to poor prognosis, defined as death (n=3) or oxygen therapy required for more than five days (n=20). Parental education, parental employment, and time to access healthcare facilities were not associated with prognosis. Of the Respiratory Index of Severity in Children (RISC) score, the RISC-Malawi score, the Lambaréné Organ

Dysfunction Score (LODS), and the WHO severity classification, the RISC score was the clinical scoring scale that performed best at predicting prognosis (area under the receiver operating characteristics [AUROC] 0.75), especially in infants <12 months of age (AUROC 0.80). Nevertheless, none of the single clinical signs were sufficiently robust as prognostic predictors to allow clinical decision-making for case management. Results of routinely-ordered laboratory testing, including white blood cells, platelets, erythrocyte sedimentation rate, and C-reactive protein, were not associated with prognosis. Plasma levels of IL-8, sTREM-1, sTNFR1, Angpt-2, and sFlt1 were significantly higher in children that progressed to poor prognosis; differences remained statistically significant after adjusting for selected potential confounding factors, except for IL-8 plasma levels. The best biomarkers for predicting poor prognosis were sFlt1 (AUROC 0.71), sTNFR1 (AUROC 0.69), and sTREM-1 (AUROC 0.68). The addition of any of these three biomarkers significantly improved the prognostic performance of the clinical sign lower chest retractions or the RISC score. The quantification of sTNFR1 allowed accurate (AUROC 0.90) identification of children ≥12 months of age with pneumonia who were at risk of fatal outcome or required long duration of oxygen therapy, and performed better than clinical characteristics or scoring scales. Furthermore, the addition of a clinical sign to this biomarker did not significantly increase its performance.

Conclusions

- The RIBhuC study findings allow a comprehensive characterization of children with pneumonia admitted at the National Referral Hospital in Bhutan.
- The children with fatal outcome were all referred from health centres far from Thimphu and were in a critical condition when they reached the study hospital.
- The proportion of partially immunized children according to age and the number of children with malnutrition were not negligible.
- Bacterial aetiology was infrequent, with only two confirmed cases of pneumonia caused by *Streptococcus pneumoniae*, while viruses were found in a considerable proportion of children. These microbiological findings coincide more with the aetiological profile of pneumonia in children from high-income countries and highlight the advanced stage of the epidemiologic transition that Bhutan seems to have reached.

- This study provided baseline information on the status of pneumococcal nasopharyngeal carriers and the pneumococcal serotypes among Bhutanese children admitted with clinical pneumonia before the introduction of the 13-valent pneumococcal conjugate vaccine in the country. We found that this vaccine could theoretically have averted up to 58% of the pneumococcal infections.
- It remains challenging to identify children with pneumonia that require antibiotics based on current clinical and laboratory parameters. Indeed, we did not identify a specific clinical sign that was suggestive of radiological pneumonia. Three commonly-used inflammatory biomarkers were moderately predictive for radiological pneumonia (proxy for bacterial pneumonia), of which procalcitonin presented the best diagnostic performance.
- Our findings confirm that immune and endothelial activation markers have the potential to inform risk-stratification and clinical decision-making in children with pneumonia.

The way forward

- The findings of this thesis need to be translated into easily implementable recommendations for policy change so as to improve the care of children with pneumonia and to reduce mortality associated with this disease. As most of the children recruited to this study lived in the capital, all the findings of this study might not be applicable to the rest of the country, which presents a wide diversity in terms of geography, climate, wealth, alimentation, and access to care. In addition, the RIBhuC study took place in the National Referral Hospital, and outcomes of children with pneumonia might differ when managed at primary or secondary healthcare level in more remote areas.
- The proportions of partially immunized and wasted children suggest that there is room for improvement regarding the national immunization coverage and the implementation of preventive measures against malnutrition.
- Generating further and continuous local data on pneumonia aetiology is strongly recommended to confirm the RIBhuC findings at the national level, to develop tailored programmes, and to monitor the prevalence of the main causative

pathogens. Fostering robust pneumonia surveillance, focusing on the burden and aetiology of the disease in children under five years of age, is essential. A good start might be to strengthen the existing national surveillance system on acute respiratory infections to improve data availability and reliability.

- It is highly recommended to monitor the impact of pneumococcal conjugated vaccine, which was introduced in the routine immunization programme in January 2019, and our data will constitute a reasonable baseline comparative of the pre-vaccine epidemiology.
- Procalcitonin was found to be a good predictor of radiological pneumonia, which may encourage policymakers to incorporate this biomarker into clinical practice in Bhutan to help decision-making to start or discontinue antibiotics in children with pneumonia, leading to better clinical outcomes and reduced antibiotic overuse.
- The fact that all the children who died were referred in critical conditions from other centres suggests room for improving the early recognition of children with severe disease at secondary and primary healthcare levels for timely referral, as prompt intensive management is associated with better outcome. Clinical signs and scoring scales mainly rely on subjective assessment and present poor specificity at predicting severity and poor prognosis, leading to unnecessary referrals with subsequent healthcare facilities overload, unnecessary exposure to treatment, and misallocation of health resources. Immune and endothelial activation markers have potential to become a simple, fast, and objective tool for risk-stratification of children with pneumonia. We encourage further investigation to identify and validate a prognostic biomarker or biomarker signature that allows the risk-stratification of children with pneumonia at their first contact with health systems both in the community settings and at the hospital level. If developed as a point-of-care tool that is accurate, cheap, equipment-free, rapid, and easy to use, such a triage tool could guide healthcare workers on decision-making for referral and prioritization of care, and would likely contribute to decreasing the high mortality associated with childhood pneumonia.

Resumen

Introducción

La neumonía es la principal causa de mortalidad en niños menores de cinco años. Un niño muere de neumonía cada 39 segundos. En 2018, la neumonía fue responsable de la muerte de 800.000 niños, sumando más muertes que la malaria, la tuberculosis, el virus de la inmunodeficiencia humana y el sarampión juntos. Aunque la neumonía afecta a la población infantil de todo el mundo, la principal carga sigue estando concentrada de forma desproporcionada en los países de ingresos bajos y medianos del sudeste asiático y el África subsahariana. En las últimas décadas se han realizado esfuerzos mundiales innegables que han conducido a avances considerables en la reducción de la morbilidad y la mortalidad asociadas a esta enfermedad. Sin embargo, a menos que el mundo acelere los esfuerzos para abordar específicamente y de forma más proactiva esta enfermedad, 3,2 millones de niños morirán innecesariamente de neumonía entre 2020 y 2030.

Los desafíos para enfrentar esta enfermedad son múltiples. En primer lugar, caracterizar la etiología de la neumonía sigue siendo un desafío que aún no se ha abordado adecuadamente. Los virus son la principal causa de neumonía en niños menores de cinco años, siendo el virus respiratorio sincitial el patógeno más comúnmente identificado en este grupo de edad. *Streptococcus pneumoniae* y *Haemophilus influenzae* tipo b son las principales causas bacterianas de neumonía infantil. Hoy en día, los casos de neumonía causada por estas bacterias se pueden prevenir con vacunas conjugadas altamente efectivas, y su incidencia ha disminuido drásticamente en entornos donde estas vacunas se han implementado adecuadamente. La gran diversidad de patógenos que puede causar neumonía implica que no existe un único tratamiento ni una única vacuna para tratar y prevenir esta enfermedad. Además, la identificación de virus respiratorios en la nasofaringe de niños con neumonía requiere una interpretación cuidadosa. La distinción entre portador nasofaríngeo y agente causal es difícil, y la detección de virus respiratorios no implica causalidad ni excluye una infección bacteriana. Existe una creciente evidencia que muestra una superposición de la etiología viral y bacteriana en las infecciones respiratorias y que sugiere la importancia de su interacción en la patogenia de la neumonía. Además, los estudios sobre la etiología de la neumonía realizados en diferentes entornos han reflejado hallazgos contradictorios, que limitan su

generalización a otros países y regiones y contribuyen a las dificultades para desentrañar las principales etiologías causantes de la neumonía infantil.

En segundo lugar, no existe una definición estandarizada de neumonía y no existe un criterio clínico simple o una herramienta diagnóstica que permita la detección precisa y, por lo tanto, el diagnóstico de la enfermedad. La superposición de signos y síntomas entre la neumonía y muchas otras enfermedades comunes pediátricas como la malaria, la anemia grave, la sepsis y las cardiopatías congénitas, conduce a dificultades a la hora de diferenciar estas enfermedades y traduce la baja especificidad de las características clínicas para el diagnóstico de neumonía. Los criterios de diagnóstico clínico de la neumonía, como los propuestos por la Organización Mundial de la Salud (OMS), son muy sensibles para detectar a todos los niños que se beneficiarían de tratamiento antibiótico, pero insuficientemente específicos. Sin embargo, distinguir entre la etiología viral o bacteriana de la enfermedad constituye un desafío adicional. Los criterios clínicos no permiten distinguir a los niños con neumonía bacteriana que requieren antibióticos de aquellos que presentan un cuadro autolimitado sin necesidad de ellos. Esto conduce a un sobretratamiento de los casos de neumonía con antibióticos, con potenciales implicaciones en la aparición de resistencia antimicrobiana. Los criterios radiológicos se desarrollaron para aumentar la especificidad en la identificación de neumonía bacteriana y se siguen utilizando como estándar de referencia tanto en la práctica clínica como en la investigación. Sin embargo, la radiografía de tórax es una herramienta imperfecta para discriminar entre neumonía bacteriana y viral con el fin de decidir si el niño requiere tratamiento con antibióticos. Frente a la dificultad para identificar el patógeno causante, la complejidad para determinar causalidad, y el rol no resuelto de las coinfecciones, se hace necesario un enfoque novedoso para reducir la alta mortalidad asociada con la neumonía infantil.

La identificación temprana de los niños con neumonía grave permite instaurar un tratamiento rápido y más agresivo conduciendo así a un mejor pronóstico y a una reducción de la mortalidad. Por lo tanto, se impone la necesidad de un reconocimiento temprano de los niños susceptibles de progresar a una enfermedad grave desde su primer contacto con el sistema de salud, con el fin de apoyar a los profesionales sanitarios en la toma de decisión para la derivación temprana, la priorización de la atención y la necesidad de hospitalización o de ingreso en cuidados intensivos. Para ello,

se han desarrollado varias escalas pronósticas basadas en características clínicas y pruebas de laboratorio simples. Sin embargo, estas escalas carecen de sensibilidad y especificidad y no han sido debidamente validadas para la neumonía infantil.

Se están investigando cada vez más los biomarcadores de respuesta del huésped en diferentes ámbitos clínicos para la neumonía y otras enfermedades infecciosas. En concreto, se valora su uso en el diagnóstico, diagnóstico diferencial etiológico, estratificación del riesgo, gravedad de la enfermedad y orientación para el inicio y la interrupción de tratamiento con antibióticos. Los biomarcadores de respuesta del huésped son mediadores de las vías de respuesta endotelial, inmunológica e inflamatoria, que son activadas en la patogenia de infecciones potencialmente mortales, independientemente de su etiología. Pueden cuantificarse en la sangre y presentan el beneficio de ser medidas precisas y reproducibles. Un biomarcador como herramienta que cumpla con los criterios ASSURED de la OMS (asequible, sensible, específico, fácil de usar, rápido y robusto, sin necesidad de equipo y entregable a usuarios finales, de sus siglas en inglés) que permita estratificar el riesgo de niños con neumonía y que se pueda usar tanto a nivel hospitalario como a nivel comunitario podría tener un impacto significativo. La proteína C reactiva y la procalcitonina se utilizan comúnmente en el diagnóstico de enfermedades infecciosas en países de ingresos altos. El uso de la procalcitonina para decidir la necesidad de antibióticos en niños con neumonía ha reducido el uso de antibióticos en países de ingresos altos sin impactar negativamente en los resultados clínicos. Sin embargo, estos biomarcadores inflamatorios son una herramienta raramente disponible en el lugar de asistencia del paciente en países con recursos limitados. Además, no hay evidencia suficiente sobre su rendimiento en países de ingresos bajos y medianos que presentan un contexto epidemiológico diferente, tales como factores demográficos y enfermedades coexistentes. No obstante, hay hallazgos prometedores de otros biomarcadores investigados en países de ingresos bajos y medianos. Por ejemplo, en Uganda, el incremento del ratio angiopoyetina-2/angiopoyetina-1 en niños con neumonía se asoció con la gravedad de la enfermedad y con un mayor riesgo de mortalidad. Aunque se necesitan más investigaciones para validar su valor diagnóstico y pronóstico, estos biomarcadores emergentes parecen prometedores para contribuir a reducir la morbimortalidad asociada con la neumonía infantil.

En los países de ingresos bajos y medianos con recursos sanitarios limitados, el diagnóstico y el tratamiento de la neumonía en niños menores de cinco años es un verdadero desafío. Además, la epidemiología y los patógenos causantes de la neumonía infantil difieren a través del mundo. La caracterización de la neumonía y de sus desafíos en un país como Bután puede arrojar luz sobre las estrategias más efectivas para garantizar la supervivencia infantil. En Bután, donde la neumonía es responsable del 15% de las muertes en niños menores de 5 años y del 27% de las muertes post-neonatales, mejorar la atención de esta enfermedad tendrá un impacto importante en la salud infantil.

El Reino de Bután es un pequeño país en el Himalaya oriental, con una población de alrededor de 800.000 personas. Este país de predominio montañoso se eleva desde unos 100 m en la frontera sur con la India hasta las altas cumbres del Himalaya por encima de los 7500 m al norte. La capital, Thimphu, se sitúa a una altitud de 2334 m. El clima varía con la altitud, desde tropical en el sur hasta alpino con inviernos muy fríos en el norte. Bután está clasificado como un país de ingresos medianos bajos. Los servicios esenciales de salud son gratuitos para los ciudadanos butaneses tanto para la medicina moderna como para la medicina tradicional. El sistema de salud se basa en la atención primaria y es accesible hasta en las zonas más remotas del país a pesar de las dificultades geográficas.

Hipótesis

- La escasez de datos epidemiológicos, clínicos y etiológicos dificulta una caracterización exhaustiva de la neumonía infantil en Bután.
- La neumonía infantil sigue siendo un importante problema de salud pública en Bután.
- Las bacterias causan alrededor del 25% de las neumonías en niños hospitalizados, siendo *Streptococcus pneumoniae* el principal agente infeccioso. Los virus se detectan con frecuencia en la nasofaringe de los niños hospitalizados con neumonía, siendo el virus respiratorio sincitial y el rinovirus los más comunes.
- La mayoría de los serotipos neumocócicos que presentan los niños hospitalizados con neumonía se pueden prevenir mediante las vacunas antineumocócicas conjugadas disponibles.

- Las características clínicas no tienen una buena capacidad predictiva en relación con los hallazgos radiológicos.
- Ciertos biomarcadores de respuesta del huésped son útiles para distinguir la neumonía bacteriana de la viral y también para identificar a los niños susceptibles de cursar con un mal pronóstico.

Objetivos

El objetivo principal de esta tesis fue identificar y reducir las lagunas de conocimiento sobre la epidemiología, la etiología, la presentación clínica y los hallazgos radiológicos de la neumonía infantil en Bután para caracterizar esta enfermedad y contribuir a su comprensión en el contexto local. Esta tesis también tuvo como objetivo evaluar el rol diagnóstico y pronóstico de ciertos biomarcadores por si solos, combinados o en adición a escalas de puntuación clínica para estratificar el riesgo de los niños hospitalizados con neumonía y predecir su resultado clínico.

Métodos

El primer artículo señala la necesidad de realizar investigación a nivel local y comenta los desafíos específicos encontrados en un país como Bután para llevarla a cabo.

El segundo artículo es una revisión sistemática que resume el conocimiento actual sobre la neumonía infantil en Bután y que identifica las lagunas de conocimiento en este campo. Se utilizaron los resultados de esta revisión y las carencias de conocimiento para enfocar los objetivos del estudio RIBhuC (del inglés Respiratory Infections in Bhutanese Children).

Se detallan los principales hallazgos del estudio RIBhuC en los **artículos 3 a 6** de esta tesis. El estudio RIBhuC se llevó a cabo entre el 1 de julio del 2017 y el 30 de junio del 2018 en el Hospital Nacional de Referencia Jigme Dorji Wangchuck, en Thimphu. Se reclutó prospectivamente a todos los niños entre 2 y 59 meses ingresados por neumonía clínica según los criterios de la OMS, siempre que los padres o cuidadores aceptaran participar en el estudio. Al ingreso, se realizó un examen físico completo incluyendo mediciones antropométricas y toma de signos vitales. Se recogieron datos demográficos y clínicos mediante entrevistas con los familiares y a partir del expediente médico. Se realizó una radiografía de tórax anteroposterior en las primeras 24 horas del ingreso y se

clasificaron los niños según los criterios radiológicos de la OMS. Se recogieron muestras de sangre en el momento del reclutamiento, o lo antes posible después del reclutamiento, para análisis de hematología y bioquímica, cultivo bacteriano, e identificación de *Streptococcus pneumoniae* mediante la reacción en cadena de la polimerasa en tiempo real a partir de dos gotas de sangre recogidas en papel de filtro. También se midieron los niveles plasmáticos de once biomarcadores de respuesta del huésped, incluyendo seis marcadores de activación endotelial e inmune: la interleucina-6 (IL-6), la interleucina-8 (IL-8), el receptor de activación soluble expresado en células mieloides-1 (sTREM-1), el receptor soluble del factor de necrosis tumoral 1 (sTNFR1), la angiopoyetina-2 (Angpt-2), y la tirosina quinasa-1 soluble similar a fms (sFlt1). Finalmente, se recogieron muestras respiratorias mediante lavado nasofaríngeo para la identificación molecular de 17 virus respiratorios y 4 bacterias atípicas, así como para la detección y tipificación capsular neumocócica.

El tercer artículo describe el perfil etiológico y las características demográficas y clínicas de esta cohorte de niños butaneses ingresados con neumonía clínica.

El cuarto artículo presenta la prevalencia de portadores nasofaríngeos neumocócicos y los serotipos neumocócicos circulantes entre los niños butaneses ingresados con neumonía clínica antes de la introducción de la vacuna antineumocócica conjugada en el país. Comparamos la prevalencia y tipos de virus respiratorios entre niños con y sin colonización nasofaríngea neumocócica para contribuir a la comprensión de la interacción entre la colonización nasofaríngea neumocócica y las coinfecciones virales.

El quinto artículo describe los hallazgos radiológicos y evalúa las diferencias en cuanto a características demográficas, etiológicas, clínicas y niveles de biomarcadores según las características radiológicas. En este artículo, también se evalúa la utilidad de biomarcadores para diferenciar entre neumonía bacteriana y viral, considerando el hallazgo de neumonía radiológica (condensación, derrame pleural o ambos) como indicador de neumonía bacteriana.

El sexto y último artículo de esta tesis evalúa el rendimiento de características clínicas, pruebas de laboratorio, escalas de puntuación clínica y biomarcadores para estratificar el riesgo pronóstico de los niños con neumonía clínica en el momento del ingreso y predecir su resultado clínico.

Resultados principales

El primer artículo de esta tesis expone lo importante que es contar con datos locales fiables que permitan informar políticas basadas en la evidencia. Los principales desafíos para desarrollar y llevar a cabo una investigación local en Bután son la falta de conciencia de que la investigación es necesaria; la falta de motivación, de conocimientos metodológicos, de capacidad analítica y de valoración crítica; la escasa priorización de fondos para fines de investigación; y el foco prioritario de los profesionales de la salud hacia la medicina asistencial, la cual representa una carga de trabajo considerable.

La revisión sistemática (**segundo artículo**) incluyó 44 artículos y documentos que resumimos cualitativamente. Concluimos que la neumonía sigue siendo responsable de una alta morbimortalidad en niños en Bután. El sistema de vigilancia nacional en vigor se centra en la identificación del virus influenza, aunque recientemente se ha introducido la monitorización de otros virus respiratorios. Encontramos muy pocos o ningún dato que describiesen las características epidemiológicas, clínicas, radiológicas y pronósticas de la neumonía infantil en Bután, o de su etiología.

El estudio RIBhuC incluyó a 189 niños con neumonía clínica definida según los criterios de la OMS, hospitalizados en el Hospital Nacional de Referencia Jigme Dorji Wangchuck, lo que corresponde al 11,9% de todos los ingresos pediátricos durante el periodo del estudio.

El tercer artículo describe las características demográficas, clínicas y microbiológicas de esta cohorte**.** Seis niños murieron, lo que corresponde a una tasa de letalidad del 3,2%. Estos seis niños fueron remitidos desde otros centros sanitarios lejos de la capital, y llegaron en estado crítico. La duración mediana de hospitalización fue de cuatro días. Más de la mitad (53,4%) de la cohorte eran lactantes menores de 12 meses de edad. El 75% de los niños estaba correctamente inmunizado de acuerdo con el calendario vacunal por edad, mientras que el 25% restante sólo estaba parcialmente inmunizado. Un niño de cada 10 presentaba desnutrición. De forma contra intuitiva, el menor número de ingresos hospitalarios se registró durante el invierno (21 de diciembre al 20 de marzo), que corresponde a la estación más fría en Bután. La mayoría de los niños (79,4%) presentó neumonía grave, según los criterios de la OMS. Al ingreso, la mitad de los niños presentaba taquipnea, las tres cuartas partes presentaban hipoxemia (definida como

SpO2 <90%) y el 54,3% presentaba tiraje subcostal o intercostal inferior. De las 149 radiografías de tórax legibles, el 53,0% eran normales, el 26,2% se clasificaron como neumonía con consolidación, derrame, o ambos (criterio principal de la evaluación de la existencia de la neumonía según los criterios de la OMS, de aquí adelante referido como "neumonía radiológica") y el 20,8% como "otros infiltrados". Se detectó crecimiento bacteriano no relacionado con contaminación en 8/148 (5,4%) de los hemocultivos, con solo dos casos de *Streptococcus pneumoniae*. Se detectó *Bordetella pertussis* mediante métodos moleculares en tres niños, de los cuales un lactante de cinco meses que falleció. La detección de virus en las secreciones de las vías respiratorias superiores fue común, con al menos un virus detectado en 103/115 (89,6%) niños, siendo infecciones mixtas en el 34,0% de los casos. Los virus más comúnmente aislados fueron el virus respiratorio sincitial (52/115; 45,2%), el rinovirus (42/115; 36,5%) y los virus parainfluenza humano (19/115; 16,5%).

El cuarto artículo muestra que 62,8% (76/121) de los niños reclutados eran portadores nasofaríngeos del neumococo. Se identificaron treinta serotipos diferentes (o grupos de serotipos cuando no fue posible diferenciarlos). La mitad de los niños portadores estaban colonizados con al menos dos y hasta cinco serotipos diferentes. Basándonos en hallazgos de estudios anteriores, consideramos los serotipos 1, 3, 4, 5, 7F, 14, 18C y 19A como altamente invasivos y nos referimos a los serotipos restantes como "menos altamente invasivos". La mayoría de los niños presentaron serotipos menos altamente invasivos, de los cuales los más frecuentes fueron 7B/C o 40 (43,4%), 6A/B (15,8%) y 23F (7,9%). Aproximadamente un tercio de los niños presentaron serotipos altamente invasivos, de los cuales los más frecuentes fueron los serotipos 14 (11,8%), 3 (6,6%) y 1 (6,6%). Más de la mitad de los niños (57,9%) presentaba al menos un serotipo incluido en la vacuna antineumocócica conjugada 13-valente, y la mitad de los niños presentaba al menos un serotipo incluido en cualquiera de las dos vacunas conjugadas antineumocócicas 10-valentes disponibles comercialmente. Detectamos una proporción similar de coinfección viral entre niños colonizados por neumococos en la nasofaringe (62/70; 88,6%) y los no colonizados (36/40; 90,0%). El rinovirus fue menos común entre los portadores neumocócicos (20/70; 28,6% versus 19/40; 47,5%; p = 0,046). No encontramos otras diferencias significativas en la detección de otros virus.

En el quinto artículo, evaluamos la asociación entre signos clínicos, agentes patógenos y biomarcadores con los hallazgos radiológicos de los 149 niños con radiografías de tórax de buena calidad (26,2% con neumonía radiológica). No encontramos diferencias significativas en la identificación de virus respiratorios entre niños con diferentes hallazgos radiológicos. De los niños con neumonía radiológica, y comparado con los niños con radiografía normal o con "otros infiltrados", una mayor proporción de niños refirieron síntomas (64,1% versus 38,5%, p = 0,007) o fiebre (42,1% versus 21,3%, p = 0,045) durante al menos cinco días antes del ingreso, y una mayor proporción de niños presentaron neumonía grave según los criterios clínicos de la OMS (92,3% versus 75,5%, p = 0,033). No se identificó ningún signo clínico predictivo de neumonía o ausencia de neumonía radiológica. Sin embargo, la velocidad de sedimentación globular y los niveles plasmáticos de proteína C reactiva y procalcitonina se asociaron a la neumonía radiológica, incluso después de ajustar por varios factores de confusión. El biomarcador con mayor poder discriminatorio para identificar la neumonía radiológica como identificador de etiología bacteriana fue la procalcitonina, con una sensibilidad del 72,0% y una especificidad del 66,2%. Ninguno de los marcadores de activación endotelial e inmune mostró buena capacidad de discriminación para la neumonía radiológica.

El sexto artículo evaluó el rendimiento de biomarcadores, solos o en combinación con características clínicas o escalas de puntuación clínica para estratificar el riesgo de los 118 niños cuyos biomarcadores se han cuantificado. Un total de 23/118 (19,5%) niños progresaron a un mal pronóstico, definido como muerte (n = 3) o necesidad de oxigenoterapia durante más de cinco días (n = 20). La educación de los padres, el empleo de los padres, y el tiempo que tardan las familias para acceder a los centros de salud no se asociaron con mal pronóstico. Consideramos cuatro escalas clínicas: la RISC (del inglés Respiratory Index of Severity in Children), la RISC-Malawi, la puntuación de disfunción de órganos de Lambaréné (LODS), y la clasificación de gravedad de la OMS. La puntuación RISC fue la escala clínica con mayor poder predictivo pronóstico (área bajo la curva ROC [AUROC] 0,75), especialmente en lactantes <12 meses (AUROC 0,80). Sin embargo, ninguno de los signos clínicos de manera individual presentó un poder predictor suficientemente bueno como para permitir la toma de decisiones clínicas. La elevación de biomarcadores que se suelen usar de forma rutinaria, como los leucocitos, las plaquetas, la velocidad de sedimentación globular, y la proteína C reactiva, no se asoció

con mal pronóstico. Los niveles plasmáticos de IL-8, sTREM-1, sTNFR1, Angpt-2, y sFlt1 fueron significativamente más elevados en los niños que progresaron a mal pronóstico, siendo las diferencias estadísticamente significativas después de ajustar por posibles factores de confusión excepto para IL-8. Los biomarcadores con mayor poder predictivo pronóstico fueron sFlt1 (AUROC 0,71), sTNFR1 (AUROC 0,69), y sTREM-1 (AUROC 0,68). La combinación de cualquiera de estos tres biomarcadores con la presencia de tiraje de pared torácica inferior o con la escala RISC mejoró significativamente el rendimiento pronóstico de este signo o escala clínica. La cuantificación al ingreso de sTNFR1 en niños ≥12 meses con neumonía permitió identificar a aquellos con riesgo de muerte o que requirieron más de cinco días de tratamiento con oxígeno. Además, sTNFR1 en este grupo de edad mostró un rendimiento mayor que el de las características clínicas o escalas de puntuación, y su combinación con un signo clínico no aumentó significativamente su rendimiento.

Conclusiones

- El estudio RIBhuC proporcionó hallazgos que permiten una caracterización integral y detallada de los niños ingresados con neumonía en el Hospital Nacional de Referencia en Bután.
- Los niños que fallecieron fueron derivados de centros de salud alejados de Thimphu y llegaron al Hospital de Referencia Nacional en condiciones críticas.
- La proporción de niños parcialmente inmunizados acorde a su edad y el número de niños con desnutrición no fueron despreciables.
- La etiología bacteriana fue poco frecuente, con solo dos casos confirmados de infección por *Streptococcus Pneumoniae*, mientras que se detectaron virus respiratorios en una proporción considerable de niños. Estos hallazgos microbiológicos se asemejan al perfil etiológico de la neumonía infantil en países de ingresos altos, sugiriendo que Bután parece haber alcanzado una etapa avanzada en su transición epidemiológica.
- Este estudio informa de la magnitud de los portadores nasofaríngeos neumocócicos y revela los serotipos neumocócicos circulantes entre los niños butaneses ingresados con neumonía antes de la introducción de la vacuna

antineumocócica conjugada 13-valente en el país. Esta vacuna podría haber evitado, en teoría, hasta el 58% de las infecciones neumocócicas.

- El uso de las características clínicas o de laboratorio para identificar a niños con neumonía que requieren antibióticos sigue siendo un gran desafío. De hecho, no encontramos ningún signo clínico que sugiriera neumonía radiológica. Tres biomarcadores inflamatorios de uso común fueron más elevados entre los niños con neumonía radiológica (proxy de neumonía bacteriana), de los cuales la procalcitonina presentó el mejor rendimiento diagnóstico.
- Nuestros hallazgos confirman que los marcadores de activación endotelial e inmune tienen el potencial de informar la estratificación de riesgo y la toma de decisiones clínicas en niños con neumonía.

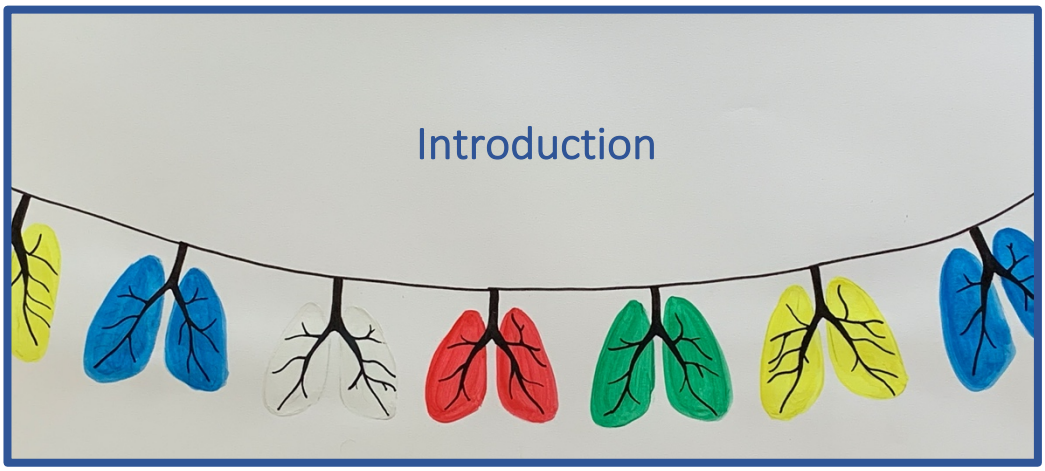
Consideraciones finales

- El siguiente paso consiste en traducir los hallazgos de esta tesis en recomendaciones de fácil implementación para elaborar políticas que contribuyan a la mejoría de la atención de los niños con neumonía y reduzcan la mortalidad asociada a esta enfermedad. La mayoría de los niños reclutados en este estudio vivían en la capital, por lo que es posible que no todos los hallazgos se apliquen al resto del país que es muy diverso en términos de geografía, clima, situación económica, alimentación y acceso a la atención sanitaria. Además, el estudio RIBhuC se llevó a cabo en el Hospital Nacional de Referencia, y el desenlace de los niños con neumonía podría ser diferente en niños tratados a nivel de atención primaria o secundaria en las áreas más remotas del país.
- La proporción de niños parcialmente inmunizados y desnutridos sugiere que se pueden optimizar la cobertura nacional de inmunización y la implementación de medidas preventivas contra la desnutrición.
- Se recomienda una recogida continua de datos locales sobre la etiología de la neumonía para confirmar los hallazgos del presente estudio a nivel nacional, con el fin de monitorizar la prevalencia y las tendencias temporales de los principales patógenos causales para poder así desarrollar programas focalizados y basados en la evidencia. Es esencial fomentar una vigilancia sólida de esta enfermedad en niños menores de cinco años que se centre en su incidencia y su etiología. Un

buen comienzo podría ser fortalecer el ya existente sistema nacional de vigilancia sobre infecciones respiratorias agudas para obtener datos fiables.

- Se recomienda monitorizar el impacto de la vacuna antineumocócica conjugada, introducida en el calendario vacunal nacional desde enero del 2019. Nuestros datos reflejan la situación epidemiológica pre-vacunal, constituyendo una fuente de referencia razonable para su comparación.
- La procalcitonina mostró ser un buen predictor de neumonía radiológica, lo que podría alentar a los legisladores a contemplar su incorporación en la práctica clínica en Bután para ayudar a la toma de decisiones (comenzar o suspender antibióticos) con el fin de obtener mejores resultados clínicos y reducir el uso excesivo de antibióticos.
- El hecho de que todos los niños que murieron fueron derivados en condiciones críticas desde otros centros sugiere que se puede mejorar la capacidad de reconocimiento temprano de enfermedad grave en los niveles de atención primaria y secundaria para una derivación temprana, ya que el manejo intensivo temprano se asocia con mejores resultados. Los signos clínicos y las escalas de puntuación se basan principalmente en una evaluación subjetiva y son poco específicos para predecir la gravedad y el mal pronóstico. Resulta en derivaciones innecesarias con la consiguiente sobrecarga de trabajo, exposición innecesaria a antibióticos, y asignación incorrecta de recursos sanitarios. Los marcadores de activación endotelial e inmune tienen un gran potencial para convertirse en una herramienta sencilla, rápida y objetiva para la estratificación de riesgo en los niños con neumonía. Con este trabajo animamos a que haya más estudios de investigación cuyo objetivo sea el de identificar y validar un biomarcador o una combinación de biomarcadores pronósticos que permitan estratificar el riesgo en niños con neumonía desde su primer contacto con los sistemas de salud, tanto en la comunidad como a nivel hospitalario. Un biomarcador desarrollado como herramienta de uso en el lugar de asistencia del paciente que fuera preciso, barato, sin necesidad de equipos adicionales, rápido y fácil de usar constituiría una herramienta de triaje que ayudaría en la toma de decisiones para la derivación y la priorización de la atención. De esta manera seguramente contribuiría a disminuir la alta mortalidad asociada con la neumonía infantil.

Introduction



Justification for the thesis

Pneumonia might be perceived as a harmless disease. Dealing with this disease might seem simple: prevention, diagnosis, and treatment. However, pneumonia remains the leading infectious cause of deaths in children under five years of age globally. Often absent from the agenda of global health priorities, this forgotten disease was recently mentioned as a "global cause without champions" (1). The challenges to address this disease are multiple. There is no standardized definition of pneumonia. There are no simple clinical criteria or diagnostic tools that allows accurate detection of the disease and identification of children who develop severe pneumonia with poor prognosis. Distinguishing between viral or bacterial aetiology is complex. There is a wide range of causative pathogens that require different treatments. No single effective vaccine is sufficient for preventing pneumonia.

Fortunately, there are effective strategies to reduce the burden of this disease. While we need an increased global investment for better implementation of effective interventions for reducing the burden of childhood pneumonia, we also need reliable data for tracking progress and better allocation of resources. A lack of data will likely lead to inadequate policies and practice. To assess how national programmes are progressing towards the reduction of the disease burden, adequate surveillance systems should be in place that are capable of measuring the burden of the disease, describing its epidemiological trends, and determining the underlying pathogens involved. As risk factors and causative pathogens of childhood pneumonia differ widely globally, it is imperative to obtain reliable local data for adequate and targeted interventions. One country that exemplifies the dearth of data regarding the characteristics of childhood pneumonia is the Kingdom of Bhutan. This thesis aims to collect data to better characterize childhood pneumonia in Bhutan and to contribute to the understanding of this disease in a local context in a low- and middle-income country (LMIC) with resource constraints.

The Severe Acute Respiratory Infection (SARI) surveillance was started in Bhutan in 2012 and is mainly focused on monitoring the influenza virus in the Bhutanese population (2). Despite the efforts made to conduct the SARI surveillance, there is scarce information regarding the aetiology of respiratory illness in children in Bhutan (3). This thesis will help strengthen the SARI surveillance by reporting all cases between 2 to 59 months of age

that meet the SARI definition. The information gained from this thesis can be used to determine priorities and strategies to prevent pneumonia and hospitalization due to pneumonia among children in Bhutan and to influence development and implementation of such preventive strategies in other countries with similar epidemiological characteristics.

Antimicrobial treatment is indicated for pneumonia of suspected bacterial aetiology. However, viral pneumonia does not respond to antibacterial drugs. Understanding the relative contribution of the different pathogens that cause pneumonia in Bhutan and the patterns of antimicrobial resistance is useful for developing effective policies regarding the management of pneumonia in Bhutan and in neighbouring countries.

Conjugate vaccines against *Haemophilus influenzae* type b and *Streptococcus pneumoniae* provide effective prevention against death from pneumonia. The *Haemophilus influenzae* type b conjugate vaccine has been available for over 30 years but was only included in Bhutan's immunization programme in 2011. The 13-valent pneumococcal conjugate vaccine (PCV) was not part of the immunization programme during the time of this research study, but has since been introduced (January 2019). A global effort has been initiated to estimate the pneumococcal burden of disease and predominant serotypes in different regions. Still, obtaining reliable local data is crucial to help determine the potential impact of available conjugate vaccines, especially in LMICs without the Global Alliance for Vaccines and Immunizations support, which is the case of Bhutan (4,5). Beyond advocacy for pneumococcal vaccine introduction, surveillance is necessary to evaluate the impact of vaccine introduction on the prevalence of serotypes.

Diagnostic and prognostic biomarkers are routinely used in many high-income countries, at the hospital level, to guide antibiotic therapy and management decisions. Understanding their performance and potential public health utility in a cohort of well-characterized pneumonia patients in Bhutan is relevant.

Pneumonia worldwide

The global burden of pneumonia

Thirty-nine seconds. One child dies of pneumonia every 39 seconds. Pneumonia is the leading infectious cause of deaths in children under five years of age (6). In 2018, it was responsible for around 15% of global deaths in this age group, which translates into 800,000 child deaths (6–9). This is more than the deaths in children due to malaria, tuberculosis, human immunodeficiency virus (HIV), and measles combined (6,7,10,11); and is equivalent to the total population of Bhutan (12).

Pneumonia affects children worldwide, but disparities persist across regions and countries, with the main disease burden concentrated in LMICs (9,13,14) (Figure 1). In 2015, the World Health Organization (WHO) African and the South-East Asia Regions accounted for over 75% of global deaths attributed to pneumonia (8).

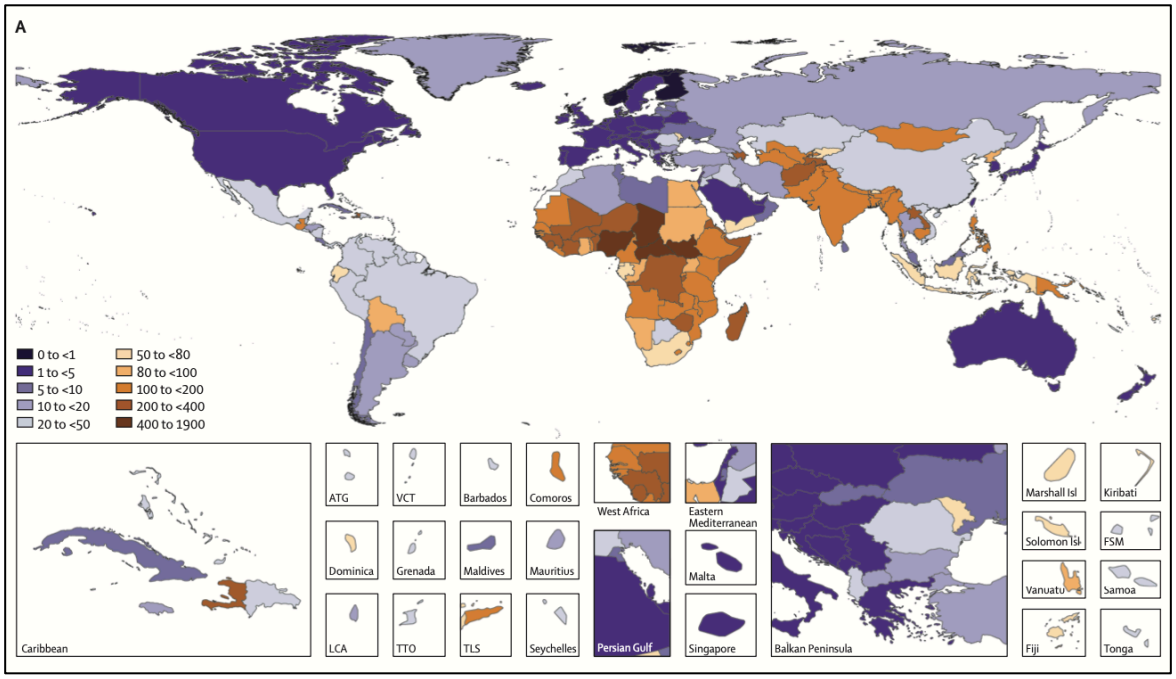


Figure 1. Global distribution of lower respiratory infections mortality among children under five years of age.
The legend shows the lower respiratory infections mortality rate per 100,000 children.
Source: Troeger 2020 (9). Reprinted with permission.

For the last half-century, pneumonia has been a major cause of medical attendance in healthcare facilities and hospital admissions in LMICs (15). In the 1980s, the WHO and the United Nations Children's Fund (UNICEF, originally known as the United Nations

International Children's Emergency Fund) estimated that acute respiratory infections (ARI) constituted between 30% to 50% of paediatric outpatient visits, and between 10% and 30% of hospital admissions (16). In 2015, it was estimated that between 86 and 226 million children under five years of age suffer annually from clinical pneumonia (8).

Unless global efforts to tackle childhood pneumonia accelerate, 3.2 million of children will die from this disease between 2020 and 2030 (17). Strong global commitment and increased investments are essential to reduce the global burden of childhood pneumonia.

Global strategies to tackle childhood pneumonia

Despite the high burden of childhood pneumonia, significant efforts to prevent and control the disease have been made since the 1970s, and considerable progress has been achieved in the reduction of the morbidity and mortality associated with the disease. In the 1970s, ARI and other infectious diseases accounted for a large proportion of deaths among children under the age of five years. Despite some of these diseases being preventable with available vaccines, immunization coverage was very low (18,19). In 1974, the WHO launched the Expanded Programme on Immunization (EPI) to make vaccines available to every child worldwide (19). This strategy successfully led to increasing immunization coverage. In LMICs, vaccine coverage for three doses of diphtheria, tetanus, pertussis, and poliomyelitis in children during their first year of life increased from less than 5% prior to the start of the programme, to over 50% in 1988, and over 85% in 2010 (19,20). This translated into a decreased incidence of the targeted diseases and decreased child mortality related to these diseases including pneumonia. A standardized immunization schedule was developed for the six vaccines originally included in the EPI programme: measles, pertussis, diphtheria, tetanus, tuberculosis, and poliomyelitis. Since then, additional vaccines have been incorporated into the routine immunization schedule of most countries, such as hepatitis b and *Haemophilus influenzae* type b. While some of the targeted pathogens cause primary pneumonia, for example *Haemophilus influenzae* type b, other vaccine-preventable diseases can lead to pneumonia secondary to complications from the diseases. Overall, millions of cases of the targeted diseases have been prevented with immunization, resulting in a considerable decreased incidence of children with pneumonia (20).

Around the same time in the mid-1970s, the WHO recognized the control of ARI as an essential component of primary healthcare programmes. Several expert groups were formed to take action in this field (13). A decade later, the WHO developed a programme for controlling ARI in children (13). This programme was initiated in response to the concern about the high mortality rate associated with ARI in LMICs, in parallel with the increased knowledge for understanding ARI and the development of effective interventions for reducing these infections; for example, the recognition that many cases were caused by bacteria, and that available preventive (immunization) and therapeutic (antibiotics and supportive treatment) measures were effective in averting deaths associated with ARI. A joint statement by the WHO and UNICEF developed the basic principles of the ARI control programme, which consisted of three major components. The first component of the programme consisted of case management, which includes early recognition of ARI by families and healthworkers, supportive treatment, antimicrobial therapy, and timely referral to a higher level of care. The second component consisted of health education and community involvement for ensuring the effectiveness of case management. This was comprised of education on the causes of ARI, education for the recognition of ARI and for identification of danger signs to differentiate moderate and serious respiratory illness from mild disease, education for reducing parental smoking and indoor pollution, promotion of prenatal care and breastfeeding, and other actions to be taken at the community level. The third component focused on preventive measures, mainly immunization against diphtheria, pertussis, measles, and tuberculosis, through strengthening the EPI activities (13,16). The aim of this programme was to foster national and international action so that simple activities addressing the components of the programme were implemented, starting at the primary healthcare level (13). During the 1980-90s, 16 other disease-specific programmes were developed and implemented, such as programmes for the prevention and control of diarrhoea, malaria, and malnutrition (13).

Integrated approaches to fight childhood pneumonia

Although these vertical individual disease control programmes contributed to reducing child mortality, they were not obtaining the expected reduced mortality results, and too many children were still dying of preventable and treatable diseases in the 1990s. In 1995, ARI (mostly pneumonia), diarrhoea, measles, malaria, and malnutrition (or any

combination of these conditions) were responsible for 70% of deaths among children under the age of five years in LMICs, translating to about 8 million deaths (21). Furthermore, it was recognized that there was an overlap of symptoms and signs between these common diseases. Indeed, fast breathing could be the result of pneumonia, malaria, or severe anaemia, requiring different treatment. Therefore, a more integrated approach was needed to assess children as a whole, rather than on a single illness, to further reduce child mortality. To respond to this challenge, the WHO and UNICEF created the global strategy of Integrated Management of Childhood Illness (IMCI) in the 1990s (22). The strategy focuses on the well-being of the child as a whole. IMCI promotes the early recognition of all co-existing conditions, as well as a simple and algorithm-based systematic approach for their management (22,23). Children are assessed, classified, and treated accordingly. For example, a child presenting with cough or difficulty breathing will be assessed for a series of signs such as fast breathing, chest indrawing, or wheezing; then classified as 'cough or cold', 'pneumonia', or 'severe pneumonia or very severe disease' based on the signs assessment; and managed (choice of antimicrobial treatment and need of referral) according to the classification (23). IMCI also promotes healthy growth and development, as well as preventive interventions including breastfeeding and vaccination. IMCI has been implemented in more than 75 countries. It is an effective and low-cost strategy with success in reducing under-five mortality and improving quality of care by healthworkers (23,24).

Another milestone in the integrated approach to fighting child mortality was the establishment of the Millennium Development Goals (MDGs). In 2000, 198 countries committed to the eight MDGs with the aim to improve the lives of the world's poorest people and to eradicate extreme poverty by 2015 (25). The MDG4 aimed to reduce the under-five mortality by two-thirds between 1990 and 2015. Affordable interventions identified by the WHO for reaching this goal included prevention and case management of pneumonia, diarrhoea, and sepsis; care for newborns and their mothers; infant and young child feeding; vaccines; malaria control; and prevention of HIV and acquired immunodeficiency syndrome (AIDS) and care of people living with HIV or AIDS. The WHO promoted four main strategies to deliver these interventions: IMCI, the EPI, infant and young child feeding, and appropriate home care and timely treatment of complications for newborns (25). During the MDG period, countries that adopted these strategies

realized a 51% reduction in mortality and 30% reduction in the incidence of childhood pneumonia. The world was close to achieving MDG4, with a reduction of the global under-five mortality rate by more than half (from 90 to 43 deaths per 1000 live births) between 1990 and 2015 (25).

However, in 2015, the under-five deaths remained unacceptably high, especially those related to preventable causes. The Sustainable Development Goals (SDGs) were established in the continuity of the MDGs, with child survival as a continued focus of the agenda (26). Of the 17 new goals set for 2030, the SDG3 focuses on ensuring healthy lives and promoting well-being for all at all ages. Reducing the morbidity and mortality associated with pneumonia is integrated into several targets and indicators, such as reducing the under-five mortality, reducing 'other' communicable diseases, reducing the number of deaths and illnesses from household and ambient air pollution, providing universal health coverage, and supporting "the research and development of vaccines and medicines for the communicable diseases that primarily affect developing countries" (26).

Recent strategies and action against childhood pneumonia

In the last decade, a few global initiatives have been developed to combat pneumonia. The WHO and UNICEF launched the Global Action Plan for Pneumonia and Diarrhoea (GAPPD) in 2013 with a proposed cohesive approach to ending preventable child deaths from pneumonia and diarrhoea by 2025 (27). The GAPPD initiative promotes interventions that have been proven to be effective for controlling pneumonia and diarrhoea in children, integrated into a "Protect, Prevent and Treat" framework:

- "Protecting children by establishing and promoting good health practices from births" by ensuring exclusive breastfeeding for six months, adequate complementary feeding, and vitamin A supplementation;
- "Preventing children from becoming ill from pneumonia and diarrhoea" by ensuring universal coverage of immunization, hand hygiene, household and ambient air pollution reduction, and HIV prevention; and
- "Treating children who are ill from pneumonia and diarrhoea with appropriate treatment" by improving care seeking and referral, providing case management

at the health facility and community level, and by ensuring antibiotics and oxygen (27).

The Stop Pneumonia Initiative was created to provide “a voice for communities who suffer from the devastating consequences of the disease and who lack access to lifesaving interventions” (28). Spearheaded by the International Vaccine Access Center at the Johns Hopkins Bloomberg School of Public Health, Stop Pneumonia strives to raise awareness about pneumonia; to promote interventions for pneumonia integrated into the *Protect, Prevent and Treat* framework; and to generate action to combat pneumonia including continued donor investment.

The Every Breath Counts coalition, which is composed of 40 members from United Nations agencies, businesses, donors and non-governmental organizations, is the world’s first public-private partnership to support national governments to end preventable child pneumonia deaths by 2030 (29,30). Its overarching goal is to reduce the number of under-five deaths attributed to pneumonia to under 3 per 1000 live births by 2030, corresponding to the indicator required for succeeding in SDG3. Its work is focused on 10 African and Asian countries that have some of the highest burdens of pneumonia, where they provide support to the governments to close the critical gaps in the prevention, diagnosis, and treatment of pneumonia (29).

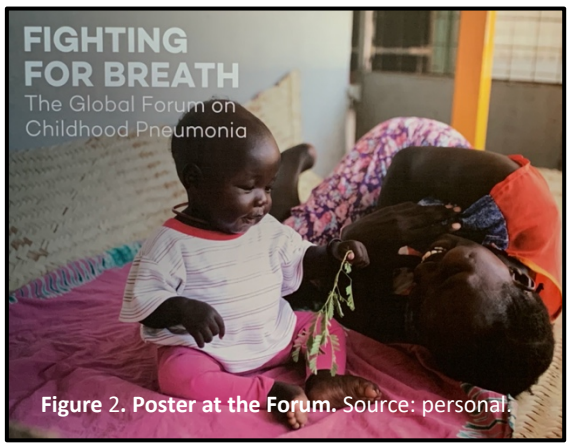


Figure 2. Poster at the Forum. Source: personal.

The Global Forum on Childhood Pneumonia held in January 2020 in Barcelona, Spain, was the world’s first conference on childhood pneumonia with a full focus on its impact in LMICs (Figure 2) (28). This meeting was a call to action to bring childhood pneumonia at the forefront of national and global health agendas. Global health leaders, non-governmental organizations, researchers, health ministers of high-disease burden countries, donors, and youth representatives were called to join forces to combat pneumonia. The parties developed actions to address this aim and committed to fulfil the GAPPD goal of reducing the number of deaths due to childhood pneumonia to less than 3 per 1000 live births and to achieve the

SDG3 to end all preventable child deaths by 2030 (28). The coalition reinforced that primary healthcare is the most efficient and effective way to achieve universal health coverage. The Pneumonia Wheel is a clear visualization of the comprehensive and integrated approach required for ending preventable child deaths from pneumonia, following the "Protect, Prevent and Treat" framework (Figure 3) (17). The three pillars of the primary healthcare constitute the foundation of this Pneumonia Wheel: (1) integrated quality health services, to ensure immunization, services for diagnosis and treatment (IMCI), and referral systems for severe cases; (2) multisectoral policies and actions to ensure nutrition and clean air to breathe; and (3) empowered people and communities, including accountability, engagement of communities in decision making for addressing their needs, and education in particular for empowering women whose role is fundamental in care-seeking and child care. Indicators across these components were established, to allow comparisons between countries and to track the progress of each country over time (17).

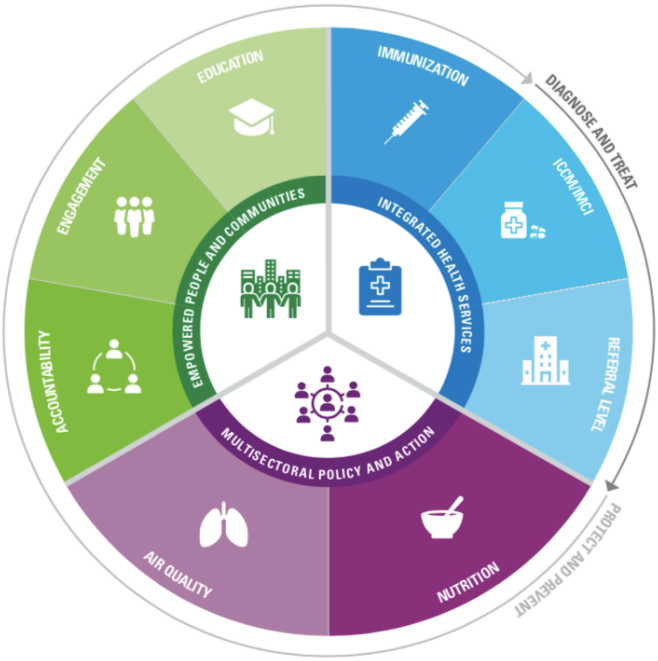


Figure 3. The Pneumonia Wheel.

Source UNICEF/Save the Children/Every breath counts 2020 (17).

Recent estimates show the persistence of global inequalities between the 30 countries with high pneumonia mortality and high-income countries for all indicators with available data (Figure 4) (17). The Chief Executive of Save the Children stated that "[Pneumonia] is a forgotten global epidemic that demands an urgent international response. Millions of children are dying for want of vaccines, affordable antibiotics, and routine oxygen

treatment. The pneumonia crisis is a symptom of neglect and indefensible inequalities in access to health care.” (10).

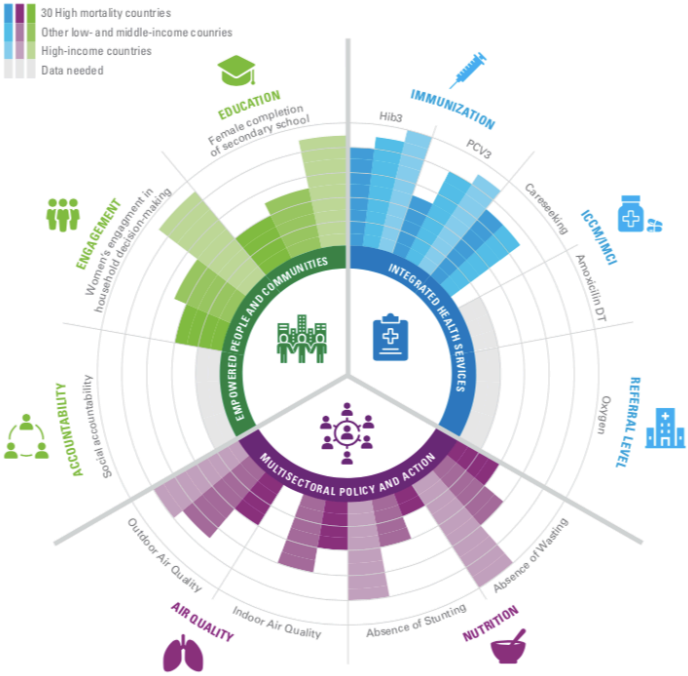


Figure 4. Indicators across components of the Pneumonia Wheel.

Source UNICEF/Save the Children/Every breath counts 2020 (17).

Clinical case definitions

WHO clinical case definition of pneumonia and classification of severity

In the early 1980s, the high burden of childhood pneumonia associated with high mortality rate led paediatricians and public health physicians to recognize the need of establishing clear diagnostic criteria for pneumonia. This was in order to identify children with pneumonia who required treatment with antibiotics, and also children with severe disease that needed referral to a higher-level health centre for additional management such as oxygen therapy (31). An ideal clinical case definition of pneumonia would require fulfilment of two main sets of criteria. First, the pneumonia case definition should be acceptable and understandable by healthcare providers worldwide to ensure optimal implementation in all settings. Second, it should be highly sensitive and specific to identify and treat all cases of pneumonia, with a special focus on children with severe disease, in order to reduce its associated mortality (31). However, determining a

definition of childhood pneumonia that is sensitive, specific, and that can be widely implemented is still a challenge. To date, no optimal gold standard definition that fulfils all of these criteria is available.

Indeed, one of the challenges of pneumonia is the lack of clinical characteristics and diagnostic criteria that are both highly sensitive and specific to detect the disease. The overlap of symptoms and signs between pneumonia and many other common diseases in children, such as malaria, severe anaemia, sepsis, and congenital heart diseases, and the subsequent difficulties in differentiating these diseases translates into low specificity of clinical characteristics for the detection of pneumonia (31–33).

In 1990, the WHO developed guidelines with recommendations for the detection and management of childhood pneumonia at first-level health facilities for countries with limited resources (31). These guidelines provided a simple standardized strategy, which consisted of classifying the severity of the illness based on the presence of clinical signs (fast breathing, chest indrawing, and general danger signs), and on applying appropriate treatment according to the severity of the disease. Children between 2 and 59 months of age with cough and cold were classified and treated accordingly:

- “No pneumonia” when the child does not present fast breathing nor chest indrawing, requiring supportive treatment only;
- “Pneumonia” when the child presents fast breathing, requiring oral antibiotic treatment for five days at home;
- “Severe pneumonia” when the child presents with chest indrawing (with or without fast breathing), requiring referral to hospital for parenteral antibiotherapy;
- “Severe pneumonia or very severe disease” when the child presents any general danger signs, requiring the first dose of oral antibiotic on the site of diagnosis and urgent referral to hospital for parenteral antibiotherapy and further evaluation and management.

These WHO guidelines on pneumonia classification and management were broadly adopted (34,35). The main objective of this strategy was to implement a clinical case definition that is sensitive; meaning a clinical case definition that does not miss the

diagnosis of pneumonia but detects most pneumonia cases for antibiotherapy and hospital management, to achieve the ultimate goal of decreasing child mortality. A systematic review evaluated the impact of using the WHO definition of non-severe pneumonia for reducing mortality (36). From nine community-based studies, the review authors found that using the WHO case-management approach reduced the total mortality by 20% (95% confidence interval [CI] 11 to 28) in infants, and by 24% (95% CI: 14 to 33) in children under five years of age.

The counterpart of the high sensitivity is the low specificity and low negative predictive value of the definition, associated with high false-positive cases. This means that many children would be classified and managed as having pneumonia, while they actually suffer from another disease. This leads to overtreatment with antibiotics and increased risk of antimicrobial resistance. In addition, the accurate diagnosis of other severe diseases such as malaria, severe anaemia or congenital heart diseases is missed, leading to inappropriate management and increased associated mortality.

Since the 1990 WHO guidance, new evidence has emerged, and case definitions were revised (35). The revised recommendations comprise simplification in the classification of pneumonia severity and changes in the choice of first-line antibiotics (Figure 5). Children between 2 and 59 months of age with cough and cold were classified as having:

- “No pneumonia” when the child does not present fast breathing nor chest indrawing;
- “Pneumonia” when the child presents with fast breathing, chest indrawing, or both, requiring home therapy with oral amoxicillin;
- “Severe pneumonia” when the child presents pneumonia with any general danger sign, requiring referral to a higher-level healthcare centre and parenteral antibiotherapy.

These definitions were incorporated into the second edition of the WHO *Pocket book of hospital care for children* (37).

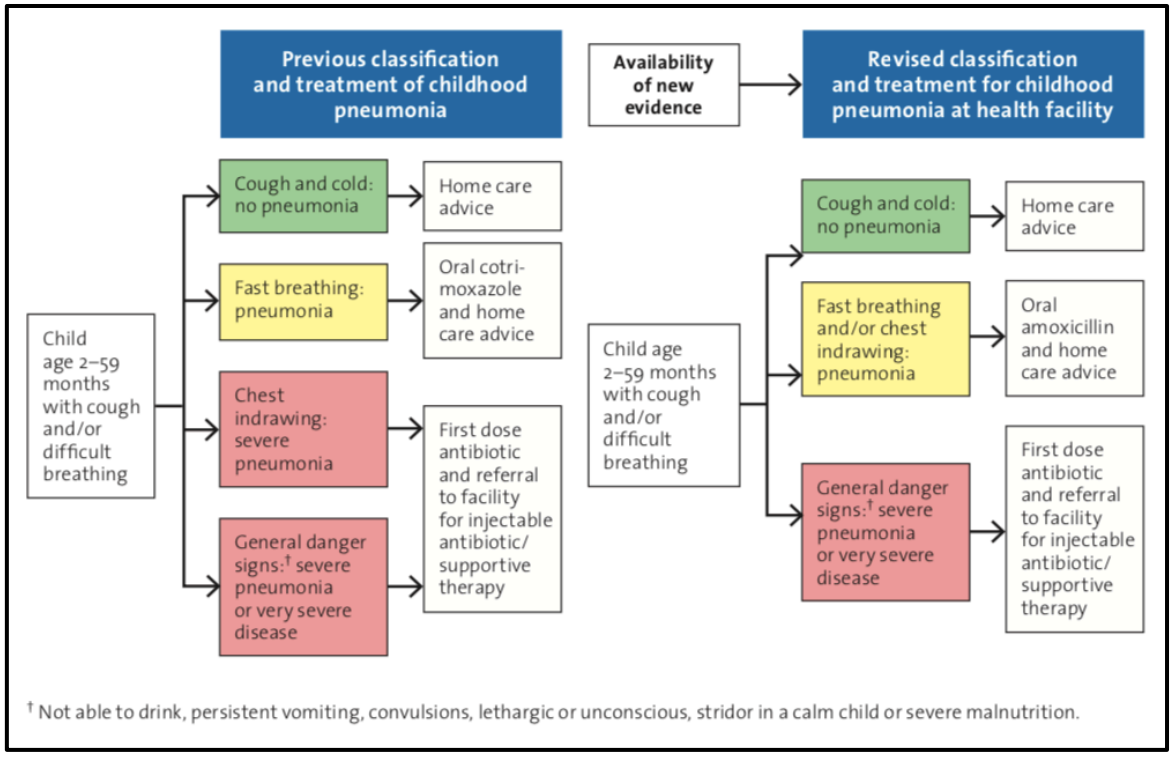


Figure 5. Comparison of previous and revised classification and treatment of childhood pneumonia at health facility.

Source: Revised WHO classification and treatment of childhood pneumonia at health facilities, 2014 (35).

These WHO clinical case definitions of childhood pneumonia apply to children aged between 2 and 59 months. Infants younger than two months present pneumonia with a larger variety of symptoms and signs than older children, and require specific management (38,39).

Other clinical case definitions

The term ARI is commonly used and implies a broad concept including both upper and lower respiratory tract infections (LRTI). Influenza, measles, diphtheria, pertussis, sinusitis, acute otitis media, nasopharyngitis, tonsillitis, epiglottitis, laryngitis, tracheitis, acute bronchitis, bronchiolitis, and pneumonia are all infections included under the umbrella of this broad term (13).

LRTI or acute lower respiratory infections (ALRI) include infection of the lung alveoli, which is pneumonia, and infection of the lower airways such as acute bronchiolitis, acute bronchitis, and whooping cough (14,40). In practice, LRTI and ALRI are often used as equivalent to the WHO-defined clinical pneumonia, and illustrate the limitations of the

WHO clinical definition in reliably distinguishing between pneumonia and bronchiolitis (41).

Pneumonia aetiology in children

Multiple infectious agents can cause pneumonia, including viruses, bacteria, and fungi. It is essential to identify these aetiological agents. At an individual level, it provides guidance for appropriate treatment (whether the child requires antibiotherapy). At a public health and research level, it gives valuable information for assessing the effectiveness of approved vaccines and helps to determine targets for the development of new vaccines.

Viruses are the main cause of pneumonia in children under the age of five years, with the respiratory syncytial virus (RSV) globally identified as the most common pathogen in this age group (42–47). Other common viral causes of pneumonia include influenza viruses, parainfluenza viruses, human rhinovirus, and human metapneumovirus (41,43,45,48).

Regarding bacterial community-acquired pneumonia, *Streptococcus pneumoniae* is the most common causative pathogen in children (46,48,49). Together with pneumonia caused by *Haemophilus influenzae* type b, those cases can nowadays be prevented with highly-effective conjugate vaccines. Their local incidence has dramatically decreased in settings where vaccines against them have been adequately implemented (50). *Staphylococcus aureus* and *Streptococcus pyogenes* are less common causes of bacterial pneumonia in children, but are often associated with severe disease (45,51,52). In some settings, particularly in sub-Saharan Africa, non-typhoidal Salmonella may also cause a considerable number of cases (52).

Mycoplasma pneumoniae and *Chlamydia pneumoniae* constitute a significant proportion of causative agents of bacterial pneumonia. Although they are more commonly detected in children older than five years of age, they are not negligible in younger children (43,45,53).

Hospital-acquired pneumonia is caused by different microorganisms than community-acquired pneumonia, with gram-negative bacteria such as *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* being significant causal pathogens (31). Other pathogens, such

as *Bordetella pertussis* and *Legionella pneumophila*, may also cause clinical episodes often indistinguishable from more 'classical' bacterial pneumonia cases.

Characterizing the aetiology of pneumonia is challenging and still not well understood for several reasons. First, the causal attribution of pathogens in the aetiology of pneumonia is unclear, and pathogens identified from nasopharyngeal samples might not infer causality of infection in the lungs. The PERCH study, a comprehensive case-control study conducted in several sites across sub-Saharan Africa and South-East Asia to determine the aetiology of childhood pneumonia, provided great insight in the understanding of causality (43). Overall, 98.9% of cases (children aged 1-59 months hospitalized with WHO-defined severe pneumonia) and 98.0% of controls (age-group-matched children in communities) had at least one pathogen detected by polymerase chain reaction (PCR) in the nasopharyngeal or oropharyngeal specimens. Furthermore, there was no clear distinction in prevalence of pathogens between cases and controls, except for a few pathogens (43). Therefore, a pathogen identified by molecular methods in the nasopharynx might be a coloniser in an asymptomatic child, residual nucleic acid from previous infection, or a causative pathogen. How to discern these from one another remains unclear (42,43,54,55).

Second, co-infections are a common finding in pneumonia aetiology studies, which also affects the interpretation of causality (31,42,51,56). In a recent study, one-third of pneumonia cases were caused by mixed infections, and both viral and bacterial pathogens were identified (31,51). The PERCH study also confirmed this finding (43). The interactions between organisms and the role of multiple potential pathogens in childhood pneumonia are not well-understood.

Third, pneumonia aetiology studies conducted in different settings have reported contradictory findings (43). This limits the generalizability of findings to other countries and regions, and contributes to the difficulties in understanding the key aetiological pathogens that cause childhood pneumonia.

Diagnostic approach

Diagnosing a child with pneumonia is challenging. The limitations of using clinical criteria for the diagnosis of pneumonia in children are described above. Following the

implementation of the WHO clinical definitions in the 1990s, several studies were conducted in LMICs to assess the reliability of the use of the two signs recommended to use to diagnose pneumonia: increased respiratory rate and chest indrawing. Mulholland *et al* (1992) found increased respiratory rate or chest indrawing to have a sensitivity of 77% to 81% and a specificity of 77% to 80% to diagnose pneumonia when applied by a paediatrician (57). Lower specificity was found when these signs were applied by a health worker. Simoes *et al* (1992) found similar findings when these signs were applied by nurses, with a sensitivity of 71% to 83% and specificity of 84% to 85% (58).

More recently, two systematic reviews assessed the diagnostic accuracy of clinical signs and symptoms to identify pneumonia in children (59,60). Both reviews included studies from high-income countries and LMICs, although most included studies were conducted in LMICs. Both reviews focused on children under five years of age presenting in ambulatory or hospital care and used chest radiography as a reference standard for the diagnosis of pneumonia. The two signs used as criteria in the WHO clinical case definition showed poor diagnostic accuracy. Age-related increased respiratory rate showed a pooled sensitivity of 54-62% and specificity of 59-64%, with broad confidence intervals (59,60). Chest retractions or indrawing showed a pooled sensitivity of 38-48% and specificity of 72-80%, with broad confidence intervals (59,60). The diagnostic accuracy of other signs and symptoms were assessed, but the authors concluded that no single clinical characteristic was sufficient on its own to reliably diagnose pneumonia in children (59,60).

The WHO definition for pneumonia was developed for case management purposes: whether the child requires antibiotics, hospitalization, or both. However, the ideal approach for pneumonia management would be to identify rapidly the causative pathogen so that the child can receive a tailored treatment, including appropriate antibiotherapy when needed. Yet, to date, there is no ideal gold standard to distinguish between bacterial and viral pneumonia. In 2001, the WHO identified chest radiography as the best available method for diagnosing pneumonia (61). The association between a consolidation or pleural effusion in a chest radiography and bacterial pneumonia is commonly used, especially for pneumococcal pneumonia, and radiography has been commonly used as the gold standard for pneumonia vaccine trials (62). However, and despite widespread use, radiological findings do not adequately correlate with

pneumonia aetiology. Radiological consolidations (commonly associated with bacterial aetiology) are also seen in children with viral pneumonia, and diffuse infiltrates (commonly associated with viral aetiology) can also be caused by bacteria (48,63–65).

Therefore, to date, no clinical or radiological criteria allow accurate distinction between bacterial and viral aetiology for pneumonia. Furthermore, microbiological identification of causative pathogens is complex. In addition to the above-mentioned challenges for defining causality, it is difficult to obtain a representative sample from the lower respiratory tract for microbiological testing, especially from children. Lung aspiration and bronchoalveolar lavage are invasive procedures, and sputum is difficult to obtain from children. Pleural fluid can only be collected from a small proportion of children presenting with pleural effusion, similarly through an invasive procedure. Other samples, such as blood and nasopharyngeal aspiration, are used to identify the causal agents of pneumonia but with limitations (51). The interpretation of causality from pathogens identified in the nasopharynx is complex (see 'Pneumonia aetiology in children' above), and the yield of blood culture in children with pneumonia is low (43).

Host-response biomarkers

To address the lack of a gold standard to accurately diagnose pneumonia, innovative approaches are needed. A point-of-care tool fulfilling the WHO ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable to end-users) criteria to diagnose pneumonia and to identify bacterial aetiology, would help decision-making to start or discontinue antibiotics in children with clinical pneumonia (66,67).

On the other hand, identification of children with bacterial pneumonia might not be sufficient to reduce the high mortality associated with this disease. Ginsburg *et al* (2019) found that most children with non-severe pneumonia improved without the need for antibiotics and that only a minority progressed to severe disease (68). Therefore, a point-of-care tool for the identification of children at risk of progressing to severe illness from those progressing to a self-limited disease is likely to present major benefits (68–71). This would help to identify children for early referral, to prioritize children at risk for early management, and to determine the need for admission and intensive care. Several prognostic scores have been developed for pneumonia and other severe diseases, such

as sepsis and malaria (72–74). These scores are based on clinical characteristics and simple laboratory testing. For pneumonia, these prognostic scores lack of sensitivity and specificity, and have not been widely validated (75).

Host-response biomarkers are mediators of the endothelial, immunological, and inflammatory response pathways, and are activated in the pathogenesis of life-threatening infections irrespective of their aetiology (70,76). They can be measured and quantified from the patient's blood. Compared to the assessment of clinical signs, such as the respiratory rate or chest retractions, which require trained healthcare providers, biomarkers present the benefit of objectivity, accuracy, and reproducibility. The most investigated inflammatory markers are the C-reactive protein (CRP) and procalcitonin (PCT) (75,77,78). Other inflammatory mediators that have been associated with severe infections include interleukin-6 (IL-6), interleukin-8 (IL-8), soluble triggering receptor expressed on myeloid cells-1 (sTREM-1), soluble tumour necrosis factor receptor 1 (sTNFR1), and chitinase-3-like protein-1 (75,79). Endothelial and immunological factors that contribute to the integrity of endothelial function and vascular integrity derive mainly from the angiopoietin (Angpt)-Tie-2 and vascular endothelial growth factor axes, and include Angpt-1, Angpt-2, soluble fms-like tyrosine kinase 1 (sFlt1), soluble vascular adhesion molecule-I, and soluble intercellular adhesion molecule-I (79).

Host-response biomarkers have been increasingly investigated in different clinical areas for pneumonia and other infectious diseases for diagnosis, aetiology, risk stratification and triage decision, severity of illness, and decision making for the initiation, duration, and discontinuation of antibiotic therapy (70). CRP and PCT are commonly used in the diagnosis of infectious diseases in high-income countries (78). The measurement of PCT in children with pneumonia for guiding antibiotic treatment reduces antibiotic use in high-income countries, without negative influence on clinical outcomes (80–82). However, these inflammatory biomarkers are rarely available as a point-of-care tool in resource-constrained settings. Besides, there is insufficient evidence on their diagnostic and prognostic accuracy in the epidemiological context of LMICs with different demographical factors and co-existing diseases such as malaria, HIV, and tuberculosis (69).

Nevertheless, recent works have shown positive findings from LMICs. For example, in Uganda, elevated Angpt-2/Angpt-1 ratio at presentation in children with pneumonia was associated with disease severity and an increased risk of fatal outcome (83). In Tanzania also, high levels of sTREM-1 at presentation in patients with fever were associated with an increased risk of fatal outcome (79). The combination of several biomarkers from different pathophysiological pathways or the combination of biomarkers with simple clinical scores can increase their diagnostic and prognostic performance (75,77,79). Although further investigation is needed to validate their diagnostic and prognostic value, these emerging biomarkers seem promising to contribute to reducing the morbimortality associated with childhood pneumonia.

Risk factors and preventive strategies

Several risk factors have been associated with childhood pneumonia, and it has been estimated that risk factors were responsible for 93.4% of the global mortality attributed to LRI in children (9). Risk factors include risks that predispose children to contract the disease (namely preventive risk factors) and risks that increase the probability of fatal outcome among those that have the disease (namely protective risk factors) (9,27). Various public health interventions addressing selected risk factors are effective in preventing and treating childhood pneumonia, leading to decreased morbidity and mortality (9,27,84). These factors and strategies are well-identified and are included in the Pneumonia Wheel (Figures 3 and 4).

Host-related risk factors

Child malnutrition is a well-defined protective risk factor, and includes wasting (low weight-for-height), stunting (low height-for-age), and being underweight (weight-for-age) (6,9,85) (Figure 6). Promotion of exclusive breastfeeding and other actions to reduce wasting have a major role in preventing pneumonia deaths (27,86).

Figure 6. Child with malnutrition, admitted at JDWNRH. Source: personal

Zinc deficiency has been identified as a preventive and protective risk factor (9). However, while zinc supplementation can help to prevent pneumonia (decrease in the incidence) (87), there is insufficient evidence supporting zinc supplementation as an adjunctive treatment to improve outcomes in children with pneumonia (time to recovery, time to discharge, readmission, and mortality) (88). Prematurity and low birth weight are other risk factors associated with increased mortality. Promotion and provision of good antenatal care services are effective strategies to reduce these risk factors and deaths associated with pneumonia (9,85). Finally co-morbidities, including infection with HIV, measles, malaria, diarrhoea, congenital heart diseases, and bronchopulmonary dysplasia, increase a child's risk of contracting and dying of pneumonia (85).

Environmental risk factors

Environmental factors are key risk factors amenable to prevention that contribute to deaths of children under five years of age from pneumonia and include outdoor air pollution, indoor air pollution (exposure to unprocessed solid fuels), living in crowded households, and inhalation of second-hand smoke (6,9,85,89,90). Handwashing with water and soap is an important intervention that contributes to reduced pneumonia mortality (6,9,91,92).

Maternal- and socioeconomic-related risk factors

Low maternal education and low socioeconomic status have been repeatedly identified as sociodemographic associated with greater risk for pneumonia and deaths from childhood pneumonia (17,85,93–96).

Healthcare-related risk factors

Childhood immunization with existing vaccines is the most effective strategy to prevent death from pneumonia. Immunization has played a major role in reducing child mortality attributable to pneumonia worldwide. It was estimated that immunization prevents two to three million deaths (all-cause deaths) every year (97,98). Vaccines related to the prevention of pneumonia include *Haemophilus influenzae* type b, *Streptococcus pneumoniae*, measles, pertussis, influenza, and tuberculosis. The introduction of measles

and pertussis vaccines contributed largely to a marked reduction in deaths attributed to these diseases (99).

In 2000, *Haemophilus influenzae* type b and *Streptococcus pneumoniae* were responsible for more than 50% of pneumonia deaths among children aged 1 to 59 months (99). Currently, conjugated vaccines exist to protect children against these two bacteria, with considerable impact. According to the Global Burden of Diseases, Injuries, and Risk Factors Study, conducted in 2017, increased coverage for *Haemophilus influenzae* type b and pneumococcal vaccines globally were responsible for 11.4% (*Haemophilus influenzae* type b) and 6.3% (PCV) decreases in LRI mortality among children under five years between 1990 and 2017 (9). The *Haemophilus influenzae* type b conjugate vaccine was first incorporated into national immunization programmes for infants in 1990. In the USA, the incidence of invasive *Haemophilus influenzae* type b disease decreased by more than 99% from 1987 to 1997, with a similar picture in other high-income countries (100). By 2013, the incidence of *Haemophilus influenzae* type b invasive disease decreased by more than 90% in the 184 countries that had included the vaccine in their national immunization programmes (101).

The introduction of PCV is more recent. By December 2019, 145 countries had introduced PCV into their national immunization programmes and 15 additional countries were planning to do so (102). The first PCV included seven serotypes (PCV7) and has been gradually replaced by vaccines covering an increased number of serotypes. Currently, there are two WHO prequalified vaccines that are commonly used: PCV13 (Prevenar 13®, Pfizer) and PCV10 (Synflorix®, GlaxoSmithKline) that include 13 and 10 serotypes respectively (103,104). A third vaccine that also includes 10 serotypes (Pneumosil®, Serum Institute of India) was recently prequalified by WHO in December 2019 (103). The introduction of PCV has substantially reduced both the burden of pneumococcal invasive disease and the rates of nasopharyngeal carriage by serotypes included in the vaccine (49,105–108). In 2015, PCV averted between 6 and 7.5 million pneumococcal disease episodes and saved 14 to 17 million disability-adjusted life years (109). The emergence of serotypes not included in the vaccines has been well-documented in high-income countries but little is known in LMICs (110–112). Surveillance data at a national level is important to identify serotype replacement and to assess most prevalent serotypes circulating in the population.

Other health-care related risk factors

Care seeking, good access to care, adequate case management, use of simple and standardized guidelines, adherence to treatment protocols, and timely referral to a superior level of health facilities contribute to reducing risks of pneumonia deaths (27,85,113,114).

Pneumonia in Bhutan

Geographic and sociodemographic profile of Bhutan

The Kingdom of Bhutan is a small landlocked country (38,394 km^2) located in the Eastern Himalayas, which is bordered by two giants, India and China (Figure 7). It is internationally known as the 'Land of the Thunder Dragon', or the country that established the concept of Gross National Happiness (Figure 8). Bhutan is currently classified as a lower-middle-income country (115).

Figure 8. Flag of Bhutan
Source: personal

Figure 7. Geographical location of Bhutan. Source: google maps (https://www.google.com/maps)

Elevation rises from around 100 m in the South to more than 7500 m in the North, with the capital, Thimphu, standing at 2334 m (116) (Figure 9). Over 70% of the country is

covered by forest, and most of the land is mountainous and rugged, which constitutes a real challenge for communication, road constructions, and access to health services. The climate varies with altitude, from tropical in the Southern plains to alpine with very cold winters in the North (117).

Figure 9. The Thimphu valley, the capital of Bhutan. Source: personal.

According to the most recent population and housing census, Bhutan had a total population of 779,666 in 2017, with 138,736 residents in the Thimphu *Dzongkhag* (district), which accounted for the highest population size among the 20 *Dzongkhags* in the country (12,118). The median age of the population was 28 years, and children under 15 years of age accounted for 28% of the population (119). Life expectancy at birth was 70.6 years in 2016 (120). Two-thirds of the population live in rural areas, but only 16% of the population in the Thimphu Dzongkhag lived in rural areas in 2017 (12,119).

The Bhutanese health system

Bhutan is a signatory to the declarations of Alma Ata on Primary Health Care (121). Since the 1970s, essential health services in both modern and traditional medicines are free for Bhutanese citizens based on a primary healthcare approach, as enshrined in the Constitution of Bhutan in 2008 (121,122) (Figure 10).

Figure 10. Directory panel at the National Traditional Medicine Hospital at Thimphu. Source: personal.

Since the 1950s and since the first fully graduated Bhutanese doctor returned to Bhutan in 1954, this country has made great strides in providing healthcare services to its people. The country has developed an efficient healthcare system to reach the most remote areas despite the geographical difficulties: national and regional hospitals at a tertiary level; district hospitals at a secondary level; and basic health units, sub-posts, and outreach clinics at a primary level (121) (Figure 11).

Figure 11. Basic health unit at Dangochang (Altitude 4080m). Source: personal.

National preventive strategies for childhood pneumonia

In 2018 in Bhutan, the under-five years mortality rate was estimated at 29.7 per 1000 live births, which corresponded to 385,000 deaths in this age group (123). This was lower than the under-five mortality rate globally (38.6 per 1000 live births) or in the South-East Asian Region (33.6 per 1000 live births) for the same year. In Bhutan, the infant mortality rate was 24.8 per 1000 live births (28.9 per 1000 live births globally) with 321,000 infant deaths (123).

The implementation of national strategies that target the main causes of under-five deaths is crucial for accelerating the decline in mortality rate in this age group. Activities focusing on the control of ARIs were initiated in 1987. The ARI Programme was established in 1992 with the primary intention of reducing under-five deaths due to pneumonia, and the WHO Standard Case Management Protocol for ARI was introduced in 1994 (118). Later, in 2000, Bhutan adopted and implemented the WHO-recommended IMCI strategy to address the major causes of deaths in under-five children. Health workers were trained across the country and it was estimated that the coverage of IMCI implementation reached 100% in all the districts of the country by 2011 (124) (Figure 12).

Figure 12. Health care worker in remote areas of Eastern Bhutan, ensuring implementation of national strategies such as IMCI and education across the country. Source: personal.

In the 2012 report dedicated to the identification of under-five deaths in health facilities in Bhutan, the Ministry of Health supported by UNICEF recognised the need of expanding the role of village health workers to provide antibiotics for pneumonia to help reduce child mortality attributable to sepsis and pneumonia (125). Mathematical models were developed to estimate the impact of different interventions for reducing the under-five mortality rate and to identify priority child health interventions that would result in maximum impact. It was estimated that oral antibiotics for the management of pneumonia would contribute to preventing 16% of additional deaths in under-five children (125).

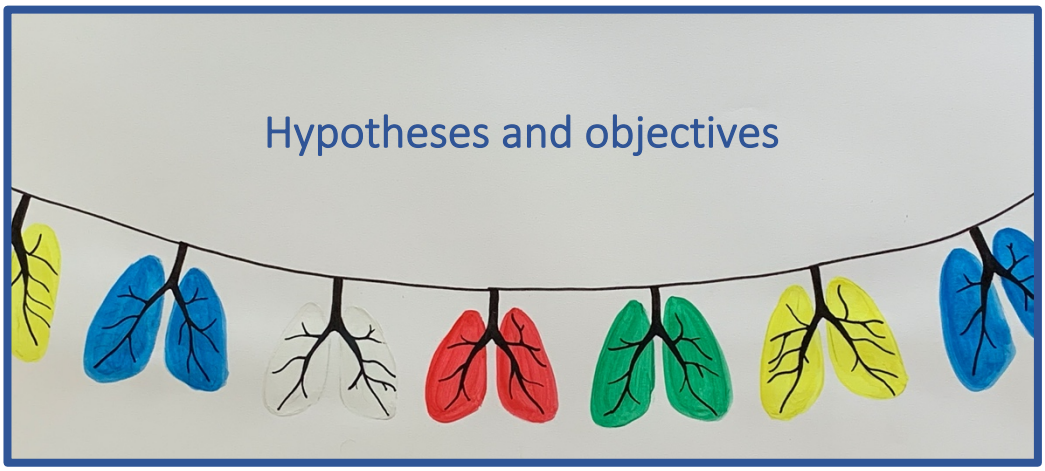
Bhutan launched the EPI in 1979, and universal childhood immunization was achieved in 1990 (126). Since then, vaccines against 14 antigens have been included in the EPI (127,128). The measles vaccine was introduced in 2006, the conjugate *Haemophilus influenzae* type b vaccine is routinely administered since 2011, and PCV13 was introduced in the childhood immunization schedule in January 2019 (127,128). National immunization coverage has been maintained over 90% since 2005, and over 95% since 2016 (121,127).

The burden of acute respiratory infections in Bhutan

Despite the aforementioned efforts, ARI seem to remain a major public health challenge in Bhutan. ARI have been rated as the principal diseases affecting the Bhutanese population since 2003 and remain one of the ten highest cause of mortality since 2010 (all age groups) (118,129,130). Aligned with global data, pneumonia was estimated to be responsible for 15% and 27% of under-five and post-neonatal deaths, respectively (131,132). In 2017, 3.6 deaths per 1000 live births were attributed to ALRI in children between 1 and 59 months of age (123).

Although the incidence of childhood pneumonia has been recorded with a decreasing trend in the last ten years, the number of outpatient visits and hospitalizations attributed to pneumonia constitutes a considerable burden to the health system (118,133).

Hypotheses and objectives



Hypotheses

1. The dearth of epidemiological, clinical, and aetiological data hampers a comprehensive characterization of childhood pneumonia in Bhutan.
2. Childhood pneumonia remains a major public health problem in Bhutan. This disease causes a high mortality rate and is responsible for a high proportion of paediatric hospitalizations.
3. Bacterial infection accounts for around 25% of cases of children admitted with pneumonia, which translates into radiological endpoint pneumonia. The proportion of positive blood cultures is much lower, around 5%. The main bacterial pathogen that causes pneumonia in children is *Streptococcus pneumoniae*.
4. Over 75% of children present at least one viral pathogen in their nasopharynx. The most common viruses detected in children hospitalized with pneumonia are RSV and rhinovirus.
5. Most pneumococcal serotypes that circulate among children hospitalized with pneumonia are preventable by PCV.
6. Clinical characteristics are poor at predicting radiological outcomes. Host-response biomarkers levels are higher in children with radiological endpoint pneumonia.
7. Sociodemographic characteristics and access to care are associated with prognosis of the disease.
8. Host-response biomarkers present promising performance to identify children at risk of poor prognosis.

General objectives

To describe the epidemiology, aetiology, clinical presentation, and radiological findings of WHO-defined clinical pneumonia among Bhutanese children aged 2 to 59 months and to assess the prognostic role of host-response biomarkers to help clinicians and policymakers in decision-making on prevention and management strategies of childhood pneumonia in Bhutan.

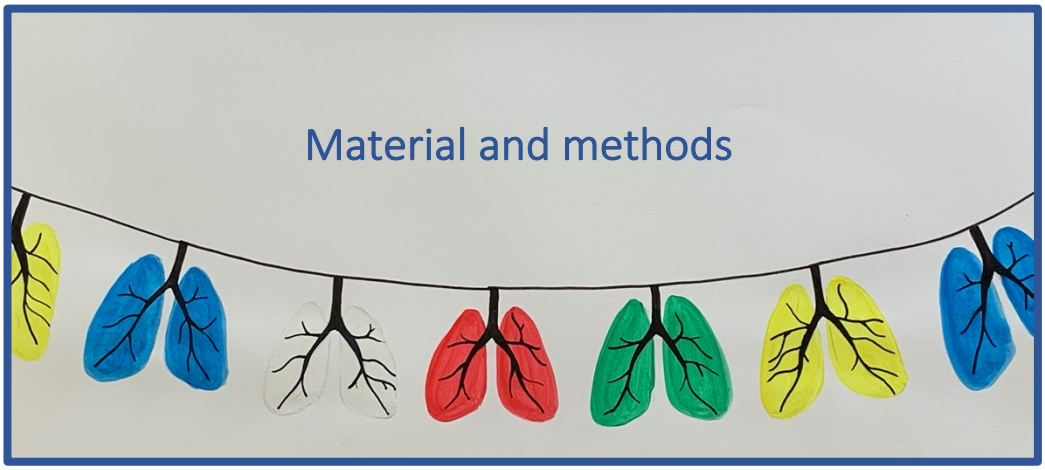
Specific objectives

1. To understand the challenges of conducting clinical research in Bhutan.
2. To identify the knowledge gaps around childhood pneumonia in Bhutan, with regards to the burden of the disease, the aetiology, clinical and radiological characteristics, associated health determinants, and national surveillance systems and preventive strategies.
3. To determine the number of children between 2 and 59 months of age admitted at Jigme Dorji Wangchuck National Referral Hospital (JDWNRH) with WHO-defined clinical pneumonia and severe pneumonia during a one-year period.
4. To describe the demographic and clinical characteristics of children between 2 and 59 months of age admitted with clinical pneumonia, and their progression until discharge.
5. To determine the main pathogens causing pneumonia in children between 2 and 59 months of age that require hospitalization.
6. To determine the prevalence of pneumococcal nasopharyngeal carriage (PNC) and to identify the pneumococcal serotypes circulating among Bhutanese children admitted with clinical pneumonia before the introduction of PCV in the country.
7. To describe the association between PNC and viral co-infections among children admitted with clinical pneumonia.
8. To describe radiological findings of children between 2 and 59 months of age admitted with clinical pneumonia, and to explore differences in radiological

outcomes by demographic characteristics, aetiology, clinical characteristics, and host-response biomarker levels.

9. To evaluate the utility of aetiological biomarkers in discerning between bacterial and viral pneumonia.
10. To assess the association between sociodemographic characteristics and access to care (time to access healthcare facilities) with pneumonia prognosis and mortality.
11. To evaluate the performance of inflammatory, immune, and endothelial activation markers alone, combined, or in addition to clinical scoring scales to risk-stratify children hospitalized with pneumonia and predict their outcome.

Material and methods



Study design

The Respiratory Infections in Bhutanese Children (RIBhuC) study was a hospital-based study conducted prospectively over 12 consecutive months from 1 July 2017 to 30 June 2018 at the JDWNRH in Thimphu.

Study area and population

The study was conducted in Thimphu, the capital of Bhutan. The geographic and sociodemographic profile of the country has been described in the introduction of this thesis. Thimphu is located in the western region of the country, standing at an elevation between 2248 and 2648 m (116,134). Temperatures range from -3ºC in winter to 22ºC in summer, coinciding with the monsoon that brings precipitations of around 350mm in July (117). The city extends over an area of 15 km long and 3 km wide (134) (Figure 13). The 2017 census registered 138,736 residents in the Thimphu *Dzongkhag* (district), of which 114,551 were in the city of Thimphu (12). The child population (up to the age of 15 years) accounted for 23.7% of the overall population in the district (12).

Figure 13. JDWNRH (red circle) in Thimphu. Source: personal.

JDWNRH is the apex tertiary care hospital in Bhutan. It constitutes the main health centre for children in the capital and is the centre of reference for the entire country. The hospital has 38 paediatrics beds: 29 in the paediatric ward, four in the paediatric high dependency unit, and five in the paediatric intensive care unit (PICU). Children are admitted to the paediatric department up to the age of 12 years, and there is a separate neonatal unit. Every year, approximately 1200 children are hospitalized to the paediatric department. Children are admitted from the outpatient department, the emergency department, or directly to the ward when referred from other centres (Figure 14).

Figure 14. The paediatric outpatient department at JDWNRH. Source: personal.

Selection criteria

All children aged 2 to 59 months admitted at JDWNRH with a diagnosis of pneumonia or severe pneumonia according to the WHO clinical definitions were eligible for recruitment (35) (Box 1). The advantage of using broadly-adopted WHO definitions is to compare our study findings with those that already exist in the literature on childhood pneumonia. We excluded newborns and infants aged less than 2 months because they have different characteristics from older infants and children. We also excluded children hospitalized in the preceding seven days in order to exclude hospital-acquired pneumonia, children with evidence of a foreign body in the respiratory tract, and those for whom the principal reason for hospital admission was a non-respiratory illness or a condition that was not

caused by a respiratory illness. We did not exclude children known to have underlying pulmonary tuberculosis or other chronic respiratory diseases.

***Pneumonia*:**

- History of cough or reported breathing difficulty, AND
- Increased respiratory rate OR chest indrawing.

***Severe pneumonia*:**

- History of cough or reported breathing difficulty

AND at least one of the following:

- Oxygen saturation < 90% or central cyanosis,
- Severe respiratory distress (e.g., grunting, very severe chest indrawing),
- Signs of pneumonia with a general danger sign: inability to breastfeed or drink, lethargy or reduced level of consciousness, convulsions.

Increased respiratory rate is defined according to age as follows:

- Respiratory rate ≥ 50 breaths per minute in children aged 2 to 11 months.
- Respiratory rate ≥ 40 breaths per minute in children aged 12 to 59 months.

Box 1. WHO definitions of pneumonia and severe pneumonia used as inclusion criteria (35)**.**

Recruitment process and data collection

From 1 July 2017 to 30 June 2018, the study co-investigators identified potential participants with the collaboration of the paediatricians, paediatrics residents, and nurses, from the outpatient department, the emergency room, the PICU, and the paediatric ward. Case screening was done 24 hours per day and seven days per week during the one-year period. If an eligible participant was missed during the night, we assessed the child for recruitment the following morning. We recruited all eligible children provided parents or caregivers consented in writing to participate in the study.

On admission, we assigned a study identification number to each participant and we performed a comprehensive physical examination including anthropometric measurements (weight and height) and vital signs. We collected demographic and clinical data from medical records (including the child health book for data such as prematurity and immunization status) and through family interviews. We conducted interviews in English or Dzongkha (the official and national language of Bhutan). For parents who did

not understand English or Dzongkha, but understood other languages such as Sharchop, Nepalese, or Hindi, interviewers fluent in the given language collected the data.

We collected blood samples, nasal swab, and nasopharyngeal washing (NPW) upon enrolment or as soon as possible after enrolment and before the initiation of antibiotics. Prior to the start of the recruitment period, the principal investigator informed all the nurses on the recruitment process and trained them on how to collect nasal swab and NPW, following standard procedures (Figure 15).

Figure 15. Nurses working in the PICU (left) and the paediatric ward (right) who assisted with sample collection from participants. Source: personal.

When a child was identified for recruitment, but blood had been already collected for clinical management, no further blood sampling was conducted. However, if another blood analysis was clinically indicated, we obtained additional blood for the specific purposes of the study. We collected and analysed the NPW samples solely for research purposes, while the remaining data and samples collected were used for clinical management. We carefully recorded the date and time of sample collection for all children. We performed an antero-posterior chest radiography within 24 hours of admission using either a digital machine (model IDC DR. 1590x 3C, Eureka) or an analogue one (model KH/HD/STANDIX-31667, Siemens), depending on availability. We collected fluid from pleural effusion when clinically indicated, and additional information of potential diagnostic interest available throughout the duration of hospitalization, such as computerized thoracic scans or cerebrospinal fluid investigation. Children were clinically managed and discharged as per the criteria of the treating paediatricians and were followed-up by one of the study investigators in terms of outcome determination. Discharge diagnoses were coded using the International Classification of Diseases, 10th Revision (135).

We collected all data using standardized questionnaires built into a tablet-based data capturing system, specifically designed for the study. We presented the recruitment algorithm used throughout the study in Figure 16 and we summarized the data collection process in Figure 17.

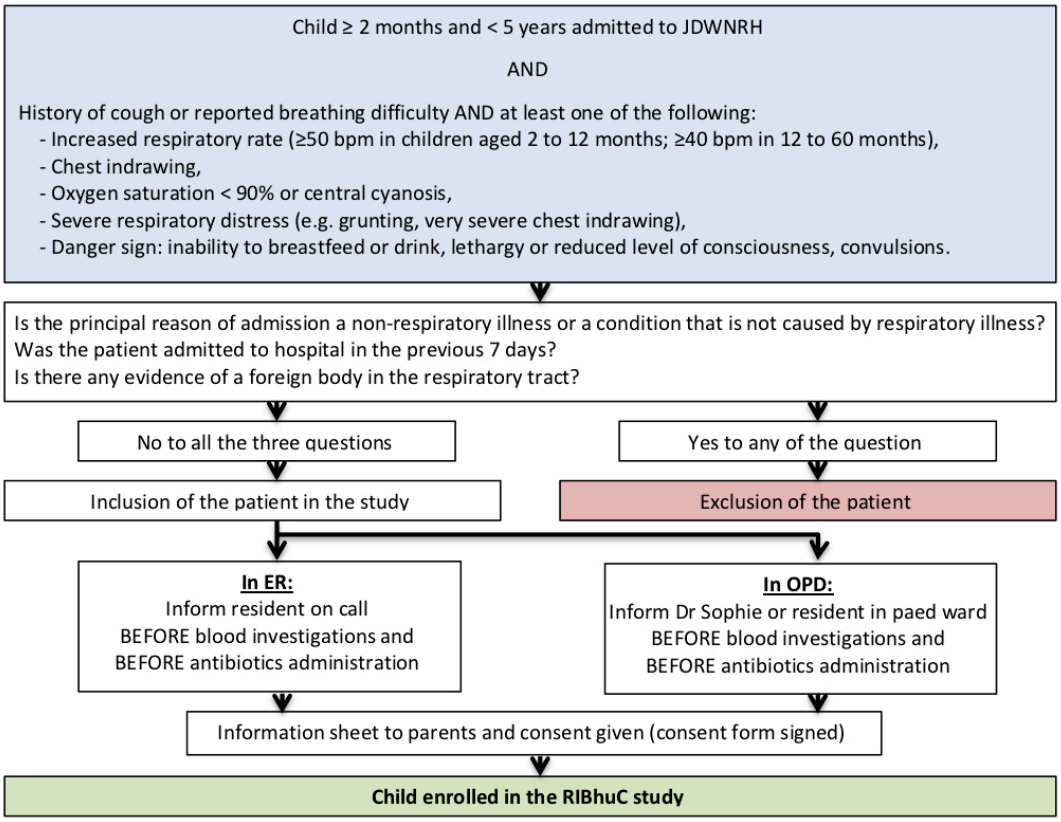
Chest radiograph interpretation

We followed the WHO protocol used in clinical trials of PCV for the interpretation of chest radiographs (62). Readers first judged the quality of the film as uninterpretable or interpretable (the latter stratified as suboptimal or adequate) and then assessed all interpretable radiographs. Significant pathology was defined as the presence of consolidation, other infiltrates, and/or pleural effusion. End-point radiologically-confirmed pneumonia was defined as consolidation, pleural effusion, or both on any hemithorax. Two study paediatricians independently interpreted the radiographs. In the study protocol, we planned an additional external quality control measure, whereby a paediatric radiologist would read a random sample of 10% of the chest radiographs. As we found substantial discordance among the two primary readers, the paediatric radiologist read again all the chest radiographs using the WHO criteria. We accepted this last reading as the final interpretation for analysis. All readers were blinded to clinical and laboratory findings.

Evaluation of the epidemiology, aetiology and clinical presentation of community-acquired pneumonia among children under five years of age admitted to the JDW National Referral Hospital in Thimphu, Bhutan

RIBhuC Study

RECRUITMENT ALGORITHM



To be done/collected	In ER	In OPD	In PICU or paed ward
Anthropometry	No	Yes, by nursing staff	Yes, if not yet done
Vital signs	Yes, by nursing staff	Yes, by nursing staff	Yes, if not yet done
Blood sample BEFORE antibiotics, following table in the lab summary sheet	Yes, by nursing staff	Yes, by nursing staff	Yes, if not yet done
Fill lab summary sheet	Yes, by resident	Yes, by Dr Sophie*	Yes, if not yet done
Child examination	Yes, by resident	Yes, by Dr Sophie*	Yes, if not yet done
Nasal swab BEFORE antibiotics	No	No	Yes, by nursing staff
Nasopharyngeal washing BEFORE antibiotics	No	No	Yes, by nursing staff
Chest radiography (digitized)	Yes	Yes	Yes, if not done yet
Questionnaire	No	No	Yes, by study investigator**
*or resident from paed ward ** Dr Sophie, Dr Dinesh, Dr Tashi T			

Contact persons: Dr Sophie Jullien (paediatrician), **Dr Dinesh Pradhan** (paed resident), **Dr Tashi Tshering,** (paed resident)

Figure 16. Recruitment algorithm

Abbreviations: bpm: breaths per minute; ER: emergency room; JDWNRH: Jigme Dorji Wangchuck National Referral Hospital; OPD: outpatient department; PICU: paediatric intensive care unit.

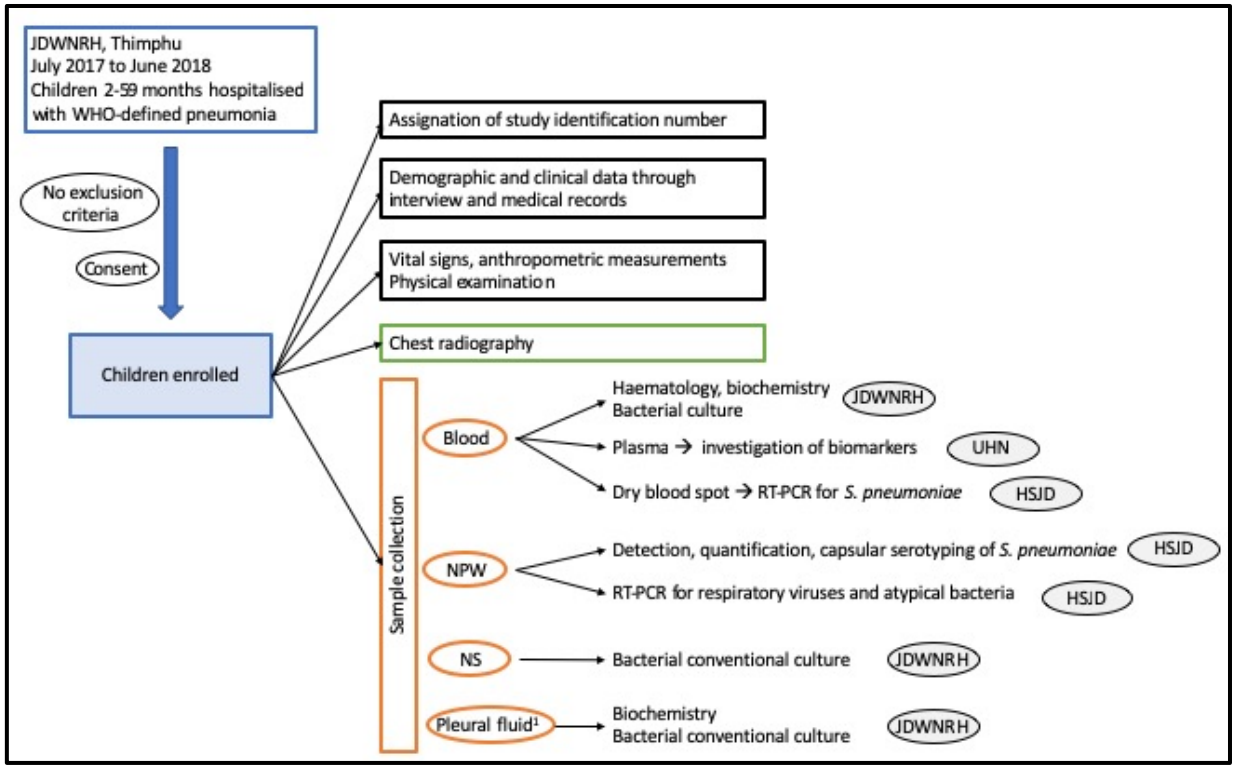


Figure 17. Summary of data collected and laboratory testing

Abbreviations: HSJD: Hospital Sant Joan de Déu (Barcelona, Spain); JDWNRH: Jigme Dorji Wangchuck National Referral Hospital (Thimphu, Bhutan); NPW: nasopharyngeal washing; NS: nasal swab; UHN: University Health Network (Toronto, Canada)
[1]Pleural fluid was collected only if clinically indicated.

Biological testing and laboratory methods

We summarized the specimen collection and laboratory testing in Figure 17.

Blood samples

We collected blood under aseptic conditions. Blood samples for haematology, biochemistry, and bacterial culture were processed and analysed at JDWNRH following the usual circuit as for any other patient admitted. We collected blood in paediatric blood culture bottles (BACT/ALERT PF, BioMérieux) according to manufacturer guidelines. We incubated the blood cultures and continuously monitored for growth using the automated blood culture system BacT/ALERT (BioMérieux). We identified bacterial isolates from positive blood cultures by colony morphology, growth requirements, and basic biochemical tests according to the standard of care at JDWNRH. We determined antibiotic susceptibility using disk diffusion using zone size interpretations from the Clinical Laboratory Standard Institute (136) (Figure 18).

Figure 18. Ragunath Sharma (co-investigator in charge of laboratory testing at JDWNRH), Sophie Jullien, and Quique Bassat (from left to right) at JDWNRH. Source: personal.

Host-response biomarkers

We collected additional blood (2mL) in an ethylenediaminetetraacetic acid (EDTA) tube for the investigation of host-response biomarkers. We centrifugated the samples at 3000 g for three minutes, and we separated and stored the plasma at -80ºC until the shipment to the Sandra Rotman Centre for Global Health in Toronto, Canada.

We quantified CRP by enzyme-linked immunosorbent assay and we measured the plasma concentration of PCT and six endothelial and immune activation biomarkers using a multiplex Luminex platform with reagents from R&D Systems (Minneapolis, MN): IL-6, IL-8, Angpt-2, sFlt1, sTREM-1, and sTNFR1 (137). We assigned to biomarker concentrations outside of the detection limits value of one-third below or above the lowest or highest limit in the standard curve, respectively.

Dry blood spot

We collected two drops of blood on filter paper, which were shipped Hospital Sant Joan de Déu in Barcelona, Spain, for identification of *Streptococcus pneumoniae* (LytA gene) by real-time polymerase chain reaction (RT-PCR).

Nasal swabs

We collected nasal swabs (AMIES transport swab) by introducing a sterile swab into one nostril, and then rotating, removing, and directly placing the swab on the transport media. Samples were cultured on 5% sheep blood and sheep chocolate agar for respiratory pathogens, at the microbiology laboratories of JDWNRH. We isolated *Staphylococcus aureus* from two samples. The technique was not adequate to isolate *Streptococcus pneumoniae*, and these findings were not included in this thesis.

Nasopharyngeal sample collection and storage

We collected respiratory secretions through NPW, using 1 to 3 mL of 0.9% saline solution with a commercial mucus extractor kit (138) (Figure 19). The specimens were sent to microbiology within 30 minutes. They were homogenized, aliquoted, and frozen at -80ºC until shipment to the Hospital Sant Joan de Déu in Barcelona, Spain.

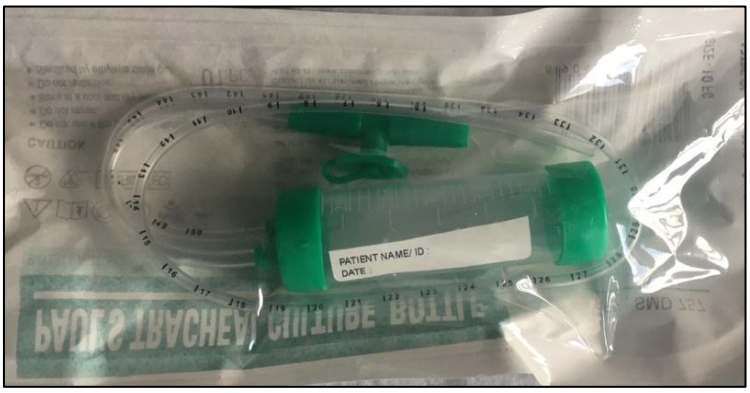


Figure 19. Mucus extractor kit used for NPW. Source: personal.

Pneumococcal detection and capsular typing from respiratory secretions

Respiratory samples were analysed at Hospital Sant Joan de Déu in Barcelona, Spain. We used NucliSENS1 EasyMag1 (bioMérieux, Marcy l'Etoile, France) for extraction of pneumococcal DNA. We performed a RT-PCR targeting the *lytA* gene of *Streptococcus pneumoniae* and the internal control targeting RNaseP of human cells for DNA amplification, using the Applied Biosystems 7500 RT-PCR System (Applied Biosystems,

CA, US) (139). We performed capsular typing of *Streptococcus pneumoniae* in all *lytA* positive samples with a fragment analysis multiplex PCR for distinguishing 40 serotypes (140).

Detection of respiratory viruses and atypical bacteria from respiratory secretions

For identification of respiratory viruses and atypical bacteria, we used the multiplex RT-PCR QIAStat respiratory panel, Qiagen, which includes 17 viral targets (adenovirus, bocavirus, coronavirus 229E/HKU1/NL63/OC43, human metapneumovirus, influenza virus A/B [A subtypes H1N1pdm09, H1, H3], parainfluenza viruses 1/2/3/4, RSV, rhinovirus) and four bacteria (*Bordetella pertussis*, *Chlamydophila pneumophila*, *Legionella pneumophila*, and *Mycoplasma pneumoniae*) (141,142).

Additional laboratory testing

On-site rapid influenza diagnostic tests (Alere BinaxNOW®) were performed as per discretion of the treating clinicians and nurses, independently of the RIBhuC study.

Pleural fluid was collected only if clinically indicated and analysed following usual procedures at JDWNRH.

If the treating physicians suspected tuberculosis or HIV, pertinent laboratory tests were requested as per Bhutanese national guidelines.

Transfer of biological samples

Frozen samples (nasopharyngeal washing and plasma) and filter papers with dry blood spot were shipped in two batches from JDWNRH to the reference centres in Barcelona, Spain, and Toronto, Canada, through an external international courier company. Packaging and transfer were performed following international standards (Figure 20).

Figure 20. RIBhuC samples packed for shipment. Source: personal.

Data management and statistical analysis

The principal investigator entered the data collected from the questionnaires into a computerized password-protected database (ODK Aggregate version 1.4.13) with study identification number (143). We limited errors in data entry by pre-defining ranges for every value. We used Stata™ v.16.0 (StataCorp, College Station, Texas, USA) for data analysis (144). We detailed the statistical analyses in the methods section of each article.

Ethics statement

Ethics approval

The study protocol was approved by the Research Ethics Board of Health, Ministry of Health, in Thimphu in March 2017 (protocol number PO/2016/086), and by the research ethics committee from the Hospital Clínic in Barcelona (HCB/2017/0741).

Study information and consent form

The principal investigator, the co-investigators, or the resident on call approached the parents or caregivers of the children that fit the inclusion criteria to provide oral and written (English or Dzongkha) information about the study. For parents or caregivers who did not understand English or Dzongkha, but other languages such as Sharchop, Nepalese, or Hindi, we ensured that the study information was provided by a healthcare staff member who spoke and understood the same language as them. It was made clear to the families that the study was voluntary, and that any information given or collected would be anonymous and non-identifiable. All the parents or caregivers were given at least 24 hours to consider partaking in the study, as per the declaration of Helsinki guidelines (145). We obtained written informed consent from the parents or the caregivers of all participants.

Overview of the articles included in the thesis

This thesis includes **six highly complementary articles** related to childhood pneumonia in Bhutan.

The **first article** is a commentary highlighting the challenges of conducting research and working full-time as a clinician in Bhutan.

The **second article** is a systematic review that summarized all available data on childhood pneumonia in Bhutan. The review provides an overview of the burden of the disease, the aetiology, risk factors, clinical and radiological characteristics, prognosis, national surveillance systems in place, and national preventive strategies. Pneumonia is the major cause of deaths among children after the neonatal period. Summarizing available data on pneumonia within the Bhutanese context allowed the identification of knowledge gaps, which is the first step to pinpoint the research needed to reduce the burden and mortality associated with this disease. Reliable data are essential for the implementation and monitoring of evidence-based management and national preventive strategies. The findings of this review provided guidance for conducting the RIBhuC study, which aimed to close the knowledge gap on childhood pneumonia in Bhutan.

Articles 3 to 6 report the findings of the RIBhuC study, which recruited Bhutanese children between 2 and 59 months of age admitted with clinical pneumonia during 12 consecutive months. The **third article** describes the aetiological profile and the demographic and clinical characteristics of the children recruited in this study.

The **fourth article** aimed to determine the prevalence of PNC and to identify the pneumococcal serotypes circulating among Bhutanese children admitted with clinical pneumonia before the introduction of PCV in the country. This article also identified and compared respiratory viruses among children with and without pneumococcal nasopharyngeal colonization, to contribute to the understanding of the interplay between PNC and viral co-infections.

The **fifth article** describes the radiological findings of the RIBhuC cohort and the differences in radiological outcomes by demographic characteristics, aetiology, clinical characteristics, and host-response biomarker levels. This article also evaluated the utility of host-response biomarkers in discerning between bacterial and viral pneumonia, taking

radiological endpoint pneumonia (consolidation, pleural effusion or both) as a proxy for bacterial aetiology.

The **sixth and last article** of this thesis assessed the performance of a wide range of clinical characteristics, laboratory testing, clinical scoring scales, and host response biomarkers to identify children with clinical pneumonia at risk of poor prognosis. This article also assessed the impact of parental education and access to care on pneumonia prognosis and mortality.


Results

ARTICLE 1

The challenges of combining clinical work with research in Bhutan: a changing status quo

Sophie Jullien

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Editorial

EDITORIAL

The Challenges of Combining Clinical Work with Research in Bhutan: A Changing Status Quo

Health research is imperative for continuously improving care, this is unquestionable. Knowing that research is costly and time consuming, can a country use the research findings from another country or does every country have to produce their own data? With the amount of studies published every day, it is essential for policy-makers and clinicians to have the knowledge and skills to identify reliable data that translates into evidence-based policies and practice. It is important to acknowledge when the findings identified by previous research can be applicable to another setting or population so that redundant research is avoided. Having said that, local data are needed for multiple purposes. To monitor progress made at a national level based on health indicators, to determine the burden of diseases and identify their characteristics, and to better describe local problems so that tailored solutions can be offered are some examples of why reliable local data are needed. 'Because local health problems often require local solutions, each country should be a producer as well as a consumer of research' reminded the WHO in 2013 [1]. However, most research is led by high-income countries and many low- and middle-income countries (LMIC) lack health research capacity. Strengthening local health research capacity in LMIC seems therefore critical for addressing health challenges locally and globally. But this is nothing new. As early as 1990, the Commission on Health Research for Development recommended the rapid expansion of country-specific health research and stated that strengthening research capacity in LMIC is 'one of the most powerful, cost-effective, and sustainable means of advancing health and development' [2]. Since then, there has been increasing calls for actions to foster health research capacity in LMIC. Although there has been some progress, many LMIC still lack sufficient health research capacity to undertake local research and to collect reliable local data to finally translate findings into policy [3]. How is Bhutan doing in this regard?

Bhutan is a landlocked country in the Eastern Himalayas, bordered by two giants, India and China. It is internationally known as the 'Land of the Thunder Dragon', or the country which established the concept of Gross National Happiness. It is currently classified as a lower-middle income country and the last census in 2017 registered a population of 735 553 [4, 5]. Universal health care is provided since the 1970s, with 'free access to basic public health services in both modern and traditional medicines' as per the Constitution of Bhutan [6]. The country has developed an efficient health system to reach the most remote areas, constituted by national, regional and district hospitals, basic health units and outreach clinics. Since the first fully graduated Bhutanese doctor returned to Bhutan in 1954, this country has made great strides in providing health care services to its people. The first and only University of Medical Sciences in the country was

inaugurated in 2014, partly to solve the shortage of specialist doctors, which remains a concern. It currently offers postgraduate residency training in several specialties. Did the health research expand as fast as the development of health care services?

Although publications may not reflect the exact number of research projects conducted in the country, it gives a rough approximation. A Pubmed enquiry with the search term 'Bhutan' on 22nd of February 2019, identified 744 publications. By selecting the 'observational study' and 'clinical trial' filters, the number of publications was reduced to four and two respectively, of which half were actually not conducted in Bhutan. The Bhutan Health Journal (BHJ) was launched in 2015 and is published biannually [7]. Most of the studies published in the BHJ are conducted by Bhutanese researchers addressing local health topics. Although limited, the amount and nature of publications in the seven numbers of this journal reflect a much more positive condition of the research conducted in Bhutan. Most of the research are cross-sectional studies and case reports. Most of the authors are part of national institutions such as the Ministry of Health and the University of Medical Sciences. Aware of the importance of research, the new curriculum for the postgraduate residency programme requires the students to design and conduct a research project. Although they face challenges, mainly when designing their project and analysing the data, the work produced by the first two batches is promising. While initiatives are taken to develop research capacity, the amount of research led by working clinicians is limited. What could be the main reasons explaining the limited research in Bhutan?

Conducting research is challenging. It requires the awareness that research is essential, and the knowledge of methodological and analytical skills. Although there are few experts in this area in Bhutan, most of the clinicians lack the necessary proficiencies when it comes to research methodology and critical appraisal. They could probably learn these skills easily. But priorities are elsewhere—clinical work. At the national referral hospital in Thimphu, doctors see up to over a hundred patients daily. Sixty to seventy children every day from Monday to Saturday is the average for a paediatrician in this hospital. Meetings, medical visits to friends and relatives once the outpatient department is closed, and oncall duties involving nights, Sundays and government holidays are added to this workload. In the last few years, the responsibility and time involved for teaching postgraduate doctors and medical students has been added to the list. Where to find the motivation for starting a research project with this busy agenda? Furthermore, research requires funding, which can become considerable depending on the project. Leading research also requires leadership competencies and collaboration with other health workers. For example, a research project that involves collecting blood samples on eligible patients presenting to the outpatient department will probably require the support of the health workers in this outpatient department if the main investigator is also doing clinical work and cannot dedicate full time to research. Thus, health workers will be asked to identify participants eligible for the study and either inform the main investigator for recruitment or carry on with the recruitment process themselves. This could include providing study information, seeking consent, and collecting blood sample as well as some basic demographic data. In the context of work overload, poor awareness of the benefits of conducting research, and limited or no funding for rewarding such tasks, this collaboration can be extremely challenging. However, correct data and sample collection are crucial to obtain reliable findings.

The health system in Bhutan has grown remarkably in the last few years. Although progress has also been made in the field of health research, there is still room for improvement. Leading research is still uncommon among clinicians. The main challenges preventing expansion of local research are the lack of awareness that research is needed, the lack of motivation, poor knowledge and methodological skills, and clinical work overload.

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ARTICLE 2

Pneumonia in Bhutanese children: what we know, and what we need to know

Sophie Jullien, Dinesh Pradhan, Quique Bassat

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Pneumonia

REVIEW Open Access

Pneumonia in Bhutanese children: what we know, and what we need to know

Sophie Jullien[1,2*], Dinesh Pradhan[3] and Quique Bassat[1,4,5,6,7]

Abstract

Background: Pneumonia is the single largest cause of death in under-five children worldwide. We conducted a systematic review to identify the knowledge gaps around childhood pneumonia in Bhutan.

Methods: We searched PubMed, ScienceDirect and Google scholar from conception to 3rd December 2018, World Health Organization, UNICEF, Bhutan's Ministry of Health and other local databases for relevant reports. We included any report describing pneumonia in Bhutanese children with regards to the burden of the disease, aetiology, related risk factors, clinical and prognostic characteristics, surveillance systems and national preventive strategies. Two review authors identified the records. We summarized the findings narratively.

Results: We included 44 records. Although with notable decreasing trends, pneumonia is still accountable for a high burden and mortality rate in Bhutanese children. The national surveillance system focuses mainly on influenza identification but has recently introduced other viral aetiology to monitor. We found very scarce or no data with regard to the bacterial aetiology, related risk factors and clinico-radiological and prognostic characteristics.

Conclusion: There is a dearth of data regarding the epidemiological, microbiological, clinical and radiological characteristics of pneumonia in children in Bhutan, leading to challenges while implementing evidence-based management and effective national preventive strategies.

Keywords: Pneumonia, Respiratory tract infections, Bhutan, Viruses, Infant, Child preschool, Epidemiology, Vaccines

Background

In 2015, pneumonia was ranked as the single biggest killer of post-neonatal children worldwide. With an estimated 15·5% attributable fraction of all deaths in children under 5 years of age, pneumonia is believed to be responsible for the deaths of around 900,000 children every year [1, 2]. The main burden remains disproportionately concentrated in low- and middle-income countries (LMIC) in Southeast Asia and sub-Saharan Africa, where pneumonia is one of the most frequent triggers of health facility consultation, and one of the most common causes of hospitalization, representing a huge load for the overburdened and fragile health care systems [3].

The world has achieved considerable progress in reducing child mortality in the last two decades [4], and reductions in pneumonia-attributable mortality are partly responsible for such a massive public health achievement [1]. Various interventions have shown to be effective to prevent and treat childhood pneumonia, including exclusive breastfeeding for 6 months, prevention of childhood malnutrition, childhood immunization, reduction of household air pollution, hand washing, and use of simple and standardized guidelines [5–7]. Although substantial progress has been achieved, the toll of preventable deaths related to pneumonia remains intolerably heavy. Better implementation of the strategies shown to be effective to prevent and treat childhood pneumonia seems crucial to further reduce the burden of pneumonia. Once implemented, adequate national surveillance systems are required to evaluate their progress towards the reduction of the disease burden. To this end, it is imperative to collect reliable local data to determine the burden of the disease, the main pathogens involved, risk factors contributing to the disease, and to describe epidemiological trends. However, reliable local data are often scarce or entirely missing in LMIC, where the main burden of pneumonia remains concentrated,

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leading to challenges when it comes to evaluating the preventive strategies implemented [8].

Bhutan is a small country (38,394 km^2) locked in the Himalayas between India and China [9]. Elevation rises from around 100 m to more than 7500, with the capital, Thimphu, standing at 2334 m [10]. It is currently classified as a lower-middle income country [11]. The population was estimated at 779,666 in 2017 and life expectancy at birth was 70·6 years in 2016 [12, 13]. Two thirds of the population live in rural areas [14]. Essential health services in both modern and traditional medicines are free for Bhutanese citizens, as guaranteed by the Constitution, based on a primary health care approach [15]. Health services are offered across the country through secondary and tertiary level hospitals, basic health units, subposts and outreach clinics [12].

The WHO-recommended Integrated Management of Childhood Illnesses (IMCI) strategy was adopted and implemented in Bhutan in 2000, as a strategy to address the major causes of deaths in under-five children. Health workers were trained across the country and the coverage of IMCI implementation reached 100% in all the districts of the country by 2011 [16]. The strategy promotes the early recognition of different syndromes (including pneumonia) in under-five children, as well as a simple and algorithm-based systematic approach for their management. Despite these efforts, acute respiratory infections (ARI) seem to remain a major public health challenge in Bhutan, whereby they were estimated to represent the fifth cause of mortality for the whole population, and to cause 15% of deaths in under-five children [12, 17]. The conjugate *Haemophilus influenzae* type b (Hib) vaccine is routinely administered since 2011 and the pneumococcal conjugate vaccine was recently introduced in the childhood immunization schedule (January 2019) [18, 19]. Reduction of risk factors, immunisation, and case management are the main approaches to disease control [3].

Summarizing the available data on the epidemiology, aetiology and clinical characteristics of pneumonia among Bhutanese children would help to better understand and characterize the impact of this major killer and would allow the identification of knowledge gaps to improve local tailored programmes to reduce the burden and mortality associated with childhood pneumonia in Bhutan.

Objectives

We conducted this systematic review in order to identify the knowledge gaps around ARI in children in Bhutan. We aimed to collect any available data with regards to the burden of the disease, the aetiology, clinico-radiological characteristics, the associated health determinants and trends over the last years, as well as the current surveillance systems and national preventive strategies.

Methods

We followed the protocol established for this review (Additional file 1: Appendix 1).

Selection criteria

We sought to include any study or primary report in which participants were children under 5 years of age (or all ages when disaggregated data were not available) in Bhutan that would report data on ARI or pneumonia, as defined by the study authors, regarding any of the following:

- burden of the disease such as incidence, prevalence, morbidity, mortality, or number of visits in health facilities;
- aetiology;
- related risk factors;
- clinical and prognostic characteristics;
- management;
- existing surveillance systems;
- national preventive strategies related to ARI or pneumonia.

We excluded documents when duplicated.

Definitions

The clinical case definition of pneumonia has been changing over the last few decades [20, 21]. As of today, there is no optimal gold standard definition available [22]. Lower respiratory infections (LRI) is a broad term that includes pneumonia and bronchiolitis, as defined in the Global Burden of Diseases, Injuries, and Risk Factors Study (GBD) [6]. This is the terminology we strived to use in this review. However, we will report data as defined by the study authors. Thereby, ARI is often used and may imply here a broader concept including both upper and lower respiratory infections.

Search methods for identification of studies

We attempted to identify all relevant reports regardless of language or publication status (published, unpublished, and in press).

Electronic searches

We conducted an electronic search of the following databases, using the search terms and strategy described in Additional file 1: Appendix 2. PubMed (1942 to 3 December 2018), ScienceDirect (1944 to 3 December 2018) and Google Scholar (up to December 2018). We manually searched all the issues of the Bhutan Health Journal for relevant publications (conception to

December 2018). We searched databases of the WHO, UNICEF, Ministry of Health in Bhutan, Royal Centre for Disease Control (RCDC), National Statistics Bureau, Jigme Dorji Wangchuck National Referral Hospital (JDWNRH) and Khesar Gyalpo University of Medical Sciences of Bhutan (KGUMSB) websites for relevant documents.

Other sources

We manually searched the reference lists of all documents identified by the above methods that met our eligibility criteria for other potentially relevant documents. We looked for unpublished theses or other unpublished documents at the libraries of the Faculty of Nursing and Public Health and Faculty of Postgraduate Medicine at KGUMSB in Thimphu, Bhutan. We contacted researchers and key stakeholders at the Ministry of Health, at the Microbiology Department at JDWNRH, and at RCDC in Thimphu, to identify additional potentially relevant unpublished data.

Data collection and analysis

Selection of studies

Two review authors (SJ and DP) independently screened the titles and abstracts of the reports identified by the electronic searches and identified in the reference lists of selected documents or in the libraries cited above, to identify eligibility criteria. Duplicate reports were removed. We retrieved the full-text articles of the records that were identified as potentially eligible. SJ and DP independently assessed the full-text articles for eligibility, using the predefined inclusion and exclusion criteria, and resolved any disagreements by discussion.

Data extraction, data management and data synthesis

SJ extracted data from the included records. DP checked all extracted data to identify any possible errors. Data describing study setting, population, methods and outcomes as well as pneumonia definitions used by the study authors were extracted. From other documents and data identified from the screened websites, we extracted additional relevant data which were classified under selected themes according to our outcomes of interest. When available, we collected data for each outcome on population characteristics, setting and methodology used. We synthesized the findings narratively with the support of graphs and tables, under different themes. We reorganised the themes based on the amount of information gathered for each of them.

Results

Results of the search

The database searches conducted up to 3rd December 2018 returned 714 records. The screening of titles and abstracts revealed 11 relevant records, for which the full-text article was retrieved. Five studies met our eligibility criteria and were included in the review. We identified 40 additional reports related to the topic through other electronic search and sources, out of which we excluded one due to duplication. Overall, we included 44 documents for qualitative synthesis (Fig. 1). Included and

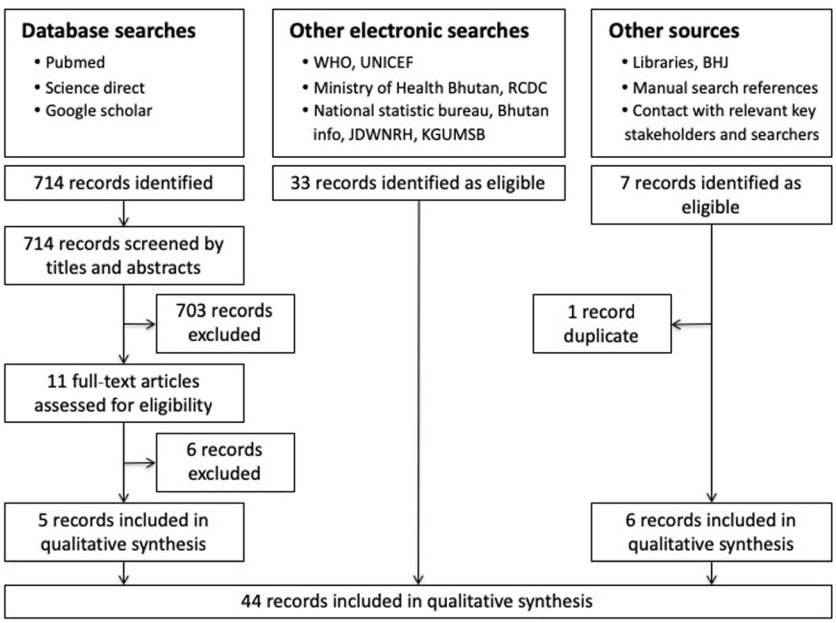

Fig. 1 Flow diagram illustrating the selection of records

excluded records with reasons for exclusion are listed in Additional file 1: Appendix 3.

We included 10 studies and 34 reports. All of them are published except one unpublished report and one medical thesis Tshering T: Incidence, risk factors, and outcome of ventilator-associated pneumonia in pediatric intensive care unit Jigme Dorji Wangchuk National Referral Hospital. A prospective hospital-based study, (unpublished observations) (see Additional file 1: Appendix 3). The medical thesis focuses on ventilator-associated pneumonia (VAP) in children admitted at the national referral hospital (JDWNRH), while all the other studies describe community acquired infections. Eight studies focused on Bhutan, while two manuscripts presented data on LRI in 195 countries including Bhutan [6, 23]. Nine out of the 10 studies were published in the last 8 years; the remaining one was published in 1995 [24]. Only two studies [24, 25] focused on children, all other studies included all age groups.

Surveillance systems for respiratory infections in Bhutan

No systematic national notifiable disease surveillance system existed in Bhutan a decade ago, albeit a few notifiable diseases were reported nationally [26]. Over the last 10 years, the RCDC initiated an influenza surveillance system among outpatients and hospitalized patients, comprising Influenza-Like Illness (ILI) and Severe Acute Respiratory Infection (SARI). This clinical and laboratory-based surveillance is carried out at 11 hospitals that were selected as sentinel sites. Each sentinel site collects random samples from few ILI cases every week, and samples from every SARI case [27]. This system works as epidemiological and virological surveillance, with the aim of monitoring the burden and trends in respiratory diseases, as well as monitoring the epidemiology of influenza viruses and other respiratory pathogens [28, 29]. Data are reported on a weekly basis and feedbacks are shared on the RCDC website every week and in quarterly bulletins [15, 28, 30]. Evaluation of the SARI surveillance platform by Thapa et al. in 2015 and 2016 identified significant underreporting of SARI cases, consequently questioning the national data ensuing from this surveillance system [31].

Burden and seasonality patterns of acute respiratory infections in Bhutan

We summarized the main findings on the burden of ARIs in Bhutan in Additional file 1: Appendix 4, Table 4A.

The incidence of pneumonia in Bhutan has exhibited a decreasing trend in the last 10 years, from 1479 cases per 10,000 children under 5 years of age in 2008 to 809 in 2017 [12, 32]. Pneumonia cases, number of outpatient visits attributed to pneumonia in hospitals and in basic health units, and number of inpatients attributed to pneumonia in under-five children have also decreased in the last 10 years in term of both absolute and relative numbers (Additional file 1: Appendix 4, Table 4B).

According to the quarterly bulletins published by the RCDC, the national surveillance system reported a total of 172,833 cases of respiratory illness in 2018 [30] Additional file 1: Appendix 4, Table 4C.

One study aimed to estimate influenza-associated respiratory hospitalization rates in Bhutan [31]. There were 11,782 hospital discharges coded with respiratory diagnosis in 2015 across Bhutan, and 13,697 in 2016. Six district hospitals identified as sentinel sites that were included in their analysis reported 3138 respiratory hospitalizations, of which 45% were among under-five children. The highest influenza-associated hospitalization rates were found among children under five: 182 (95% CI: 153 to 210) and 532 (95% CI: 473 to 591) per 100,000 persons in 2015 and 2016, respectively.

Looking at the most updated available data, the influenza sentinel surveillance reported 1214 cases of ILI per 10,000 hospital visits and 29 cases of SARI per 100 hospital admissions in 2018 [30] (Additional file 1: Appendix 4, Table 4D).

Finally, one record looked at VAP in children admitted in the paediatric intensive care unit in 2017. Out of the 92 included patients, 13 were diagnosed as VAP, resulting in VAP incidence of 14·1% and in VAP incidence density of 44·9/1000 ventilator days.

Regarding the seasonality pattern of ARI, Fig. 2 shows the number of ILI and SARI cases reported weekly in the last 2 years. There are two main peaks each year, around the weeks 20 and 35 in 2017 and around the weeks 11 and 34 in 2018.

Mortality related to acute respiratory infections and pneumonia in Bhutanese children

We summarized the main findings on the mortality related to ARIs in Additional file 1: Appendix 5, Table 5A.

Since 2015, WHO ranked pneumonia as the leading individual cause of under-five mortality worldwide, and Bhutan is not an exception. In 2016 ARI was estimated to be responsible for 15 and 27% of under-five and post-neonatal deaths, respectively. Table 1 summarizes changes in pneumonia-attributable mortality between the years 2000 and 2016 globally and for Bhutan [2].

The GBD study estimated an increasing LRI mortality rate from 51 to 80 per 100,000 under-five children in 2015 to 100 to 249 in 2016 [6, 23].

Focusing on data collected under the term “pneumonia”, this disease was ranked as the fifth main cause of mortality among the Bhutanese population in 2017 [12]. The estimates of deaths for different major causes in Bhutanese children between one and 59 months of age showed a

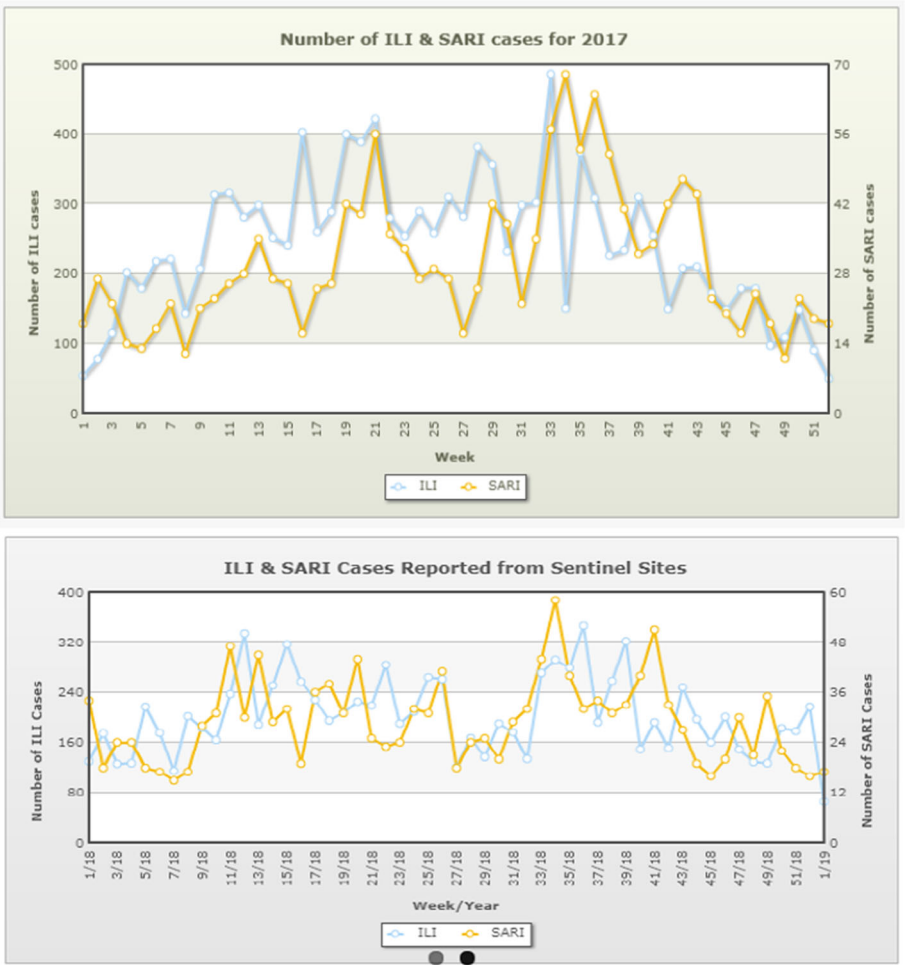

Fig. 2 Number of ILI and SARI cases in 2017 and 2018. Reproduction permitted. Source: RCDC Fluview weekly reports [30]

decline from 2000 to 2010 in the number of deaths attributed to pneumonia (See Additional file 1: Appendix 5, Table 5B). However, pneumonia remains the single biggest cause of post-neonatal child deaths, causing 27·8% of the overall number of deaths in 2010 [25]. A hospital-based study conducted between 2009 and 2011 collected causes of under-five mortality in Bhutan. Authors reported that pneumonia accounted for 45% of deaths in children from one to 59 months, but underlined that their analysis provided data on child mortality happening in health facilities, which is likely to overestimate the true value [25]. Finally, the scarce data available on VAP in Bhutanese children describe five deaths (38·5%) out of the 13 cases of VAP included in the study.

Table 1 Estimates of child deaths due to acute respiratory infections. Source: UNICEF [2]

		Total under 5 deaths	Total post-neonatal death	Under 5 deaths due to ARI			Post-neonatal death due to ARI[a]		
				Absolute number	Rate (per 1000 live births)	%	Absolute number	Rate (per 1000 live births)	%
Global	2000	10,050,919	6,047,778	1,774,025	14	18	1,466,299	11	24
	2016	5,649,439	3,036,996	878,829	6	16	719,413	5	24
Bhutan	2000	1280	768	278	17	22	242	15	32
	2016	468	206	69	5	15	56	4	27

Abbreviations: *ARI* acute respiratory infections
[a]Calculated as (Under5 deaths due to ARI – neonatal deaths due to ARI)

Aetiology of ARI

We found scarce data regarding the aetiology of ARI in Bhutan, most of which focused on influenza virus, mainly collected through the national surveillance system. We identified one paper investigating *Streptococcus pneumoniae*, and one thesis reporting on bacterial isolates for VAP.

Wangchuk et al. summarized epidemiological data on influenza from November 2008 to 2011, which includes the pandemic influenza A(H1N1)pdm09 period [33]. Influenza strains circulating prior to the pandemic period included A/H1 and A/H3, with A (H1N1)pdm09 remaining a dominant strain for almost 1 year after the pandemic period, when it was replaced by A/H3. Influenza B was present throughout and after the pandemic period. During the pandemic period, 2149 samples were collected with results shown in Additional file 1: Appendix 6, Table 6A.

The RCDC publishes "Fluview", a weekly summary of the ILI and SARI surveillance [30]. Every week, each of the 11 hospitals identified as sentinel sites collect samples from between four and six cases of ILI, and from each cases of SARI. The respiratory samples collected at the sentinel sites are firstly tested by Real Time Polymerase Chain Reaction for Flu A/H1, Flu A/H3, Flu A/H5N1, Flu A/H7N9, Flu A/unsub and Flu B. For those samples negative to any type of influenza, there is a further attempt to identify respiratory syncytial virus (RSV) and human metapneumovirus (HMPV). Recently, identification of further viruses also includes adenovirus and parainfluenza viruses 1, 2 and 3, reported in the last two quarterly bulletins (from mid 2018) (personal communication). In 2018, RSV was identified in a considerable number of cases, while HMPV, adenovirus and parainfluenza viruses were isolated in few cases only [30].

Tshokey et al. presented the serotypes and antibiotic susceptibility of *S. pneumoniae* isolates collected at the JDWNRH in Thimphu in 2014 and 2015 [34]. Fifteen different serotypes were identified out of 21 confirmed pneumococcal isolates, collected from several sources (five were from blood sample and two from sputum) (Additional file 1: Appendix 6, Table 6B). Authors also looked at the serotype coverage from the available pneumococcal vaccines and calculated that the 7-valent and 10-valent vaccines would cover 26·7% and 40·0% of the identified serotypes respectively, while the 13-valent and 23-valent would cover 53·3% of them.

Unpublished data provided by the Department of Microbiology from the same hospital, JDWNRH in Thimphu, described the number of pneumococcal isolates and their antibiotic susceptibility in 2016. Only nine isolates were identified from blood samples, and 12 from respiratory samples, all of them fully susceptible to penicillin (Additional file 1: Appendix 6, Table 6C).

Finally, there were 18 bacterial isolates from endotracheal aspirates of 13 children with VAP reported in the medical thesis. *Klebsiella* spp. [8] and *Escherichia coli* [4] were the most common pathogens identified, followed by *Acinetobacter* spp. [2], *Enterococcus* [2], *Staphilococcus aureus* [2] and *Pseudomonas* spp. [1]. Antibiotic susceptibility was not reported for these bacterial isolates.

Risk factors for ARI

One study published in 1995 described risk factors of ARI [24]. This prospective cohort study of 113 children born in 1990 found a statistically significant reduction in the incidence of both diarrhoea and respiratory tract infections associated with breastfeeding. Tashi and colleagues also looked at risk factors for prediction of VAP among a limited number of patients. Amongst the medical co-morbidities, disorders of the central nervous system, trauma and sepsis were statistically significant in the development of VAP.

The GBD study calculated modelling estimates in under-five LRI mortality attributable to change in risk factors between 2000 and 2016. In Bhutan, it was estimated that changes related to childhood wasting, stunting and overweight have reduced LRI-related mortality by 9·41%, 3·56% and 4·45% respectively. Similarly, changes related to handwashing have reduced LRI-related mortality by 0·38%, breastfeeding by 1·29%, second-hand smoking by 0·23%, household air pollution by 7·52%, adequate antibiotic treatment by 3·01%, zinc deficiency by 0·29% and Hib vaccination by 6·44%. The only assessed risk factor that was attributed to an increase in LRI-related mortality was ambient particulate matter pollution, with a 7·33% increase [6].

Clinico-radiological characteristics, prognosis and management of ARI

We did not find any study describing the clinical or radiological characteristics, prognosis and management of Bhutanese children suffering from community-acquired pneumonia or ARI. The only data available suggest that in 2010, 74·2% of children under five with suspected pneumonia were taken to an appropriate healthcare provider, and that treatment with antibiotics was given in 48·7% of the cases with suspected pneumonia [35]. Tashi and colleagues presented 13 children with VAP. Most of them had important co-morbidities, including sepsis [7], congenital heart disease [6] and central nervous system infection [5] as primary diagnosis. The median duration of stay in the intensive care unit was 28 days while it was 5 days for children without VAP.

National preventive strategies

Activities for ARI control were initiated in 1987. The ARI Programme was instituted in the erstwhile Department of Health in 1992 and activities were intensified in 1993 with the primary intention of reducing under-five deaths due to pneumonia. WHO Standard Case Management Protocol for ARI was introduced in 1994, while IMCI was implemented in 2000 [12].

In the report dedicated to the identification of under-five deaths in health facilities in Bhutan, the Ministry of Health supported by UNICEF recognised the need of expanding the role of village health workers to provide antibiotics for pneumonia in order to help reduce child mortality attributable to sepsis and pneumonia [25]. Models were developed to estimate the impact of different interventions for reducing the under-five mortality rate, and so as to identify priority child health interventions that would result in maximum impact. It was estimated that oral antibiotics for the management of pneumonia would contribute to prevent 16% of additional deaths in under-five children [25].

A health economics analysis was conducted to determine the cost-utility of 10- and 13-valent pneumococcal conjugate vaccines (PCV10 and PCV13) compared to no vaccination in Bhutan [36]. It suggested that PCV13 and PCV10 could prevent 30 and 18 deaths respectively in the vaccinated population. If the proportion of the vaccinated population is over 80%, which is likely to be the case in Bhutan, the indirect effect of the vaccine to the unvaccinated population, known as herd protection, could prevent 12 and 10 deaths for PCV13 and PCV10 respectively. Authors concluded that both PCVs would be cost-effective in Bhutan, thus recommending their inclusion in the immunization programme. This has recently been approved by the Bhutanese government, with PCV13 being introduced into the routine vaccination programme in January 2019. The seasonal influenza vaccine is not routinely recommended for at-risk population. Thapa et al. recently published estimations of the number of hospitalizations due to influenza with the aim of informing the Ministry of Health of the seasonal influenza vaccine could be assessed [31]. The benefits of its introduction among recommended target groups are currently being explored by the Bhutanese government.

Discussion

According to national estimates, ARI and pneumonia are a significant national public health concern in Bhutan. Although pneumonia trends in this country have followed the decreasing trends observed globally, ARI is still responsible for high hospitalisation rates and a significant outpatient burden. Children under five are the most affected group. This is of particular concern, as pneumonia is the single predominant cause of death both in Bhutan and worldwide in this age group. However, there is no robust and comprehensive data in Bhutan describing this illness. Indeed, we did not identify data that would provide a thorough understanding on the aetiology of ARI in Bhutanese children, nor help us characterize this syndrome better. In addition to the scarcity of local data, global estimates might be questionable. Indeed, GBD estimates showed an increase in the LRI mortality rate from 51 to 80 per 100,000 under-five children in 2015 to 100 to 249 in 2016, which is unexpected and doubtful [6, 23]. There were inconsistencies between different local sources with respect to the trend of the burden and mortality of the disease over the years.

Most of the data we identified regarding the aetiology of ARI and pneumonia are from the national ILI and SARI surveillance. This relatively new surveillance system has made great efforts in the last decade, being able to publish weekly epidemiological and virological data from the whole country, through a sentinel surveillance system. Up to now, identification of RSV, HMPV, adenovirus and parainfluenza viruses is attempted only for samples negative for influenza virus. However, targeted screening leads to underreporting of other pathogens, and failure to adequately describe or identify mixed viral infections, commonly occurring in ARI [37, 38]. This issue has been detected and testing all viruses will soon be performed in all the collected samples independently of influenza identification (personal communication). In spite of the moderately good performance of the national surveillance system in place, underreporting appears to be common, questioning therefore the accuracy of the national data provided.

Overall, there is a lack of knowledge regarding the causing pathogens of childhood pneumonia in Bhutan. The national surveillance data showed an increased number of ILI and SARI at the end of the cold season, and during the monsoon. However, no studies were found describing in detail the viral aetiology of ARI in children, in spite of the importance that viruses are known to play in the aetiology of ARI in this age group. Additionally, data on the bacterial aetiology of ARI in Bhutan are also very limited, coming from studies with very low sample sizes [34], from a study on nosocomial VAP, or from limited hospital-based microbiological surveillance efforts. We did not find any study describing other important causative pathogens of respiratory infections in children such as *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and *Legionella pneumophila*. We also did not find any study looking at other causes of LRI-like syndrome in children, such as bronchitis, which would fall under the terminology of pneumonia as defined by the WHO. Some of these admissions could be due to non-infectious causes such as hyperreactive

airways and contribute to the burden of what is overall classified under the broader umbrella of ARI. Altogether, there are considerable knowledge gaps regarding the aetiology of ARI in Bhutanese children that need to be addressed in a well conducted prospective aetiological study, as a better understanding of the causes of this deadly syndrome, which is crucial for better and more tailored management and preventive strategies. Moreover, comprehensive local data on resistance patterns of causative agents is also needed to effectively design antibiotic management and prevent the emergence and spread of antimicrobial resistance.

Vaccines are possibly one of the greatest public health tools and a well-established strategy to prevent pneumonia and reduce the number of deaths attributed to this illness. Immunization is free of charge for the Bhutanese population. Overall, the country presents high immunization coverage rates, over 95% since 2010, and estimated as 94·4% in 2017 [15]. We found no comprehensive data describing the circulating pneumococcal serotypes in the Bhutanese population, and very limited data describing the burden of pneumococcal diseases in Bhutanese children. Following the recent introduction of PCV13 in the routine immunization programme, it would be highly recommended to monitor the impact of the vaccine. This could be achieved through surveillance system or prospective study to determine circulating pneumococcal serotypes, to estimate the burden of the disease including number of hospitalisations attributed to pneumonia and pneumonia-related mortality, and to assess the interplay between *S. pneumoniae* and viral respiratory infection.

With a small population of less than a million of persons, an elevation ranging from 100 to 7500 m with consequent variety of climate, and a free health care system, Bhutan presents considerable different characteristics compared to the neighbouring countries. Elevation, climate, population density and free health care are all likely to play a role on the epidemiology and aetiology of childhood pneumonia. Therefore, although some data could be extrapolated from neighbouring countries to characterise this disease, we believe that local data are required to fill the knowledge gap on pneumonia in Bhutanese children, given the unique context of this country.

Conclusions

To our knowledge, this is the first systematic review that gathers evidence from a variety of sources on ARI and pneumonia in Bhutanese children and their significant importance in terms of attributable childhood morbidity and mortality. This is a critical step for summarizing all that is currently known about ARI in Bhutan based on existing data, but also points out the many existing knowledge gaps, which should be addressed so that this syndrome can be better characterised as to its aetiology, epidemiology, patients' clinico-radiological characteristics and prognostic factors. Data is needed at a national level, as factors that influence the outcomes such as aetiology and patient characteristics vary geographically. Efforts should be made towards the development of research strategies in order to identify causative agents of pneumonia in Bhutan, as well as health determinants and prognostic factors of the illness, so that adequate control measures can be established in the country.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s41479-019-0065-x.

Additional file 1: Appendix 1. Protocol. **Appendix 2.** Detailed search strategy. **Appendix 3.** List of included records and excluded studies with reasons. **Appendix 4.** Burden of acute respiratory infections in Bhutan. Table 4A. Summary of findings on burden of acute respiratory infections in Bhutan. Table 4B. Incidence and burden of pneumonia in under-five children in Bhutan from 2008 to 2017. Table 4C. Cases of respiratory illness in 2017 and 2018. Table 4D. Incidence of ILI and SARI from 2016 to 2018. **Appendix 5.** Mortality related to acute respiratory infections and pneumonia in Bhutan. Table 5A. Summary of findings on mortality related to acute respiratory infections. Table 5B. Estimates of deaths by cause in children aged 1 to 59 months for Bhutan, from 2000 to 2010. **Appendix 6.** Aetiology of acute respiratory infections. Table 6A. Positivity of samples by age and virus sub-type from 11th June 2009 to 8th August 2010. Table 6B. Pneumococcal serotypes and antibiotic susceptibility from samples collected at JDWNRH in 2014 and 2015. Table 6C. Number of pneumococcal isolates from samples collected at JDWNRH in 2016

Abbreviations

ARI: Acute Respiratory Infections; BHJ: Bhutan Health Journal; GBD: Global Burden of Diseases, Injuries, and Risk Factors Study; Hib: Haemophilus influenza type b; HMPV: Human metapneumovirus; ILI: Influenza-like illness; IMCI: Integrated Management of Childhood Illnesses; JDWNRH: Jigme Dorji Wangchuck National Referral Hospital; KGUMSB: Khesar Gyalpo University of Medical Sciences of Bhutan; LMIC: Low- and middle-income countries; LRI: Lower respiratory infections; PCV10: 10-valent pneumococcal conjugate vaccines; PCV13: 13-valent pneumococcal conjugate vaccines; RCDC: Royal Centre for Disease Control; RSV: Respiratory Syncytial Virus; SARI: Severe Acute Respiratory Infections; UNICEF: United Nations International Children's Emergency Fund; VAP: Ventilator-Associated Pneumonia; WHO: World Health Organization

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Authors' contributions

SJ and QB conceived the idea of the manuscript. SJ elaborated the protocol, ran the literature search and wrote the manuscript. SJ and DP performed the screening of the reports returned by the literature search. SJ extracted the data of the included reports and DP double checked all the extracted data

from the original reports. DP and QB appraised the manuscript and contributed to it by revising the different versions. All authors read and approved the final manuscript.

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Availability of data and materials
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Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Publisher's Note

Appendices - Pneumonia in Bhutanese children: what we know, and what we need to know

Appendix 1. Protocol

Selection criteria

We will look to include any type of study or any other source that collect data about:

P: children under 5 years of age in Bhutan or all age groups if disaggregated data not available.

I: Acute respiratory infections (ARI) or pneumonia

O: data on:

- burden of ARI such as incidence, prevalence, number of visits in health facilities, and number of hospital admissions; and mortality related to ARI
- aetiology of ARI
- risk factors related to ARI
- clinical description, prognosis and management of ARI
- surveillance systems and national preventive strategies related to ARI

Exclusion criteria: duplication, or when full texts of abstracts not available/found.

Search methods for identification of studies

Information sources

We will look for potential eligible studies and reported through electronic search of:

- Pubmed, Google Scholar, Science Direct online databases,
- WHO and UNICEF websites,
- Bhutan Health Journal,
- Official reports of Ministry of Health (MoH) in Bhutan, National Statistics Bureau, Royal Centre for Disease Control (RCDC), Bhutan Info, Khesar Gyalpo University of Medical Sciences of Bhutan (KGUMSB) and Jigme Dorji Wangchuck National Referral Hospital (JDWNRH).

We will also look for other sources to identify potential eligible documents, such as:

- Unpublished theses and reports at KGUMSB and JDWNRH,

- Contact with relevant researchers on this area with access to reports not available in the public domain (MoH, JDWNRH, RCDC, etc.)
- Manual check of references of retrieved documents.

Search query terms

For Pubmed, we will use the following search strategy: ((respiratory infections[MeSH Terms] OR pneumonia[MeSH Terms]) OR (acute respiratory infection[Title/Abstract] OR pneumonia[Title/Abstract])) AND (Bhutan[MeSH Terms] OR Bhutan[Title/Abstract]). For ScienceDirect and Google scholar, we will use the combination of the following terms: *Acute respiratory infection*, *Pneumonia*, *Pulmonary infection*, *Bhutan and Bhutanese*. We will seek guidance from experts in this field to help improve our established search terms and strategy.

Language: no restriction

Date range: from available data according to different sources, relevant to the data collected, to the date of the conduct of the search strategy.

Data collection and analysis

Selection of studies

Two review authors will independently screen the results from our search, from looking at the titles and abstracts, and by checking all the cited sources. We will then retrieve the full-text articles of the potential eligible studies. We will finally assess the full-text articles for eligibility, following our inclusion and exclusion criteria.

Data extraction and data synthesis

Two review authors will extract data from the included documents. We will extract data on setting, population, methods and definitions used by the authors. We will collect the outcomes and gather them under different themes:

1. Description of surveillance systems and national preventive strategies in place
2. Burden of ARI
3. Description of the aetiology of ARI
4. Determinants and risk factors of ARI
5. Description of the clinical characteristics and clinical management of ARI

We do not aim to do any metaanalysis of quantitative data. We will summarise our findings in a narrative way, with the support of graphs and tables.

Appendix 2. Detailed search strategy

Pubmed

Search	Query
#5	Search (#3 AND #4)
#4	Search (#1 OR #2)
#3	Search ((Bhutan[MeSH Terms]) OR Bhutan) OR Bhutanese
#2	Search (((acute respiratory infection) OR pneumonia) OR pulmonary infection) OR lower respiratory tract infection
#1	Search (respiratory infections[MeSH Terms]) OR pneumonia[MeSH Terms]

ScienceDirect

Search terms
Acute respiratory infection AND Bhutan
Acute respiratory infection AND Bhutanese
Pneumonia AND Bhutan
Pneumonia AND Bhutan
Pulmonary infection AND Bhutan
Pulmonary infection AND Bhutanese

Google Scholar
Bhutan Acute respiratory infection
Bhutan Pneumonia
Bhutan Pulmonary infection

Appendix 3. List of included records and excluded studies with reasons

Included records

Studies	Bohler 1995 (1)
	Dorji 2018 (2)
	GBD 2018 (3)
	GBD 2017 (4)
	Gupta 2012 (5)
	Tshering 2018 (in press)
	Thapa 2018 (6)
	Tshokey 2017 (7)
	Wangchuk 2013 (8)
	Wangchuk 2011 (9)
Reports	Annual Health Bulletins, MoH, from 2009 to 2018 (10)
	Bhutan Health System Review 2017 (11)
	Bhutan Health Situation Trend (12)
	BMIS 2010, UNICEF (13)
	EPI Factsheet Bhutan 2016 (14)
	ILI-SARI Guidelines 2014, MoH (15)
	ILI-SARI Guidelines 2012, MoH (16)
	JDWNRH isolates and susceptibility (unpublished data)
	NEWARS Guidelines 2014 (17)
	RCDC bulletins, weekly Fluview (2 bulletins) (18)
	RCDC bulletins, quarterly (12 bulletins from 2016, 2017 and 2018) (18)
	RCDC monthly epidemiology report (18)
	UNICEF – ARI data per country (19)

Excluded studies

Study identification	Reason for exclusion
Caini 2018 (20)	Wrong outcome. Authors looked at the distribution of influenza subtypes within age strata in 29 countries, with no disaggregated data from Bhutan.
Caini 2016 (21)	Wrong outcome. This is a retrospective study of epidemiological data, which includes surveillance data from 30 countries.
Fisher 2014 (22)	This is a review of the global burden of influenza as a cause of cardiopulmonary morbidity and mortality, including Bhutan. There is no original data, and the few data reported about Bhutan are from studies included in our systematic review (duplicated data).
Rutvisuttinunt 2017 (23)	Wrong outcome. Assessment of viral methods in samples negative while tested with conventional methods for influenza.
Roth 2015 (24)	Wrong outcome. Systematic review addressing acute respiratory infections case definitions in South Asia.
Zhou 2016 (25)	Wrong population. This study analysed retrospectively 121 clinical respiratory samples that were negative by several molecular tests or immunofluorescence assays for identification of viral pathogens with other methods. However, only four of these samples were from Bhutan from 2009 and 2010, the rest of them were from Thailand, Philippines and Nepal.

Appendix 4. Burden of acute respiratory infections in Bhutan

Table 4A. Summary of findings on burden of acute respiratory infections in Bhutan

Indicators	Population	Date	Main findings	Source and ref
Incidence, prevalence				
Incidence of pneumonia[a]	<5 years	2008 2017	1479 cases per 10,000 children 809 cases per 10,000 children	MoH, annual health bulletin (10,26)
Pneumonia cases[a]	<5 years	2008 2017	10,626 (5·4% among total morbidity) 5875 (3·7% among total morbidity)	MoH, annual health bulletin (10,26)
ARI cases	All ages; <5 years	2017	171,880 cases 34,835 (20·3%) children	Notifiable disease system (10)
'Respiratory illness'[b]	All ages	2018	172,833 cases	RCDC, quarterly bulletins (18)
Hospital visits and inpatients				
Number of inpatients attributed to pneumonia[a]	<5 years	2008 2017	2554 (31·5% among total hospital inpatients) 1372 (12·3% among total hospital inpatients)	MoH, annual health bulletin (10,26)
Outpatient visits attributed to pneumonia in hospitals[a]	<5 years	2008 2017	3610 (3·3% among total outpatient visits) 1506 (1·8% among total outpatient visits)	MoH, annual health bulletin (10,26)
Outpatient visits attributed to pneumonia in BHUs[a]	<5 years	2008 2017	4462 (5·5% among total outpatient visits) 2924 (4·8% among total outpatient visits)	MoH, annual health bulletin (10,26)
Respiratory hospitalization rate (discharges coded with respiratory diagnosis)	All ages	2015 2016	11,782 cases 13,697 cases	National hospital-based surveillance (6)
	All ages; <5 years	2016	3138 respiratory hospitalizations 45% were among children <5 years	Sentinel sites: 6 district hospitals (6)
Influenza-associated respiratory hospitalization rates	All ages	2015 2016	50 per 100,000 persons (95% CI 45 to 55) 118 per 100,000 persons (95% CI 110 to 127)	6 district hospitals identified as sentinel sites (6)
	<5 years	2015 2016	182 per 100,000 persons (95% CI 153 to 210) 532 per 100,000 persons (95% CI 473 to 591)	
Hospital visits and admissions[c]	Al ages	2018	1214 cases of ILI per 10,000 hospital visits 29 cases of SARI per 100 hospital admissions	RCDC, influenza sentinel surveillance (18)
VAP	Children between 1 month and 12 years	2017	92 children admitted in the paediatric intensive care unit were included 13 children diagnosed as VAP. VAP incidence: 14·1% VAP incidence density: 44·9/1000 ventilator days.	Thesis, JDWNRH (27)

Abbreviations: ARI: acute respiratory infections; BHUs: basic health units; CI: confidence interval; ILI: influenza-like illness; JDWNRH: Jigme Dorji Wangchuck National Referral Hospital; MoH: Ministry of Health; RCDC: Royal Centre for Disease Control; SARI: severe acute respiratory infections; VAP: ventilator-associated pneumonia.

[a]Detailed data for each year are given in Table 4B.

[b]More details in Table 4C.

[c]More details in Table 4D.

Table 4B. Incidence and burden of pneumonia in under-five children in Bhutan from 2008 to 2017.

Source: Annual Health Bulletins, Ministry of Health (10)**.**

	Pneumonia incidence (per 10,000 CYAR)	**Pneumonia cases** (% among total morbidity)	**Pneumonia outpatient visits in hospitals**[a] (% among total outpatient visits in hospitals)	**Pneumonia hospital inpatients** (% among total hospital inpatients)	**Pneumonia outpatient visits in BHUs** (% among total outpatient visits in BHUs)
2008	1479	10626 (5·4%)	3610 (3·3%)	2554 (31·5%)	4462 (5·5%)
2009	1031	7850 (3·9%)	2863 (2·6%)	1750 (21·0%)	3237 (3·8%)
2010	1135	9204 (5·1%)	2877 (2·9%)	2390 (26·2%)	3937 (5·3%)
2011	974	7975 (4·7%)	2642 (2·7%)	1711 (19·4%)	3622 (5·7%)
2012	1204	9939 (5·4%)	2809 (2·7%)	2322 (22·2%)	4807 (7·1%)
2013	1080	8953 (4·9%)	2730 (2·6%)	1451 (17·6%)	4772 (7·0%)
2014	1138	9446 (4·8%)	2924 (2·7%)	1651 (15·8%)	4873 (6·5%)
2015	905	7488 (4·0%)	1881 (1·8%)	1537 (12·7%)	3935 (5·6%)
2016	991	8134 (4·4%)	1989 (1·9%)	2117 (16·3%)	3909 (6·0%)
2017	809	5875 (3·7%)	1506 (1·8%)	1372 (12·3%)	2924 (4·8%)

Abbreviations: BHU: basic health unit. CYAR: children year at risk.
[a]These data exclude the cases from the national referral hospital and from two Indian military hospitals.

Table 4C. Cases of respiratory illness in 2017 and 2018. Source: RCDC quarterly bulletins (18)**.**

	2017				**2018**			
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
ARI (all ages)	41,022	39,809	43,536	39,878	37,655	49,376	45,273	37,845
SARI (all ages)	548	567	1021	65	531	988	850	315
Total respi-ratory illness	41,570	40,376	44,557	39,943	38,186	50,364	46,123	38,160
	166,446[a]				172,833			

Abbreviations: ARI: acute respiratory infections; SARI: severe acute respiratory infections

Note: There is a small difference between the 171,880 cases of ARI reported in the annual health bulletin in 2017 with the numbers calculated from the four bulletins available in the RCDC website corresponding to 2017: 166,446 total number of respiratory illnesses and 164,245 cases of ARI.

Table 4D. Incidence of ILI and SARI from 2016 to 2018. Source: RCDC quarterly bulletins (18)**.**

	2016	**2017**	**2018**
ILI per 10,000 hospital visits (all ages)	Not available	1610	1214
SARI per 100 hospital admissions (all ages)	32	28	29

Abbreviations: ILI: influenza-like illness; SARI: severe acute respiratory infections.
[a]Data available only for 6 months of the year.

Appendix 5. Mortality related to acute respiratory infections and pneumonia in Bhutan

Table 5A. Summary of findings on mortality related to acute respiratory infections

Indicators	Date	Main findings	Source and ref
Under-five deaths	2015	51 to 80 per 100,000	GBD (3,4)[a]
	2016	100 to 249 per 100,000	GBD (3,4)[a]
	2016	15% attributable to ARI	UNICEF (19)
Post-neonatal deaths (between 1 and 59 months)	2000[b] 2010	230 (28·0%) deaths attributable to pneumonia 120 (27·8%) deaths attributable to pneumonia	Gupta for the MoH (5)
	2009 to 2011	45% attributable to pneumonia	Hospital-based[c] (5)
	2016	27% attributable to ARI	UNICEF (19)
Children between 1months and 12 years	2018	5 deaths over a year among children with VAP	JDWNRH (27)
All population	2017	5th main cause of death ('pneumonia')	MoH, annual health bulletin (10)

Abbreviations: GBD: Global Burden of Diseases, Injuries, and Risk Factors Study; MoH: Ministry of Health; VAP: ventilator-associated pneumonia.

[a]The GBD study provides data on the burden of LRI in 195 countries, including Bhutan. The GBD team used literature review and country-level covariates to produce modelled estimates such as LRI mortality, LRI incidence, hospital admissions due to LRI, risk factors for LRI mortality and LRI burden attributed to four high-burden aetiologies. However, disaggregated data per country are not available for all these estimates.

[b]Detailed data for each year from 2000 to 2010 are given in Table 5B.

[c]Data on child mortality happening in health facilities (at the national referral hospital, the two regional referral hospitals and seven of the district hospitals), which is likely to overestimate the true value.

Table 5B. Estimates of deaths by cause in children aged 1 to 59 months for Bhutan, from 2000 to 2010. Source: Gupta for the Ministry of Health in Bhutan (5)**.**

Year	All deaths <5 yrs	Deaths in 1-59 months	1-59 months							
			Diarrhoea	Measles	Injury	Malaria	AIDS	Meningitis	Other conditions	Pneumonia
2000	1359	820	162 (19.8%)	30 (3.7%)	99 (12.1%)	8 (1.0%)	0 (0.0%)	62 (7.6%)	228 (27.9%)	230 (28.0%)
2001	1300	779	151 (19.3%)	54 (6.9%)	62 (8.0%)	8 (1.1%)	0 (0.0%)	57 (7.3%)	227 (29.1%)	221 (28.4%)
2002	1230	726	143 (19.7%)	10 (1.4%)	63 (8.7%)	9 (1.2%)	0 (0.0%)	53 (7.3%)	231 (31.8%)	218 (30.0%)
2003	1204	717	135 (18.9%)	6 (0.9%)	64 (8.9%)	4 (0.6%)	1 (0.1%)	54 (7.6%)	237 (33.0%)	215 (30.0%)
2004	1182	710	129 (18.2%)	6 (0.8%)	65 (9.2%)	3 (0.4%)	1 (0.1%)	50 (7.0%)	244 (34.3%)	213 (30.0%)
2005	1134	680	118 (17.3%)	5 (0.8%)	64 (9.4%)	2 (0.4%)	1 (0.1%)	48 (7.0%)	240 (35.3%)	202 (29.7%)
2006	1078	641	107 (16.6%)	3 (0.4%)	62 (9.7%)	2 (0.4%)	1 (0.2%)	41 (6.4%)	235 (36.7%)	190 (29.7%)
2007	1007	586	93 (15.9%)	0 (0.0%)	58 (9.9%)	1 (0.2%)	1 (0.2%)	37 (6.3%)	224 (38.2%)	172 (29.4%)
2008	934	527	78 (14.8%)	0 (0.1%)	52 (9.9%)	0 (0.1%)	2 (0.4%)	32 (6.0%)	211 (40.1%)	151 (28.6%)
2009	864	471	67 (14.1%)	1 (0.2%)	47 (10.0%)	2 (0.3%)	2 (0.4%)	24 (5.2%)	196 (41.6%)	133 (28.2%)
2010	811	432	57 (13.2%)	5 (1.3%)	44 (10.2%)	1 (0.1%)	3 (0.7%)	21 (5.0%)	181 (41.8%)	120 (27.8%)

Appendix 6. Aetiology of acute respiratory infections

Table 6A. Positivity of samples by age and virus sub-type from 11th June 2009 to 8th August 2010. Reproduction permitted. Source: Wangchuk 2013 (8)**.**

Subtype	Age median (in years)	Total cases	Age group, % 0–5	6–20	21–35	36–50	>50
A/H1	25·0	23	17·4	4·3	47·8	17·4	13·0
A/H3	22·5	47	11·4	31·8	45·5	6·8	4·5
A(H1N1)pdm09	18·0	487	6·2	57·4	27·7	7·1	1·5
Flu B	15·0	154	12·9	60·5	21·8	2·7	2·0
Total	18·0	711	8·4	54·6	28·3	6·5	2·2

Table 6B. Pneumococcal serotypes and antibiotic susceptibility from samples collected at JDWNRH in 2014 and 2015. Source: Tshokey et al (7)**.**

Type of sample	Eye secretions (10) Blood (5) Throat frotis (2) Sputum (2) Ascitic fluid (1) Pus (1)
Serotypes identified[a]	Three 10A Two 1, 6B and 19F One each of 4, 6A, 6C, 7C, 7F, 9V, 15B, 19A, 33C, 38, 41 and non-typeable
Antibiotic susceptibility	All isolates sensitive to penicillin, chloramphenicol, and ceftriaxone 9.5% of isolates resistant to erythromycin, and 38.1% to cotrimoxazole.

[a]Although 37 isolates were first identified as *S. pneumoniae*, only 21 were confirmed to be *S. pneumoniae* and were serotyped.

Table 6C. Number of pneumococcal isolates from samples collected at JDWNRH in 2016. Source: personal communication, unpublished.

Type of sample	Number of isolates	Antibiotic susceptibility (%)			
		Penicillin	Erythromycin	Cotrimoxazole	Chloramphenicol
Blood	9	100	89	17	88
Respiratory	12	100	83	-	-

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ARTICLE 3

Pneumonia in children admitted to the national referral hospital in Bhutan: a prospective cohort study

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Pneumonia in children admitted to the national referral hospital in Bhutan: A prospective cohort study

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ABSTRACT

Objectives: The study aim was to describe the etiological profile and clinical characteristics of pneumonia among children hospitalized in Thimphu, Bhutan.
Methods: This prospective study enrolled children aged 2–59 months admitted to the Jigme Dorji Wangchuck National Referral Hospital with World Health Organization (WHO)-defined clinical pneumonia. Demographic and clinico-radiological data were collected through questionnaires, physical examination, and chest radiography. Blood samples and nasopharyngeal washing were collected for microbiological analysis including culture and molecular methods.
Results: From July 2017 to June 2018, 189 children were enrolled, of which 53.4% were infants. Pneumonia-related admissions were less frequent over the winter. Chest radiographies were obtained in 149 children; endpoints included pneumonia in 39 cases (26.2%), other infiltrates in 31 (20.8%), and were normal in 79 children (53.0%). Non-contaminated bacterial growth was detected in 8/152 (5.3%) blood cultures, with only two cases of *Streptococcus pneumoniae*. Viral detection in upper respiratory secretions was common, with at least one virus detected in 103/115 (89.6%). The three most-commonly isolated viruses were respiratory syncytial virus (52/115; 45.2%), rhinovirus (42/115; 36.5%), and human parainfluenza virus (19/115; 16.5%). A third of patients with viral infections showed mixed infections. Case fatality rate was 3.2% (6/189).
Conclusion: Respiratory viral infections predominated among this cohort of WHO-defined clinical pneumonia cases, whereas bacterial aetiologies were uncommon, highlighting the epidemiologic transition that Bhutan seems to have reached.

Abbreviations: BiPAP, bilevel positive airway pressure; CPAP, continuous positive airway pressure; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; GCS, Glasgow coma scale; Hb, haemoglobin; IQR, interquartile range; JDWNRH, Jigme Dorji Wangchuck National Referral Hospital; LMICs, low- and middle-income countries; NPW, nasopharyngeal washing; PCV, pneumococcal conjugate vaccine; PICU, Paediatric Intensive Care Unit; RR, respiratory rate; RSV, respiratory syncytial virus; RT-PCR, real-time polymerase chain reaction; SD, standard deviation; WAZ, weight-for-age *Z*-score; WBC, white blood cells; WHO, World Health Organization.

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Background

Pneumonia is the single largest cause of mortality in children aged under five years, causing an estimated 15.5% of all deaths in children under five years of age, and over 800,000 paediatric deaths annually (Liu et al., 2016; UN IGME, 2018). Most of these lives could be saved through more effective and equitable health system interventions, combining prevention, early and accurate diagnosis, and treatment (Walker et al., 2013; Rambaud-Althaus et al., 2015). The main pneumonia burden remains disproportionately concentrated in low- and middle-income countries (LMICs) in Southeast Asia and sub-Saharan Africa (Walker et al., 2013). Pneumonia deaths are decreasing, but more slowly than for other major causes of mortality, and too slowly to achieve the Sustainable Development Goal ambition of "ending preventable child deaths" by 2030 (United Nations, 2018).

Risk factors and causative pathogens of childhood pneumonia differ across the world. Obtaining reliable local data, including the burden of the disease, epidemiological trends, and the determination of the main pathogens involved, is imperative to help develop targeted interventions. Therefore, adequate surveillance systems are required to monitor the effectiveness of national strategies implemented towards the reduction of the disease burden. However, the lack of local data and weak surveillance systems in many LMICs hamper an adequate knowledge of the epidemiology and aetiology of childhood pneumonia in those settings where reliable data are most needed.

One country that exemplifies the dearth of data regarding childhood pneumonia is the Kingdom of Bhutan (Jullien et al., 2020), a small country locked in the Himalayas, with an estimated population of 779,666 in 2017 (Department of Information Technology, 2016; Ministry of Health, 2018). In this predominantly mountainous country, elevation rises from around 100 m in the southern foothills to over 7500 m in the northern Himalayan range, with the capital, Thimphu, standing at 2334 m (Central Intelligence Agency, 2019). The climate varies with the altitude, from tropical in the southern plains to alpine with very cold winters in the North. In Thimphu, the temperature ranges from −3 °C in winter to 22 °C in summer on average, coinciding with the monsoon that brings precipitations of around 350 mm in July (Climate-data org, 2019). Bhutan is classified as a lower-middle income country as of 2020 (The World Bank, 2020). Essential health services in both modern and traditional medicines are free for Bhutanese citizens, based on a primary healthcare approach (World Health Organization, 2017).

We conducted this prospective hospital-based observational study to describe the epidemiology, aetiology, and clinical and radiological presentation of World Health Organization (WHO)-defined pneumonia among children aged between 2 and 59 months admitted to the Jigme Dorji Wangchuck National Referral Hospital in Thimphu.

Box 1. WHO definitions of pneumonia and severe pneumonia used as inclusion criteria (World Health Organization, 2014).

Pneumonia:
- History of cough or reported breathing difficulty, AND
- Increased respiratory rate (RR) OR chest indrawing.

Severe pneumonia:
- History of cough or reported breathing difficulty

AND at least one of the following:
- Oxygen saturation <90% or central cyanosis,
- Severe respiratory distress (e.g. grunting, very severe chest indrawing),
- Signs of pneumonia with a general danger sign: inability to breastfeed or drink, lethargy or reduced level of consciousness, convulsions.

Increased RR is defined according to age as follows:
- RR ≥ 50 breaths per minute in children aged two months or more and less than 12 months.
- RR ≥ 40 breaths per minute in children aged 12 months or more and less than 60 months.

Methods

Study design and participants

This was a prospective hospital-based study conducted for 12 consecutive months at the Jigme Dorji Wangchuck National Referral Hospital (JDWNRH) in Thimphu. The hospital has 38 paediatric beds, including five in the paediatric intensive care unit (PICU).

All children aged 2–59 months hospitalized with WHO-defined pneumonia (irrespective of severity) were eligible for recruitment (World Health Organization, 2014) (see Box 1). Children admitted in the preceding seven days or with evidence of a foreign body in the respiratory tract were excluded. Potential participants were identified during day and night by the study co-investigators with the collaboration of paediatricians, paediatric residents, and nurses from the outpatient department, the emergency room, the PICU, and the paediatric ward. If an eligible participant was missed during the night, the child was assessed and recruited the following morning. All eligible children were recruited provided parent(s) or guardian(s) consented to study participation.

Data collection

On study admission, a study identification number was assigned and a comprehensive physical examination was performed, including anthropometric measurements, vital signs, axillary temperature, and peripheral oxygen saturation in room air. Demographic and clinical data were collected from the medical records and through family interviews. Sample collection upon enrolment, or as soon as possible after enrolment, included blood samples and nasopharyngeal washing (NPW). All the nurses in the PICU and paediatric ward were trained at the beginning of the study by the lead investigator on how to collect these samples. When a child was identified for recruitment but blood had already been collected, no further blood sampling was conducted. However, if another blood analysis was clinically indicated, additional blood was obtained for the specific purpose of the study. Fluid from pleural effusion was collected when clinically indicated. All recruited patients underwent a postero-anterior chest radiography upon admission. Additional information of potential diagnostic interest, such as computed tomography scans, ultrasound, or cerebrospinal fluid investigation available throughout admission, was also collected. Children were clinically managed and discharged as per existing hospital protocols and discretion of the treating paediatricians, and were followed-up by one study investigator in terms of outcome determination. All data were collected using digitalized and standardized forms (see Supplementary material for clinical definitions and details of variables measured).

Chest radiograph interpretation

The WHO protocol used in clinical trials of pneumococcal conjugate vaccines (PCV) was followed to interpret chest radiographs (Cherian et al., 2005). In brief, readers first judged the

quality of the film (uninterpretable or interpretable, the latter stratified as suboptimal or adequate) and then classified findings for all interpretable radiographs. Significant pathology was defined as the presence of consolidation, other infiltrates, and/or pleural effusion. Endpoint radiologically confirmed pneumonia was defined as consolidation, pleural effusion, or both on any hemithorax. Initially, two paediatricians independently interpreted the radiographs. Discordant results were read by a third reader, trained in WHO criteria for interpretation of chest radiographs. An additional external quality control measure was included in the study protocol, whereby a paediatric radiologist would read a random sample of 10% of the chest radiographs. However, as substantial discordance was observed between the two primary readers, all chest radiographs were again independently interpreted by the paediatric radiologist using the WHO criteria. This last reading was accepted as final interpretation for analysis.

Biological sample testing and laboratory methods

Blood was collected under aseptic conditions following the hospital's validated standardized procedures. Blood for haematology, biochemistry, and culture was processed following standard procedures. Blood was cultured using an automated blood culture system (BacT/ALERT®). Bacterial isolates were identified by colony morphology, growth requirements, and basic biochemical tests. Antibiotic susceptibility was determined using disk diffusion in accordance with the guidelines of the Clinical Laboratory Standard Institute (CLSI, 2015).

Additionally, real-time polymerase chain reaction (RT-PCR) for LytA gene of *Streptococcus pneumoniae* in dried-spot collected blood, and host-response biomarkers in additional blood (2 mL, EDTA tube) were investigated (findings reported elsewhere) (Brotons et al., 2017). The blood samples were centrifuged at $3000 \times g$ for three minutes, and the serum was separated and stored at $-80\,^{\circ}C$.

NPW samples were homogenized and aliquots frozen at $-80\,^{\circ}C$ and subsequently shipped to Barcelona, Spain, where they were subjected to molecular screening (multiplex RT-PCR QIAStat respiratory panel, Qiagen, for 17 viral targets and four bacterial targets). NPW were also subjected to detection of pneumococcus and capsular typing (findings reported elsewhere).

Rapid influenza diagnostic tests (Alere BinaxNOW®) were performed as per discretion of the treating clinicians and nurses, independently of the current study. Investigations for active tuberculosis included Mantoux test and gastric aspirates for microscopy and GeneXpert®.

Data management and statistical analysis

The lead investigator entered data into a computerized password-protected database (ODK®) with study identification number. Errors in data entry were limited by pre-defined ranges for every value. Stata 15.1 was used for data analyses (StataCorp, 2017). Mean with standard deviation (SD) and median with interquartile range (IQR) were used to summarize normally and non-normally distributed variables respectively.

Results

Study profile and demographic characteristics

Between 1st July 2017 and 30th June 2018, 1591 children were admitted to the paediatric department of JDWNRH. Among them, 286 (18.0%) were children aged 2–59 months with respiratory symptoms, of which 189 (66.1%) were recruited (Figure 1).

The baseline characteristics of the 189 children are presented in Table 1. Median age was 10.8 months; over half of the children were infants. Most children were adequately immunized according to age. There was no known case of HIV infection. Children were mainly from the district of Thimphu, although the study included patients from 16 out of the 20 districts in Bhutan. On average, families reported that it had taken around 15 min to reach the closest healthcare facility. Twenty-seven children (14.3%) were referred from another health centre. Summer, fall, and spring each comprised around 30% of the recruited cases, while winter had the lowest number of pneumonia admissions (10.1%). October was the month with the highest number of cases (37; 19.6%) (Figure 2).

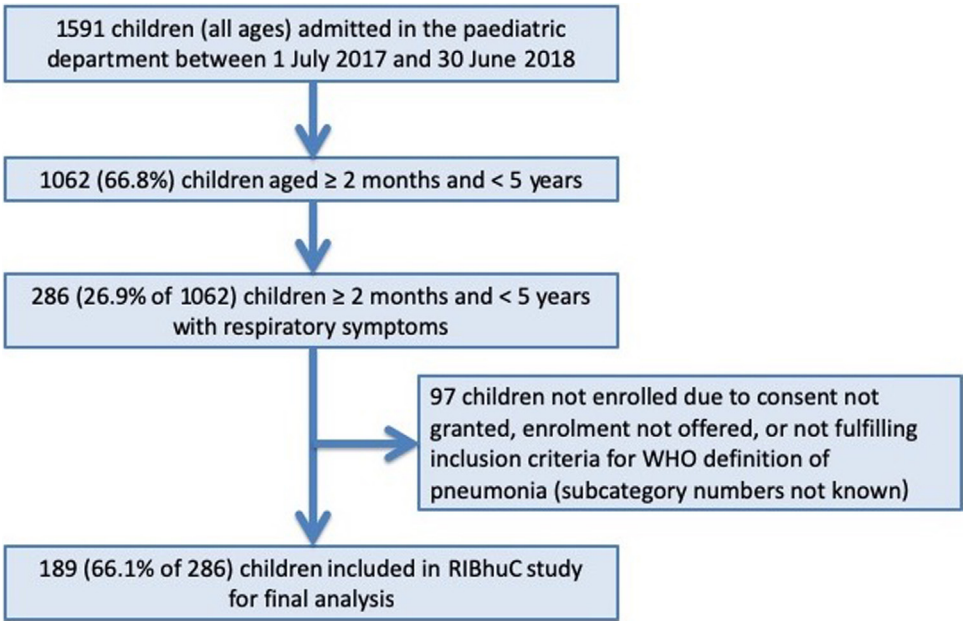

Figure 1. Study profile.

Clinical characteristics

Clinical characteristics upon admission are presented in Tables 2 and 3. Wasting (WAZ ≤ -2 SD) was detected in 17 children (9.0%). On admission, 77 children (41.2%) presented with fever, half of the children were breathing fast according to age, and three-quarters were hypoxemic. Median basal oxygen saturation was 85% (IQR 80–90) among the 173 children with available measurement in room air without oxygen therapy. On auscultation, typical lung consolidation-related sign (crackles) was most common (57.5%), followed by rhonchi (45.2%) and wheezing (25.0%).

On admission, 35.8% of the children were anaemic, 36.9% had leucocytosis, and 25.3% had neutrophilia. Two common inflammatory markers were tested at JDWNRH: C-reactive protein (CRP) with a mean of 2.06 mg/dL (SD 2.09), and erythrocyte sedimentation rate (ESR) with a mean of 24.89 mm (SD 28.02). Twenty-five

Table 1
Baseline characteristics of recruited children

Patients characteristics		*n*/*N*	%
Sex	Female	80/189	42.3
	Male	109/189	57.7
Age group	2 to <6 months	46/189	24.3
	6 to <12 months	55/189	29.1
	12 to <24 months	38/189	20.1
	24 to <36 months	20/189	10.6
	36 to <48 months	15/189	7.9
	48 to <60 months	15/189	7.9
Immunization	Fully immunized according to age	143/189	75.7
	Partially immunized according to age	43/189	22.7
	Not immunized	0/189	0
	Unknown	3/189	1.6
Preterm birth (<37 weeks of gestation)	No	174/189	92.1
	Yes	13/189	6.9
	Unknown	2/189	1.0
Co-morbidities	Known case of HIV infection	0/189	0
	Suspected case of tuberculosis	4/189	2.1
	Known underlying chronic respiratory disease	1/189[a]	0.5
Previous admission due to pneumonia	Yes	43/189	22.7
	No	143/189	75.7
	Unknown	3/189	1.6
Education	Both parents are illiterate	26/189	13.8
	Only one parent has basic (primary) education	26/189	13.8
	Both parents have basic (primary) education	78/189	41.3
	At least one parent has university education	48/189	25.4
	Unknown	11/189	5.8
Employment	Both parents are unemployed	2/189	1.1
	Only one parent is employed	105/189	55.6
	Both parents are employed	67/189	35.4
	Unknown	15/189	7.9
Number of people living in the household	$\leq$5 people living in household	117/189	61.9
	>5 people living in household	62/189	32.8
	Unknown	10/189	5.3
Exposure factors in the household	Smokers	21/189	11.1
	Non-smokers	158/189	83.6
	Smokers, unknown	10/189	5.3
	People chewing betel nut (*doma*)	115/189	60.8
	No people chewing betel nut	64/189	33.9
	People chewing betel nut, unknown	10/189	5.3
Type of heater used in the household (>1 option possible for each household)	Electrical	138/189	73.0
	Wood-burning stove (*bukhari*)	21/189	11.1
	Open fire	4/189	2.1
	Kerosene	14/189	7.4
Residency of the family	Thimphu	133/189	70.4
	Paro	15/189	7.9
	Chukha	5/189	2.7
	Wangdue	5/189	2.7
	Others	31/189	16.3
Closest health facility	JDWNRH	85/189	45.0
	Other hospital	57/189	30.2
	Basic health unit	39/189	20.6
	Unknown	8/189	4.2
Time to access healthcare facility	$\leq$15 min	107/189	56.6
	>15 but $\leq$30 min	58/189	30.7
	>30 but $\leq$60 min	6/189	3.2
	>60 min	5/189	2.7
	Unknown	13/189	6.9
Transport to access healthcare facility	Taxi	68/189	36.0
	Car	65/189	34.4
	Walk	42/189	22.2
	Public transport	1/189	0.5
	Unknown	13/189	6.9

[a] One patient was diagnosed with asthma.

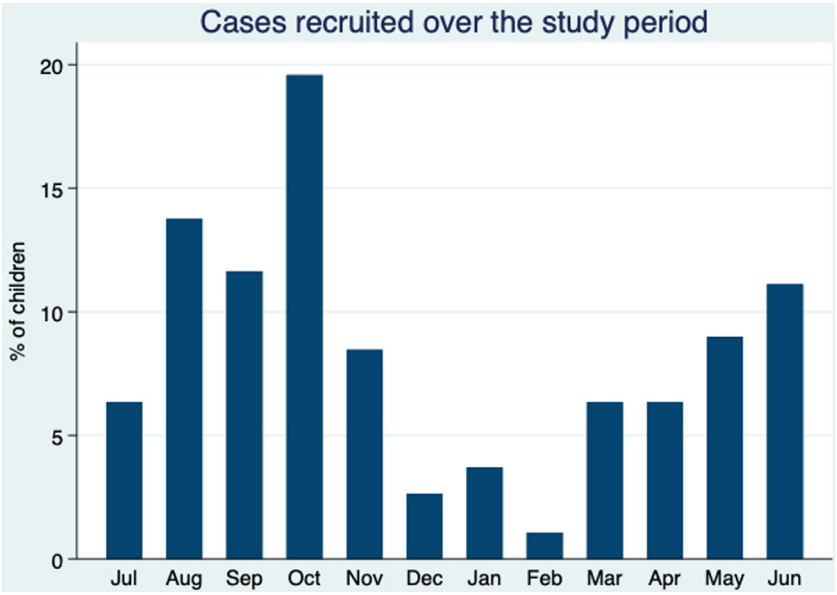

Figure 2. Proportion of pneumonia cases distributed per month.

children (13.2%) had CRP levels above the threshold (>4 mg/dL) commonly considered suggestive of high risk of bacterial infection, whereas 25 children had high ESR (≥50 mm) (Sanders et al., 2008; Bruel et al., 2011). Only four children presented with both high CRP and ESR.

Chest radiography was performed in 178/189 children (94.2%). Images were available for interpretation by the study investigators in 150 of them (84.3%). In 28 cases, children were discharged before investigators could interpret the radiography findings and the radiograph was missing. One film was judged uninterpretable. Among the final 149 readable chest radiographs, 79 (53.0%) were normal, 39 (26.2%) were classified as primary endpoint pneumonia, and 31 (20.8%) as other infiltrates.

Microbiological findings

While HIV infection was not suspected in any child by the treating physicians, active tuberculosis was suspected in 10 children (5.3%) but was not confirmed by the laboratory tests in any of them.

Blood culture was performed in 148/189 children (78.3%), of which 45 (30.4%) had received antibiotics prior to sample collection (Table 4). Thoracocentesis was performed in one child with pleural effusion. Six different pathogens were isolated among the eight non-contaminated positive blood cultures: *S. pneumoniae* (two cases), *Pseudomonas* sp. (two cases), *Escherichia coli*, *Acinetobacter* sp., *Salmonella typhi*, and *Serratia rubidaea* (one case each). Drug sensitivity results are shown in Supplementary Table 2. *S. pneumoniae* was isolated in the only sample of pleural fluid that was collected, which corresponds to the same child with positive blood culture, subsequently also confirmed by RT-PCR in blood.

NPW was collected in 129/189 children (68.3%). The NPW sample was too scarce or of bad quality to run the test in 14 children (10.9%). Among the remaining 115 children, 52 (45.2%) had received antibiotics prior to sample collection. *Bordetella pertussis* was detected in three (2.6%) children, and *Mycoplasma pneumoniae* in one (0.9%) child; *Chlamydophila pneumoniae* and *Legionella pneumophila* were not detected among respiratory samples.

At least one virus was identified in 103/115 NPW samples (89.6%) (Table 4). Viral co-infection was detected in 35/103 children (34.0%): 22 presented double infection, 10 presented triple infection, and three children were infected with four viruses. The most commonly isolated virus was respiratory syncytial virus (RSV) (52; 45.2%), followed by rhinovirus (42; 36.5%), human parainfluenza virus (19; 16.5%), and influenza virus (16; 13.9%). Coronavirus were detected in two children (1.7%). Routine rapid flu test was performed under the Influenza national surveillance programme in 32/189 children (16.9%), being positive for influenza A in seven cases, for influenza B in one case, and for co-infection of influenza A and B in one case. Analysis by RT-PCR confirmed the detection of influenza virus in 4/9 children with positive rapid flu test, and detected 10 additional cases with influenza virus.

Among children with at least one virus detected, 4/86 (4.6%) had a positive blood culture for bacteria and 24/89 (27.0%) had radiological endpoint pneumonia. Among children with no virus detected, 3/9 (23.3%) had a positive blood culture and 4/11 (36.4%) had radiological endpoint pneumonia (Supplementary Table 3). No children with influenza had a positive blood culture. However, 6/15 (40.0%) children with influenza identified in their nasopharynx had radiological endpoint pneumonia.

Lumbar puncture was not indicated in any of the children.

Evolution during admission

Children were hospitalized for a median of four days (IQR 2–6) (Table 5). Thirty children required PICU admission, with a median stay of 72 h (IQR 24–96). Three-quarters of the children were put on oxygen therapy, of which half for at least three days. Most children (72.0%) received antibiotics during admission. Antibiotics were stopped in the first two days of admission in 10 children (7.4%) and advised to be continued after discharge in 90 (66.2%). Main diagnoses given by the treating physician at discharge are shown in Supplementary Table 4. Half of the children were discharged with a diagnosis of pneumonia or bronchopneumonia. In terms of the seasonal variability of the most common clinical syndromes given by the treating physician at discharge, bronchopneumonia was mainly in fall (50.0%), bronchiolitis in spring (43.6%), and pneumonia did not show a clear seasonal pattern (Supplementary Figure 1).

Six children had a fatal outcome (case fatality rate 3.2%); all had been referred from other centres in critical condition.

Table 2
Clinical characteristics of recruited children at time of admission

		n/N	%
History of the current episode			
Duration of illness	<24 h	4/188	2.1
	≥24 to <72 h	41/188	21.8
	≥72 h to <7 days	93/188	49.5
	≥7 days	50/188	26.6
Reported fever prior to admission	No fever	29/184	15.8
	Median duration of fever, in hours (IQR)	72 (24–120)	NA
Danger sign (as per WHO definition)	Any danger sign	37/189	19.6
	Unable to drink or breastfeed	34/189	18.0
	Lethargy or reduced level of consciousness (GCS <15)	8/189	4.2
	Convulsion during the present episode[a]	2/189	1.1
Medical treatment sought prior to admission	Yes	102/186	54.8
Child started on antibiotics prior to admission	Yes	43/187	23.0
Antibiotics received prior to admission (more than one per child possible)	Amoxicillin or ampicillin	35/43	81.4
	Gentamycin	12/43	27.9
	Ceftriaxone	4/43	9.3
	Cefotaxime	2/43	4.7
	Erythromycin	2/43	4.7
	Cloxacillin	1/43	2.3
	Azithromycin	1/43	2.3
Severity of clinical pneumonia			
WHO definition on admission	WHO severe pneumonia	150/189	79.4
	WHO non-severe pneumonia[b]	19/189	10.0
	Do not meet WHO definition[c]	20/189	10.6
Severity during hospitalization	Severe pneumonia[c]	164/189	86.8
	Non-severe pneumonia[c]	25/189	13.2
Clinical examination at time of admission			
Nutritional status	No wasting (WAZ > −2 SD)	170/187	90.9
	Moderate wasting (WAZ ≤ −2 SD and > −3 SD)	10/187	5.4
	Severe wasting (WAZ ≤ -3 SD)	7/187	3.7
Vital signs	Increased respiratory rate according to age[d]	92/184	50.0
	Hypoxemia (SpO2 < 90%)	140/187	74.9
	Fever (≥37.5 °C)	77/187	41.2
	High fever (>39 °C)	9/187	4.8
Inspection	Central cyanosis	13/188	6.9
	Rhinorrhoea	63/188	33.5
	Lower chest wall indrawing	102/188	54.3
	Severe chest indrawing (supraclavicular and/or suprasternal)	22/187	11.8
	Nasal flaring	39/188	20.7
	Head nodding	2/187	1.1
	Grunting	10/188	5.3
	Deep breathing	0/188	0
	Digital clubbing	0/188	0
Auscultation	Crackles	108/188	57.5
	Rhonchi	85/188	45.2
	Wheezing	47/188	25.0
	Prolonged expiration	30/188	16.0
	Reduced air entry	17/188	9.0
	Inspiratory stridor	6/188	3.2
	Tubaric murmur	1/188	0.5
	Heart murmur	8/188	4.3
Other signs	Time for capillary refill > 2 s	7/188	3.7
	Weak peripheral pulses	7/188	3.7
	Weak central pulses	4/188	2.1
	Clinical shock	7/188	3.7
	Hepatomegaly	17/188	9.0
	Splenomegaly	2/188	1.1
	Glasgow coma score <15	8/188	4.3
	Prostration	2/188	1.1

Abbreviations: GCS: Glasgow coma scale; NA: not applicable; WAZ: weight-for-age *Z*-score.
[a] Two children presented with convulsions. One was diagnosed as febrile convulsion, while the other child was a severe case of pneumonia which led to a fatal outcome.
[b] Some children who presented with non-severe pneumonia developed hypoxemia during their hospitalization, which is a sign of severity as per the WHO definition.
[c] Twenty children (10.6%) did not strictly meet the WHO definition of pneumonia at the time of admission but were admitted to the paediatric ward with suspected pneumonia or bronchiolitis as per the clinical discretion of the treating paediatricians. Four of them developed hypoxemia during hospitalization requiring oxygen therapy and were therefore classified as severe pneumonia. None of the remaining 16 children were admitted to PICU or presented other signs of severity, and were classified as non-severe pneumonia (Supplementary Table 1).
[d] Increased respiratory rate (RR) according to age is defined as RR ≥ 50 bpm in children aged 2–12 months and RR ≥ 40 bpm in children aged ≥12 months.

NPW was not collected in three children due to the severity of their illness upon arrival. Of the other three children, one child presented a triple co-infection by *B. pertussis*, parainfluenza virus, and influenza virus. Four fatal cases were diagnosed as suffering of pneumonia, and two of bronchiolitis. Two deaths occurred within the first 24 h of admission to our centre. A summary of the main characteristics of these six children is presented in Supplementary Table 5.

Table 3
Laboratory findings on admission, blood sample

		n/N	%
Haematology			
Anaemia	Yes (Hb < 11 g/dL)	67/187	35.8
	Mild (Hb ≥ 10 and <11 g/dL)	31/187	16.6
	Moderate (Hb ≥ 7 and <10 g/dL)	35/187	18.7
	Severe (Hb < 7 g/dL)	1/187	0.5
Abnormal count of WBC (10^9/L)	Leucopenia (<5.0)	7/187	3.7
	Leucocytosis[a]	69/187	36.9
	Neutrophilia (≥70% of WBC)	47/186	25.3
	Neutropenia (<1.5)	3/186	1.6
Abnormal count of platelets (10^9/L)	Thrombocytosis (>450)	47/183	25.7
	Thrombocytopenia (<150)	2/183	1.1
Biochemistry			
Urea (mg/dL)	Urea > 40	4/116	3.4
Creatinine (mg/dL)	Creatinine > 1.2	4/117	3.4
Sodium (mEq/L)	Hyponatremia (<135)	15/119	12.6
	Hypernatremia (>145)	11/119	9.2
Potassium (mEq/L)	Hypokalemia (<3.5)	5/119	4.2
	Hyperkalemia (>5.5)	4/119	3.4
Inflammatory markers			
CRP	High CRP (>4 mg/dL)	25/178	14.0
ESR	High ESR (≥50 mm)	25/168	14.9

Abbreviations: CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; Hb: haemoglobin; WBC: white blood cells.
[a] Leucocytosis was defined as white blood cells greater than 15×10^9 cells/L for children aged between 2 and 11 months and greater than 13×10^9 cells/L for children aged between 12 and 59 months.

Table 4
Microbiological findings

	n/N	%
Invasive bacterial disease[a]		
Non-contaminated positive blood culture[b]	8/148[c]	5.4
S. pneumoniae isolated by blood culture	2/148	1.4
S. pneumoniae isolated by RT-PCR in dried blood spot sample (Ct LytA)	1/148	0.7
Non-contaminated positive pleural culture	1/1	100
S. pneumoniae isolated by pleural fluid culture	1/1	100
Viral detection		
Rapid flu test in pharyngeal swab	9/32[d]	28.0
At least one virus detected in NPW	103/115	89.6
Among children with positive virus findings in NPW		
Single viral infection in NPW	68/103	66.0
Mixed viral infection in NPW	35/103	34.0
RSV	52/115	45.2
Rhinovirus	42/115	36.5
Parainfluenza virus[e]	19/115	16.5
Influenza virus	16/115[f]	13.9
Adenovirus	8/115	7.0
Bocavirus	6/115	5.2
Human Metapneumovirus	4/115	3.5
Coronavirus (Cor229E, CorHKU1, CorNL63, CorOC43)	2/115	1.7

Abbreviations: NPW: nasopharyngeal washing; PCR: polymerase chain reaction; RSV: respiratory syncytial virus; RT-PCR: real-time polymerase chain reaction.
[a] Vials for blood culture were out of stock at the hospital for few weeks during the study period, leading to blood culture not being performed in 12 participants, although molecular screening in bloodspots in filter paper was conducted for all 10 of these children.
[b] Coagulase-negative staphylococci, and *Bacillus* spp were considered contaminants, as per our protocol.
[c] Bacterial growth was detected in 22 blood cultures, but it was attributed to contamination in 14 cases.
[d] Seven children had positive rapid flu test for influenza A, one child for influenza B, and one child for influenza A and B. Out of the seven children with rapid flu test positive for influenza A, detection of influenza A by RT-PCR in NPW was also positive in four cases, but negative in one case, and "failed/inhibited" in the remaining two cases. For the child with rapid flu test positive for influenza B and for the child with rapid flu test positive for both influenza A and B, RT-PCR in NPW was negative for both influenza A and B in both children.
[e] Parainfluenza viruses 1, 2, 3, and 4 were detected in 2 (1.7%), 1 (0.9%), 14 (12.2%), and 3 (2.6%) children respectively.
[f] Fourteen were influenza A, and two were influenza B.

Discussion

This is the first published series of comprehensive epidemiological, clinical, and microbiological data describing Bhutanese children under five years of age hospitalized with WHO-defined clinical pneumonia. Mortality related to pneumonia was 3.2%, similar to other studies from LMICs (Jroundi et al., 2014; Lazzerini et al., 2016; Bénet et al., 2017; Chen et al., 2018; O'Brien et al., 2019). Nevertheless, this remains high for Bhutan in spite of the country offering free and easily accessible healthcare services. The six children who died were referred from other health centres and reached the study hospital in critical condition.

The high proportion of infants in our study highlights that infants are particularly vulnerable and prone to hospitalization due to severe pneumonia (Fancourt et al., 2017; Chen et al., 2018; Jakhar et al., 2018). There was no child known or suspected to be infected with HIV, which is consistent with the very low number of under-five year old children infected with HIV in Bhutan (UNAIDS, 2018).

Table 5
Evolution during admission

		n/N	%
Evolution and outcome			
Hospital stay	<24 h	9/189	4.8
	≥24 to <72 h	67/189	35.4
	≥72 h to <7 days	82/189	43.4
	≥7 days	31/189	16.4
	Admission to paediatric intensive care unit	30/189	15.9
	Admission to high dependency unit	41/189	21.7
Management	Invasive mechanical ventilation	7/189	3.7
	Non-invasive mechanical ventilation	13/189[a]	6.9
	Oxygen therapy	142/189	75.1
	Antibiotics during admission	136/189	72.0
Outcome	Alive at discharge	183/189	96.8
	Death	6/189	3.2
	Transferred	1/189	0.5
	Absconded	0/189	0
	Withdrawn from the study	0/189	0

[a] Twelve children required continuous positive airway pressure (CPAP). One child was put on bilevel positive airway pressure (BiPAP) and was changed to CPAP after improvement. One child only required high flow nasal cannula oxygen.

Winter, which is the coldest season in Bhutan, surprisingly showed the lowest number of cases (10.1%); this finding differs from what is commonly seen in other settings, whereby hospitalization of childhood pneumonia tends to peak during the coldest season (Murdoch et al., 2014; Ben-shimol et al., 2015). However, this finding is consistent with those reported by the national sentinel surveillance programme for severe acute respiratory infections, and with the proportion of all-cause paediatric admissions, lower during winter (Royal Centre for Disease Control, 2018). This could be partially explained by the fact that winter coincides with the school break in Bhutan, with less contact among children; and families moving from the capital to the villages with lower population density.

Hypoxemia is a well-established predictor of severity in children with pneumonia (Duke et al., 2001; Lozano, 2001). A high proportion of children in this study (74.9%) presented with hypoxemia, which is much higher than reported in other settings (Subhi et al., 2009; O'Brien et al., 2019). We defined hypoxemia as SpO2 < 90%, which is considered appropriate for altitudes under 2500 m, as is the case with Thimphu (2334 m). This characteristic might therefore not be generalizable to Bhutanese children who live at different altitudes than that of Thimphu.

While bacterial aetiology was infrequent, viruses were identified in a considerable proportion of children. These microbiological findings coincide more with the etiological profile of pneumonia in children from high-income countries, highlighting the advanced stage of the epidemiologic transition that Bhutan seems to have reached (Omran, 2005; Prayle et al., 2011). The findings from the PERCH study, conducted in seven LMICs with routine use of PCV, are similar (O'Brien et al., 2019). Even in the absence of a deployed PCV in Bhutan (PCV was introduced only in January 2019), the burden of pneumococcal invasive disease appears to be low in children.

The low proportion of confirmed bacterial cases could be explained by several reasons. First, vaccination coverage was high, which is representative of the rest of the country, although the PCV was not in routine use during the recruitment period (WHO, 2016). Second, almost one-third of the children had received antibiotics prior to collection of blood sample, which reduces the yield of blood culture by around 45% (Berkley et al., 2005; Rhodes et al., 2010; Driscoll et al., 2017; O'Brien et al., 2019). Small blood volume is another factor known to compromise the sensitivity of blood culture (Berkley et al., 2005; Bouza et al., 2007; Driscoll et al., 2017). Blood collection is challenging in children, especially in infants. Blood volumes collected for each child were not recorded in this study but, in practice, around 1 mL was dedicated for blood culture in most cases, despite the 2–3 mL recommended in the protocol. Nevertheless, these findings confirm the low yield of blood culture in hospitalized children with pneumonia and question both the need of blood culture for uncomplicated cases of pneumonia and using blood culture as the preferred screening tool for invasive bacterial disease in children with pneumonia. Molecular methods have been found to be more sensitive than blood culture to detect pneumococcal invasive disease (Muñoz-almagro et al., 2011; Selva et al., 2013; O'Brien et al., 2019). This was not the case in this study.

B. pertussis was isolated in respiratory samples of three children. This is similar to the detection rate of around 1% of hospitalized pneumonia cases in similar studies (Jroundi et al., 2014; Barger-kamate et al., 2016). One of these three children, aged five months, had a fatal outcome. This underlines the high fatality ratio of pertussis-infected pneumonia, especially in infants who are unvaccinated, and suggests the need of intervention such as maternal vaccination to reduce morbi-mortality associated with pertussis in vulnerable populations.

Viral detection was common. The use of PCR techniques has increased the ability to detect respiratory viruses (Ruuskanen et al., 2011). However, evidence of the detection of viruses in asymptomatic individuals has raised concern about the clinical significance of these positive findings. Attribution of causality is not straightforward, as viruses can commonly be found both in symptomatic but also asymptomatic individuals (Jartti et al., 2008; Ruuskanen et al., 2011; Rudan et al., 2013; O'Brien et al., 2019). While the causative role of RSV, influenza, adenovirus, human metapneumovirus, and bocavirus in childhood pneumonia is well-established, the pathogenic role of other viruses such as rhinovirus is still questioned (Fry et al., 2007; Caracciolo et al., 2008; Ruuskanen et al., 2011; Shi et al., 2017; Jayaweera et al., 2018; O'Brien et al., 2019). Using molecular methods, rhinovirus has been shown to be the most frequent respiratory pathogen isolated in children, and its detection in asymptomatic children is significantly higher than other respiratory viruses (Kusel et al., 2006; Jartti et al., 2008; Ruuskanen et al., 2011). Nevertheless, clinical relevance of rhinovirus has been proven by the association of this virus with respiratory symptoms in children, mainly wheezing (Kusel et al., 2006; Khetsuriani et al., 2007). In our series, 27.5% of the children with rhinovirus presented with wheezing. Infection with coronavirus (Cor229E, CorHKU1, CorNL63, CorOC43) was low in the present study. Similarly, the new coronavirus (SARS-CoV-2) seems to cause a low infection rate in children (World Health Organization, 2020). The reason why coronavirus infection rate in children is low is unknown.

In addition, the interpretation of positive viral findings is challenging due to the identification of multiple co-existing viral infections (Jartti et al., 2008; Ruuskanen et al., 2011). Co-infections were common in the present study, which is consistent with the existing literature (Ruuskanen et al., 2011; Jroundi et al., 2014; Jiang et al., 2017). Considering radiological pneumonia endpoint as a proxy for bacterial pneumonia, 27.0% of children with positive NPW findings had a viral-bacterial co-infection, and 40.0% of children with influenza detected in NPW had an influenza-bacterial co-infection. The contribution of viral-bacterial co-infections is well-acknowledged in the aetiology of childhood pneumonia, particularly the interaction between influenza virus and *S. pneumoniae* (O'Brien et al., 2000; Kwofie et al., 2012; Brealey et al., 2015). The combined effect of bacteria and viruses was shown to increase the severity of the disease, and bidirectional interactions have been described: respiratory viruses leading to bacterial superinfection, and bacteria pathogens promoting respiratory viral superinfections (Brealey et al., 2015). However, there is still a lack of robustness supporting these findings.

This study has several limitations. Most children in the present study lived in Thimphu, and the microbiological findings may not be generalized to the rest of the country. Bhutan is very diverse: comprised of cities, such as Thimphu, and isolated households in very remote areas, leading to different lifestyles and environmental exposures; and also diverse in terms of altitude, with different climates and precipitations.

Conclusions

The burden of pneumonia requiring hospitalization was highest among infants. Respiratory viruses were detected in a considerable number of children, although a clear pathogenic role cannot be established. Together with the relatively low proportion of children presenting a likely bacterial pneumonia – around a quarter as per positive blood culture and radiological findings – these findings emphasize the advanced stage of the epidemiologic transition that Bhutan seems to have reached. This study is the first step to better understand the aetiology and clinicopathological characteristics of pneumonia in Bhutanese children. Henceforth, the development of targeted pneumonia interventions and hypothesis-driven research is encouraged to reduce the morbidity and mortality associated with this disease. Fostering a robust pneumonia aetiology surveillance in children under five years of age appears important and would allow the assessment of the impact of the recently introduced PCV in reducing the burden of pneumonia.

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Ethical approval

The study protocol was approved by the Research Ethics Board of Health, Ministry of Health, in Thimphu in March 2017 (protocol number PO/2016/086), and by the research ethics committee from the Hospital Clínic in Barcelona (HCB/2017/0741).

Conflict of interest

No conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at https://doi.org/10.1016/j.ijid.2020.04.017.

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Supplementary material

Definitions and details of variables measures

- Seasonality was defined according to Northern hemisphere seasonal patterns: summer from 21 June to 20 September, fall from 21 September to 20 December, winter from 21 December to 20 March, and spring from 21 March to 20 June.
- Temperature was measured axillary using a digital thermometer. Fever implied a documented axillary temperature of ≥ 37.5ºC.
- Peripheral capillary oxygen saturation was measured using Mindray VS-800 Vital Sign Monitor and Biolight BLT M800 Handheld pulse oximeter. Hypoxemia was defined as oxygen saturation in room air under 90% (Lazzerini et al. 2015).
- Nutritional status was based on the weight-for-age Z score (WAZ), generated using the 2000 Centers for Disease Control and Prevention Growth Reference (Centers for Disease Control and Prevention; Vidmar et al. 2013). Wasting was considered if WAZ score ≤ 2.
- Mechanical ventilation support was considered non-invasive when it was delivered through high-flow nasal cannula oxygen, continuous positive airway pressure (CPAP) or bilevel positive airway pressure (BiPAP), and invasive when positive pressure was delivered through an endotracheal tube. High-frequency oscillatory ventilation is currently not used in Bhutan.
- Discharge diagnoses were coded using the International Classification of Diseases, 10th Revision (WHO 2016).

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Supplementary table 1. Characteristics of children who did not fulfil the WHO definition of pneumonia on admission

Referral	Prior antibiotic	Pathological auscultation, stridor, or both	Received O_2	Antibiotic during admission	Probable reason for admission	NPW findings for virus
No	No	No	No	Yes	Fever (final diagnosis enteric fever)	Negative
No	No	Crackles	No	Yes	Pathological auscultation	Positive
No	No	Rhonchi	No	Yes	Pathological auscultation	Positive
No	No	Rhonchi	No	Yes	Pathological auscultation	Positive
No	Yes	Rhonchi	No	Yes	Pathological auscultation	Positive
No	No	No	No	Yes	Fever (final diagnosis flu A)	Failed
Yes	No	Crackles	No	Yes	Pathological auscultation	Positive
No	No	Rhonchi	No	No	Pathological auscultation	Negative
No	No	Crackles	No	Yes	Pathological auscultation	Positive
No	No	No	No	No	Fever (final diagnosis viral pneumonia and AGE)	Positive
No	No	No	No	No	Fever (final diagnosis URTI and AGE)	Positive
No	No	Crackles	No	No	Pathological auscultation	Positive
No	No	Crackles	Yes	No	Pathological auscultation	Positive
No	No	No	No	Yes	Fever (final diagnosis pneumonia)	Positive
No	No	Crackles	Yes	Yes	Pathological auscultation	Positive
No	No	Wheezing	Yes	No	Pathological auscultation	Not collected
No	No	Rhonchi	No	No	Pathological auscultation	Not collected
No	No	Crackles	No	Yes	Pathological auscultation	Positive
No	Yes	Stridor	No	Yes	Pathological auscultation	Not collected
No	No	No	Yes	Yes	Fever, hypoxemic (final diagnosis pneumonia)	Not collected

Abbreviations: AGE: acute gastroenteritis; NPW: nasopharyngeal washing; URTI: upper respiratory tract infection.

This table provides a summary of the 20 children who did not fulfil the WHO definition of pneumonia on admission, but were nevertheless admitted with suspicion of acute respiratory infection such as pneumonia or bronchiolitis. Fourteen were diagnosed with pneumonia based on pathological auscultation, three of them required oxygen therapy during admission. Another child also required oxygen therapy during admission. None of them required admission on paediatric intensive care unit or high dependency unit. The first patient was admitted with suspicion of respiratory infection, with infiltrates seen in the chest radiography by the treating physician, and therefore included in the study. During admission, *Salmonellla typhi* was isolated in blood culture, and the child was managed as enteric fever.

Supplementary table 2. Drug sensitivity pattern of bacterial isolates

Bacteria isolated by blood culture	Number of isolates	Sensitivity	Resistance
S. pneumoniae	2	Full to all tested	None
Pseudomonas spp	2	Full to all tested	None
		Ciprofloxacin Gentamicin Ceftazidime	Piperacillin
E. coli	1	Ampicillin Ceftriaxone Gentamicin Ciprofloxacin	Trimethoprim/ Sulfamethoxazole
Acinetobacter	1	Amikacin Ciprofloxacin Imipenem Gentamicin Meropenem Polymixin B	Piperacillin Ceftazidime
Salmonella typhi	1	Full to all tested	None
Serratia rubidaea	1	All tested except ampicillin	Ampicillin

Supplementary table 3. Respiratory viruses according to bacterial and radiological findings

		Blood culture for bacteria		CXR	
		Positive	**Negative**	**Endpoint pneumonia**	**Infiltrates or normal**
Virus in NPW	Positive	4/86 (4.6%)	82/86 (95.4%)	24/89 (27.0%)	65/89 (73.0%)
	Negative	3/9 (33.3%)	6/9 (66.7%)	4/11 (36.4%%)	7/11 (63.6%)
Influenza in NPW	Positive	0/15 (0%)	15/15 (100%)	6/15 (40.0%)	9/15 (60.0%)
	Negative	7/80 (8.8%)	73/80 (91.2%)	22/85 (25.9%)	63/85 (74.1%)

Abbreviations: CXR: chest radiograph; NPW: nasopharyngeal washing.

Supplementary table 4. Main diagnoses given at discharge by the treating physicians

Main diagnoses given at discharge by the treating physician (non-mutually exclusive)	n N=189	%
Pneumonia (including “lobar” and “unspecified”; excluding “viral pneumonia”)	49	25.9
Bronchopneumonia	44	23.3
Bronchiolitis	39	20.6
Lower respiratory tract infection	18	9.5
Bronchitis	10	5.3
Viral pneumonia	11	5.8
Influenza	8	4.2
Aspiration pneumonia	1	0.5
Pleural effusion	1	0.5
Upper respiratory tract infection	3	1.6
Asthma exacerbation	3	1.6
Whooping cough	2	1.1
Acute respiratory failure	2	1.1
Acute respiratory distress syndrome (ARDS)	3	1.6
Croup	2	1.1
Sepsis	9	4.8
Systemic inflammatory response syndrome	1	0.5
Viral fever	2	1.1
Measles	1	0.5
Otitis media	2	1.1
Urine tract infection	2	1.1
Congenital cardiac defects	9	4.8
Congenital laryngomalacia	1	0.5
Febrile convulsion	2	1.1
Anaemia	32	16.9
Malnutrition or failure to thrive	3	1.6
Diagnosis other than bronchiolitis, bronchitis, lower respiratory tract infection, bronchopneumonia, pneumonia, viral pneumonia, influenza, pleural effusion, croup, asthma exacerbation and whooping cough	12	6.4

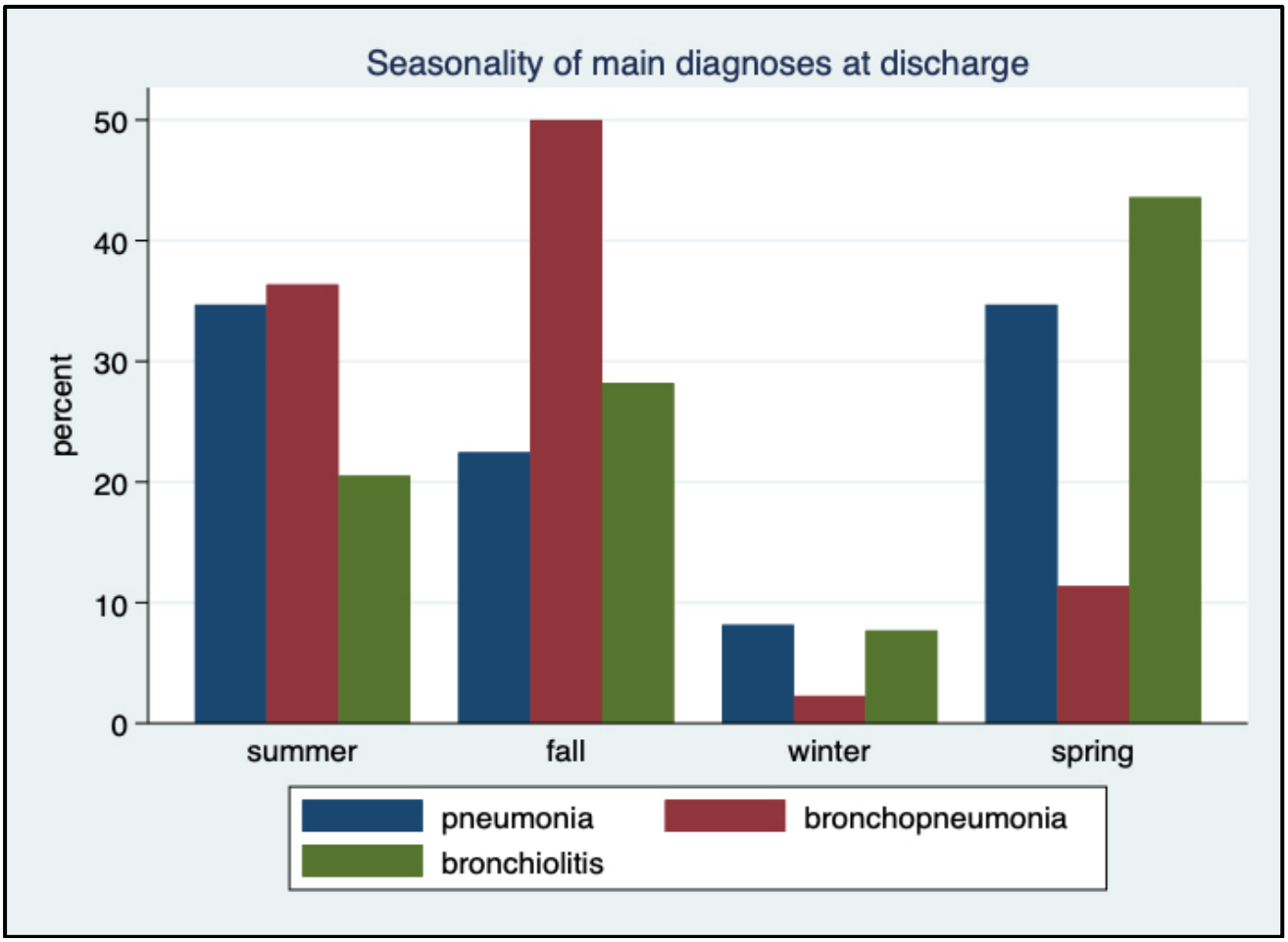
Supplementary table 5. Characteristics of children with fatal outcome

Characteristics	Child 1	Child 2	Child 3	Child 4	Child 5	Child 6
Age, months	3	48	3	5	3	7
Gender	Female	Male	Female	Male	Male	Female
Month of admission	July	April	May	June	June	June
Referred	Yes	Yes	Yes	Yes	Yes	Yes
Immunized	Partially	Totally	Totally	Partially	Partially	Partially
Malnutrition	No	No	No	No	No	No
Duration of illness prior to admission	6 days	7 days	5 days	10 days	7 days	2 days
Duration of fever prior to admission	No fever	5 days	1 day	No fever	7 days	2 days
Time to access health care facility	10 min	Unknown	60 min	Unknown	Unknown	30 minutes
Transport to access health care facility	On foot	Unknown	By taxi	Unknown	Unknown	By taxi
Parents' education	Both basic education	Unknown	Both illiterate	Only one parent basic education	Unknown	Both basic education
Parents' employment	Both employed	Only one parent employed	Both employed	Unknown	Unknown	Only one parent employed
CXR	Infiltrates	Infiltrates	Missing	Normal	Pneumonia	Infiltrates
WBC (10^9/L)	2.7	33.31	19.4	7	Missing	32.2
CRP (mg/dL)	0.8	2.2	Missing	0.1	Missing	3.2
ESR (mm)	Missing	60	2	2	Missing	30
Blood culture	Negative	Contamination	Negative	Negative	Not collected	Negative
Pathogens identified in NPW	Rhinovirus	Inhibited result	NPW not collected	Influenza A Parainfluenza B. pertussis	NPW not collected	NPW not collected
Main diagnosis	Bronchiolitis	Pneumonia	Bronchiolitis	Pneumonia	Pneumonia	Pneumonia
Time of death after admission	89 hours	84 hours	20 hours	48 hours	2 hours	72 hours

Abbreviations: CRP: C-reactive protein; CXR: chest radiography; ESR: erythrocyte sedimentation rate; NPW: nasopharyngeal washing; WBC: white blood cells

The six children who died were referred from other centres. All of them were directly admitted to paediatric intensive care unit and put on invasive mechanical ventilation if not previously started. They were all started on intravenous antibiotics prior to referral and admission to our centre. Five of them were infants. Five cases happened during spring. NPW was not collected in three children due to the severity of their illness upon arrival.

Supplementary figure 1. Seasonality of main diagnoses given at discharge



ARTICLE 4

Pneumococcal nasopharyngeal carriage among Bhutanese children hospitalized with clinical pneumonia: serotypes and viral co-infection

Sophie Jullien, Ragunath Sharma, Mimi Lhamu Mynak, Desiree Henares, Carmen Muñoz-Almagro, Quique Bassat

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Pneumococcal nasopharyngeal carriage among Bhutanese children hospitalized with clinical pneumonia: serotypes and viral co-infection

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Abstract

Background: Pneumococcal nasopharyngeal colonization (PNC) generally precedes pneumococcal disease. The purpose of this study was to determine the prevalence of PNC and to identify the pneumococcal serotypes circulating among Bhutanese children under five years of age admitted with clinical pneumonia, before the introduction of pneumococcal conjugate vaccine (PCV13) in the country. We also aimed to contribute to the understanding of the interplay between PNC and viral co-infection among this population.

Methods: This was a prospective study conducted at the Jigme Dorji Wangchuck National Referral Hospital in Bhutan over 12 consecutive months. Children aged 2 to 59 months admitted with WHO-defined clinical pneumonia were eligible for recruitment. We collected blood for bacterial culture and molecular identification of *S. pneumoniae*, and nasopharyngeal washing for screening of respiratory viruses, and for the detection and capsular typing of *S. pneumoniae* by real-time polymerase chain reaction (RT-PCR).

Results: Overall, 189 children were recruited, and PNC was tested in 121 of them (64.0%). PNC was found in 76/121 children (62.8%) and *S. pneumoniae* was identified in blood (both by culture and RT-PCR) in a single child. Respiratory viruses were detected in a similar proportion among children with (62/70; 88.6%) and without PNC (36/40; 90.0%; $p = 1.000$), but rhinovirus detection was less common among children with PNC (20/70; 28.6% versus 19/40; 47.5%; $p = 0.046$). Capsular typing identified 30 different serotypes. Thirty-nine children (51.3%) were colonised with two to five different serotypes. A third of the children presented with serotypes considered highly invasive. Over half of the children (44/76; 57.9%) were carrying at least one serotype included in PCV13.

(Continued on next page)

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Conclusions: This study provides baseline information on the status of PNC among Bhutanese children admitted with clinical pneumonia prior to the introduction of PCV13, which is valuable to monitor its potential impact. PCV13 could theoretically have averted up to 58% of the pneumococcal infections among the children in this study, suggesting a future role for the vaccine to significantly reduce the burden associated with *S. pneumoniae* in Bhutan.

Keywords: *Streptococcus pneumoniae*, Pneumonia, Colonization, Bhutan, Child preschool

Background

Streptococcus pneumoniae is a common cause of invasive bacterial disease (IBD), including pneumonia, meningitis and sepsis in children under five years of age. Although the prevalence of pneumococcal associated IBD and deaths has declined in the last two decades, mainly as a result of the parsimonious global introduction of pneumococcal conjugate vaccines (PCV), this pathogen still causes a significant burden. In 2015, *S. pneumoniae* was estimated to cause 8.9 million cases of clinical pneumonia in children aged 1 to 59 months, and 294,000 deaths among HIV-uninfected children aged 1 to 59 months. The majority of these deaths (81%) were attributed to pneumonia [1]. The vast majority of the pneumococcal burden is now concentrated in low- and middle-income countries (LMIC). In 2015, approximately 50% of all pneumococcal deaths were registered in four countries: India, Nigeria, the Democratic Republic of the Congo, and Pakistan [1].

Pneumococcal nasopharyngeal colonization (PNC) is generally considered a prerequisite for pneumococcal disease and is a source of spread between people [2, 3]. However, *S. pneumoniae* is part of the commensal nasopharyngeal flora and in most instances, PNC does not lead to disease [3]. While the association between PNC and development of acute otitis media is well recognised, its relationship with pneumonia is less strongly established [4, 5]. Other bacteria and viruses are common colonizers of the nasopharynx. The interplay between respiratory viruses and *S. pneumoniae* on the progression to the disease is still poorly understood [6, 7].

By December 2019, 145 countries had introduced PCV into their national immunization programme and 15 additional countries were planning to do so [8]. Currently, there are two WHO prequalified vaccines that are commonly used: PCV13 (Prevenar 13®, Pfizer) and PCV10 (Synflorix®, GlaxoSmithKline) that include 13 and 10 serotypes respectively [9, 10]. A third vaccine that also includes 10 serotypes (Pneumosil®, Serum Institute of India) was recently prequalified by WHO in December 2019 [9]. The introduction of PCV has substantially reduced both the burden of pneumococcal invasive disease and the rates of PNC by serotypes included in the vaccine [1, 11–14]. The emergence of serotypes not included in the vaccines has been well documented in high-income countries but little is known in LMIC [15–17], and surveillance data at a national level are important to identify serotype replacement and to assess most prevalent serotypes still circulating in the population.

Bhutan is a small country landlocked in the Himalayas, with an estimated population of 779,666 in 2017 [18, 19]. It is currently classified as a lower-middle income country [20]. The Constitution guarantees free essential health services for Bhutanese citizens, based on a primary health care approach [21]. Similar to other LMIC, pneumonia remains a major public health challenge in Bhutan, whereby the number of outpatient visits and hospitalizations attributed to pneumonia constitutes a considerable burden to the health system [22]. The conjugate *Haemophilus influenza type b* (Hib) vaccine has been routinely administered since 2011, and PCV13 was introduced in the immunization programme in January 2019 [23, 24]. Despite the burden that pneumonia represents for the country, there are scarce national data on the epidemiology and aetiology of childhood pneumonia, leading to challenges while implementing effective national preventive strategies [25]. Furthermore, while information on the pneumococcal serotypes circulating before the introduction of PCV13 is essential to monitor the impact of the vaccine, there are no data on circulating pneumococcal serotypes among the Bhutanese population [25].

We conducted this prospective study to determine the prevalence of pneumococcal carriage and to identify the pneumococcal serotypes circulating among Bhutanese children under five years of age admitted with WHO-defined pneumonia, before the introduction of PCV in the country. We also aimed to contribute to the understanding of the interplay between PNC and viral co-infections among this population.

Methods

Study design and patient enrolment

This prospective Respiratory Infection in Bhutanese Children (RIBhuC) study was conducted over 12 consecutive months at the Jigme Dorji Wangchuck National Referral Hospital (JDWNRH) in Thimphu, Bhutan. The RIBhuC study aimed to describe the epidemiology, aetiology and clinico-radiological presentation of WHO-

defined pneumonia among admitted children under five years of age. The recruitment process and data collection have been described elsewhere [26]. In brief, we recruited all children aged 2 to 59 months who were admitted at JDWNRH with a diagnosis of pneumonia (including severe pneumonia) according to the WHO definitions [27]. Pneumonia was defined as history of cough or reported breathing difficulty, together with increased respiratory rate (respiratory rate ≥ 50 breaths per minute in children aged 2 to 11 months; or respiratory rate ≥ 40 breaths per minute in children aged 12 to 59 months) or chest indrawing. Severe pneumonia was defined as history of cough or reported breathing difficulty, and at least one of the following: oxygen saturation < 90% or central cyanosis, severe respiratory distress (e.g. grunting, very severe chest indrawing), or general danger sign (inability to breastfeed or drink, lethargy or reduced level of consciousness, convulsions). Children admitted in the previous seven days were not recruited to the study in order to exclude hospital-acquired infections. Children whose principal reason for admission was a non-respiratory illness or a condition that was not caused by respiratory illness, and those with evidence of a foreign body in the respiratory tract were also excluded.

Data collection

For all eligible patients whose parents consented to participate in the study, we performed a meticulous physical examination and collected biological samples at time of admission (or as soon as possible after admission) and before initiation of antibiotics. This included blood samples for bacterial culture, full blood cell count, and biochemistry; and nasopharyngeal washing (NPW) for the identification of *S. pneumoniae*, respiratory viruses and atypical bacteria. We collected demographic and clinical data from the medical records and by interviewing the parents. A chest radiography (CXR) was indicated for each child on admission.

Specimen collection and laboratory testing

Nasopharyngeal sample collection and storage

Respiratory secretions were collected through NPW, using 1 to 3 mL of 0.9% saline solution with a commercial mucus extractor kit, and sent to the local microbiology lab within 30 min, according to the corresponding standard of procedure developed in our protocol [28]. Specimens were homogenized, aliquoted, frozen at − 80 °C, and shipped to Hospital Sant Joan de Déu in Barcelona, Spain, for centralised molecular analyses.

Pneumococcal detection and capsular typing from respiratory secretions

We performed a duplex real-time polymerase chain reaction (RT-PCR) targeting the *lytA* gene of *S. pneumoniae* and the internal control targeting RNaseP of human cells for DNA amplification, using the Applied Biosystems 7500 RT-PCR System (Applied Biosystems, CA, US) [29]. We performed capsular typing of *S. pneumoniae* in all *lytA* positive samples with a fragment analysis multiplex PCR for distinguishing 40 serotypes [30]. We considered the serotypes 1, 3, 4, 5, 7F, 14, 18C and 19A as highly invasive according to findings from other studies [31–36], and refer to the remaining serotypes as 'less-highly invasive'.

Detection of respiratory viruses and atypical bacteria from respiratory secretions

For identification of respiratory viruses and atypical bacteria, we used the multiplex RT-PCR QIAStat respiratory panel, Qiagen, which includes 17 viral targets (adenovirus, bocavirus, coronavirus 229E/HKU1/NL63/OC43, human metapneumovirus, influenza virus A/B [A subtypes H1N1pdm09, H1, H3], parainfluenza viruses 1/2/3/4, respiratory syncytial virus, rhinovirus) and four bacteria (*Bordetella pertussis*, *Chlamydophila pneumophila*, *Legionella pneumophila*, and *Mycoplasma pneumoniae*) [37, 38].

Blood collection and testing

We collected blood for haematology, biochemistry and bacterial culture. Blood samples were processed at JDWNRH following standardized procedures. Blood was cultured using an automated blood culture system (BacT/ALERT®), and bacterial isolates were identified by colony morphology, growth requirements, and basic biochemical tests. Two drops of blood were collected on filter paper and shipped to Spain for further screening of *S. pneumoniae* (LytA gene) by RT-PCR.

Chest radiography interpretation

The methods we used for the interpretation of CXR are described in detail elsewhere [26]. In brief, we followed the WHO protocol used in clinical trials of PCV [39]. Two paediatricians independently assessed each CXR, a third reader read the discordant results, and a paediatric radiologist interpreted again all CXR for additional reliability. CXRs were classified as either radiologically confirmed endpoint pneumonia (defined as consolidation, pleural effusion or both on any hemithorax), other infiltrates, or normal.

Data management and statistical analysis

The lead investigator entered data into a computerized password-protected database (ODK®) with study identification number. We limited errors in data entry by predefining ranges for every value. We used Stata 16.0 for data analysis [40]. We examined the association between

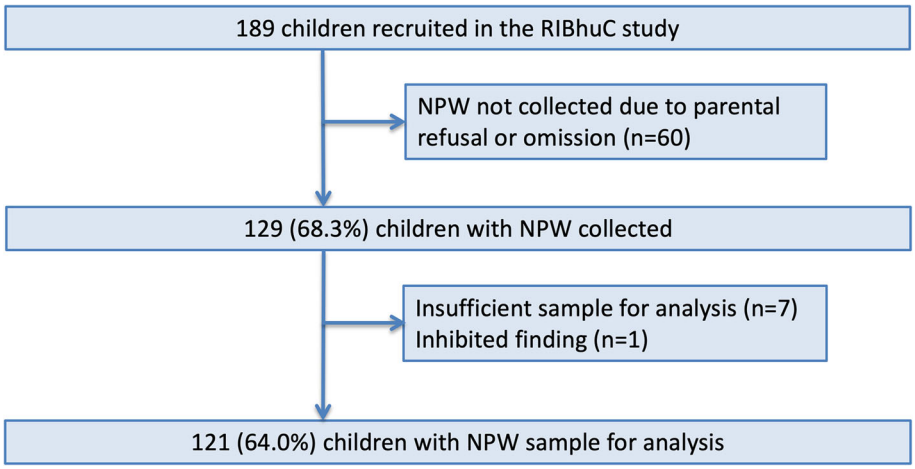

Fig. 1 Study profile. Abbreviations: NPW: nasopharyngeal washing; RIBhuC: Respiratory Infections in Bhutanese Children

pneumococcal carriage and a set of clinical signs and potential risk factors by using Chi-square or Fisher exact test (for categorical variables) and Wilcoxon rank-sum test (for continuous variables). A *p*-value less than 0.05 was considered statistically significant.

Results

Among the 189 children recruited in the RIBhuC study between 1st July 2017 and 30th June 2018 [26], NPW was collected in 129 children (68.3%). After microbiological screening for virus, there was insufficient sample for pneumococcal testing in seven children, and one sample showed an inhibited finding, leaving 121/189 cases (64.0%) for analysis (Fig. 1).

Pneumococcal nasopharyngeal carriers' prevalence and characteristics

Pneumococcal carriage was found in 76/121 children (62.8%). We reported the baseline characteristics of children by status of nasopharyngeal colonization of *S. pneumoniae* in Table 1. Among colonized children, around half of them were infants (54.0%), and this proportion of infants was similar in the non-colonized (53.3%) group. Among colonized children and compared to non-colonized ones, there was a significant higher proportion of females (38/76, 50.0%; versus 13/45, 28.9%; $p = 0.023$).

There was no significant difference in the proportion of children with at least another child under five years of age in the same household between colonized and non-colonized children (42.1% versus 31.1%; $p = 0.229$). Colonized children were less likely to have received antibiotics prior to admission (13.3% versus 31.1%; $p = 0.018$) and prior to NPW specimen collection (31.6% versus 71.1%; $p = < 0.001$).

On CXR, there was a trend for children with PNC to present less infiltrates than those without colonization (10.4% versus 29.0%), but there was no differences in the proportion of children with endpoint pneumonia (29.9% versus 26.3%; $p = 0.051$).

There were no significant differences between colonizers and non-colonizers in regard to laboratory findings (such as C-reactive protein or erythrocyte sedimentation rate), outcome, and prognosis.

Microbiological findings by pneumococcal nasopharyngeal colonization

Bacterial findings

Overall, six children had bacteria isolated by blood culture (five with PNC and one without PNC), of which only one was *S. pneumoniae* (Table 2).

S. pneumoniae was isolated by blood culture in a single child, and subsequently confirmed by molecular methods (RT-PCR in dried blood spot). That same child was found to be a pneumococcal carrier in the nasopharynx.

Association of PNC with viral co-infection

Respiratory viruses were detected in a similar proportion among children with and without PNC (62/70; 88.6% versus 36/40; 90.0%; $p = 1.000$) (Table 2). However, rhinovirus detection was more common among children without PNC (47.5% in non-colonised children versus 28.6%; $p = 0.046$), whereas respiratory syncytial virus (RSV) was more common in the colonized group, although this did not reach statistical significance (50.0% in colonized children versus 32.5%; $p = 0.075$). No further significant differences were found regarding the detection of other viruses.

Table 1 Baseline characteristics of children with and without pneumococcal nasopharyngeal colonization

	***S. pneumoniae* colonization** ***n* = 76 (62.8%)**	**No *S. pneumoniae* colonization** ***n* = 45 (37.2%)**	***p*-value[a]**
Demographic characteristics			
Gender, female	38/76 (50.0%)	13/45 (28.9%)	0.023
Age in months (median, IQR)	10.8 (7.6–23.4)	9.7 (4.0–23.0)	0.169
Age groups			0.948
Infants (< 12 months)	41/76 (54.0%)	24/45 (53.3%)	
≥ 12 months	35/76 (46.0%)	21/45 (46.7%)	
HIV infection	0/76 (0%)	0/45 (0%)	NA
Vaccination status			0.157
Fully vaccinated according to age	63/75 (84.0%)	33/45 (73.3%)	
Partially vaccinated according to age	12/75 (16.0%)	12/45 (26.7%)	
Not vaccinated	0/75 (0%)	0/45 (0%)	
At least one other child under 5 years of age in the household	32/76 (42.1%)	14/45 (31.1%)	0.229
6 people or more living in the household	30/76 (39.5%)	16/45 (36.4%)	0.736
Education			0.157
Both parents are illiterate	10/75 (13.3%)	8/44 (18.2%)	
Only one parent has primary education	17/75 (22.7%)	4/44 (9.1%)	
Both parents have primary education	28/75 (37.3%)	23/44 (52.3%)	
At least one parent has university education	20/75 (26.7%)	9/44 (20.4%)	
Employment			1.000
Both parents are unemployed	1/71 (1.4%)	1/43 (2.3%)	
Only one parent is employed	46/71 (64.8%)	27/43 (62.8%)	
Both parents are employed	24/71 (33.8%)	15/43 (34.9%)	
Season			0.677
Summer	27/76 (35.5%)	15/45 (33.3%)	
Fall	30/76 (39.5%)	16/45 (35.6%)	
Winter	5/76 (6.6%)	6/45 (13.3%)	
Spring	14/76 (18.4%)	8/45 (17.8%)	
History and severity of the current episode			
Antibiotics started prior to admission	10/75 (13.3%)	14/45 (31.1%)	0.018
Antibiotics prior to NPW collection			< 0.001
No	52/76 (68.4%)	13/45 (28.9%)	
Yes, for less than 24 h	13/76 (17.1%)	14/45 (31.1%)	
Yes, for more than 24 h	11/76 (14.5%)	18/45 (40.0%)	
Days of fever prior admission (median, IQR)	3 (1–5)	2 (0–4)	0.363
WHO severe pneumonia on admission	55/76 (72.4%)	38/45 (84.4%)	0.256
Severe pneumonia during admission	60/76 (79.0%)	39/45 (86.7%)	0.287
Radiological findings			
CXR findings			0.051
Pneumonia endpoint	20/67 (29.9%)	10/38 (26.3%)	
Other infiltrates	7/67 (10.4%)	11/38 (29.0%)	
Normal	40/67 (59.7%)	17/38 (44.7%)	
Laboratory findings			
CRP > 4 mg/dL	10/73 (13.7%)	8/42 (19.1%)	0.447

Table 1 Baseline characteristics of children with and without pneumococcal nasopharyngeal colonization *(Continued)*

	***S. pneumoniae* colonization** ***n* = 76 (62.8%)**	**No *S. pneumoniae* colonization** ***n* = 45 (37.2%)**	***p*-value[a]**
ESR ≥ 50 mm	13/69 (18.8%)	4/39 (10.3%)	0.239
Leucocytosis	34/76 (44.7%)	15/44 (34.1%)	0.253
Neutrophilia	28/76 (36.8%)	9/44 (20.5%)	0.061
Evolution and outcome			
Oxygen therapy during hospitalization	52/76 (68.4%)	34/45 (75.6%)	0.403
Duration of hospitalization			0.285
< 24 h	3/76 (3.9%)	2/45 (4.4%)	
≥ 24 to < 72 h	31/76 (40.8%)	12/45 (26.7%)	
≥ 72 h to < 7 days	29/76 (38.2%)	25/45 (55.6%)	
≥ 7 days	13/76 (17.1%)	6/45 (13.3%)	
Fatal outcome	1/76 (1.3%)	1/45 (2.2%)	1.000
Poor prognosis, simple definition (admission to PICU and/or fatal outcome)	10/76 (13.2%)	7/45 (15.6%)	0.714
Extended poor prognosis definition (admission to PICU, admission to HDU, fatal outcome, and/or hospitalization ≥7 days)	23/76 (30.3%)	15/45 (33.3%)	0.725

***Abbreviations*:** *CRP* C-reactive protein, *CXR* chest radiography, *ESR* erythrocyte sedimentation rate, *HDU* high dependency unit, *IQR* interquartile range, *NA* not applicable, *PICU* paediatric intensive care unit

[a]We examined the association between pneumococcal carriage status and the selected variables using Chi-square or Fisher exact test (for categorical variables) or Wilcoxon rank-sum test (for continuous variables non-normally distributed)

Distribution of pneumococcal serotypes

Thirty different serotypes (or groups of serotypes when it was not possible to differentiate them) were identified among the 76 children with PNC (Fig. 2). Over half of the children (39/76; 51.3%) were colonized with at least two and up to five different serotypes. The less-highly invasive serotypes 7B/C or 40 (the laboratory technique being unable to differentiate between these three serotypes) were the most common identified, being detected in 33/76 children (43.4%). The following most common less-highly invasive serotypes were 6A/B (12/76; 15.8%), 14 (9/76; 11.8%), and 23F (6/76; 7.9%). Other serotypes not included in the multiplex PCR technique used for this study were found in 10/76 children (13.2%).

Around a third of the children (24/76; 31.6%) presented with highly invasive serotypes, out of which the most common were 14 (9/76; 11.8%), 3 (5/76; 6.6%), and 1 (5/76; 6.6%). Over half of the children (44/76; 57.9%) presented at least one serotype included in PCV13, and half of the children presented at least one serotype included in any of the two PCV10 (38/76 [50.0%] for Synflorix® and 37/76 [48.7%] for Pneumosil®) (Fig. 3).

Discussion

We found a prevalence of PNC of 62.8%, which is comparable to that of other developing countries before the introduction of PCV [6, 17, 41]. Similar studies in India found a PNC prevalence ranging between 35 and 75% [41, 42]. We found no published data on PNC in children admitted with clinical pneumonia from other neighbouring countries. We used PCR to detect PNC, which is more sensitive than culture, and thereby which could lead to a higher prevalence as compared to pneumococcal carriage studies that used culture. However, the recent multicentric study conducted in nine settings over eight developing and emerging countries also used PCR and found an overall PNC prevalence of 68.1% among children admitted with suspected pneumonia, similar to our study [41]. As expected, prior administration of antibiotics in our study appeared to reduce pneumococcal carriage detection [17, 43].

The most prevalent serotypes were 7B/7C/40 (indistinguishable by laboratory technique) identified in 43.4% of the children, followed by 6A/B (15.8%), 14 (11.8%), 23F (7.9%), 3 and 1 (6.6% each). This serotype distribution is rather different from that described in similar studies. We found a much higher proportion of children with the serotype 7B/7C/40, while the proportion of children we identified with the serotypes 19F, 6A or 6B was considerably lower than in similar studies (proportion of serotypes in children sick with respiratory symptoms) [7, 17, 41, 42, 44] or among healthy children in community-based carriage studies [43, 45–48]. In the neighbouring context of India, 6A/B and 19F were also found to be the most prevalent serotypes in children admitted with clinical pneumonia, together with serotypes 14 and 23F. In a systematic review that included Indian studies looking at prevalence of serotype distribution among children with invasive pneumococcal disease prior to introduction of PCV, the most prevalent serotypes in

Table 2 Bacterial and viral findings by pneumococcal nasopharyngeal colonization

	No *S. pneumoniae* colonization *n* = 45 (37.2%)	*S. pneumoniae* colonization *n* = 76 (62.8%)	*p*-value[a]
Bacterial findings			
Positive bacterial blood culture (of any cause)	1/39 (2.6%)	5/62 (8.1%)[b]	0.401
Positive *S. pneumoniae* (blood culture)	0/39 (0%)	1/62 (1.6%)	1.000
Positive *S. pneumoniae* (dry blood spot, RT-PCR)	0/38 (0%)	1/64 (1.6%)	1.000
Positive for atypical bacteria in NPW			
Bordetella pertussis	1/40 (2.5%)	2/70 (2.9%)	1.000
Chlamydophila pneumophila	0/40 (0%)	0/70 (0%)	NA
Legionella pneumophila	0/40 (0%)	0/70 (0%)	NA
Mycoplasma pneumoniae	0/40 (0%)	1/70 (1.4%)	1.000
Viral findings			
At least one virus identified in NPW	36/40 (90.0%)	62/70 (88.6%)	1.000
Multiple (≥2) viruses identified in NPW	13/36 (36.1%)	20/62 (32.3%)	0.697
Positive for respiratory syncytial virus	13/40 (32.5%)	35/70 (50.0%)	0.075
Positive for rhinovirus	19/40 (47.5%)	20/70 (28.6%)	0.046
Positive for influenza virus	8/40 (20.0%)	8/70 (11.4%)	0.220
Positive for parainfluenza virus	5/40 (12.5%)	13/70 (18.6%)	0.408
Positive for adenovirus	3/40 (7.5%)	5/70 (7.1%)	1.000
Positive for bocavirus	4/40 (10.0%)	2/70 (2.9%)	0.188
Positive for human metapneumovirus	2/40 (5.0%)	2/70 (2.9%)	0.621
Positive for coronavirus	1/40 (2.5%)	1/70 (1.4%)	1.000

Abbreviations: *NPW* nasopharyngeal washing, *RT-PCR* real-time polymerase chain reaction
[a]We compared the proportions of the selected variables between pneumococcal colonization and non-colonization using Chi-square or Fisher exact test (for categorical variables) or Wilcoxon rank-sum test (for continuous variables non-normally distributed)
[b]One culture positive to *S. pneumoniae*

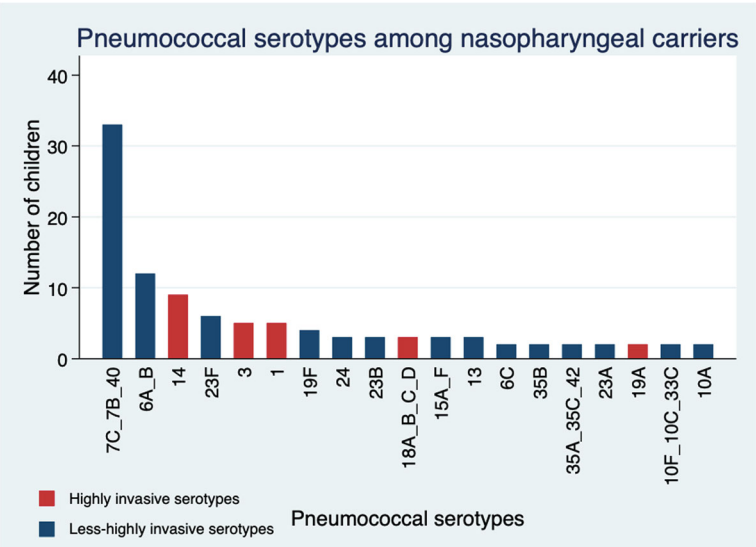

Fig. 2 Bar graphs of pneumococcal serotypes among nasopharyngeal carriers. Other serotypes were identified in one child each: high invasive (4, 5, 7F/A, and 15B/C) and non-high invasive (9 V/A, 16F, 20, 22A/F, 34, 35F/47F, and 39). Among the 12 children presenting with the serotype 6A/B, 2 cases were serotype 6A, 2 cases were serotype 6B, and it was not possible to differentiate between 6A and 6B in the remaining 8 cases

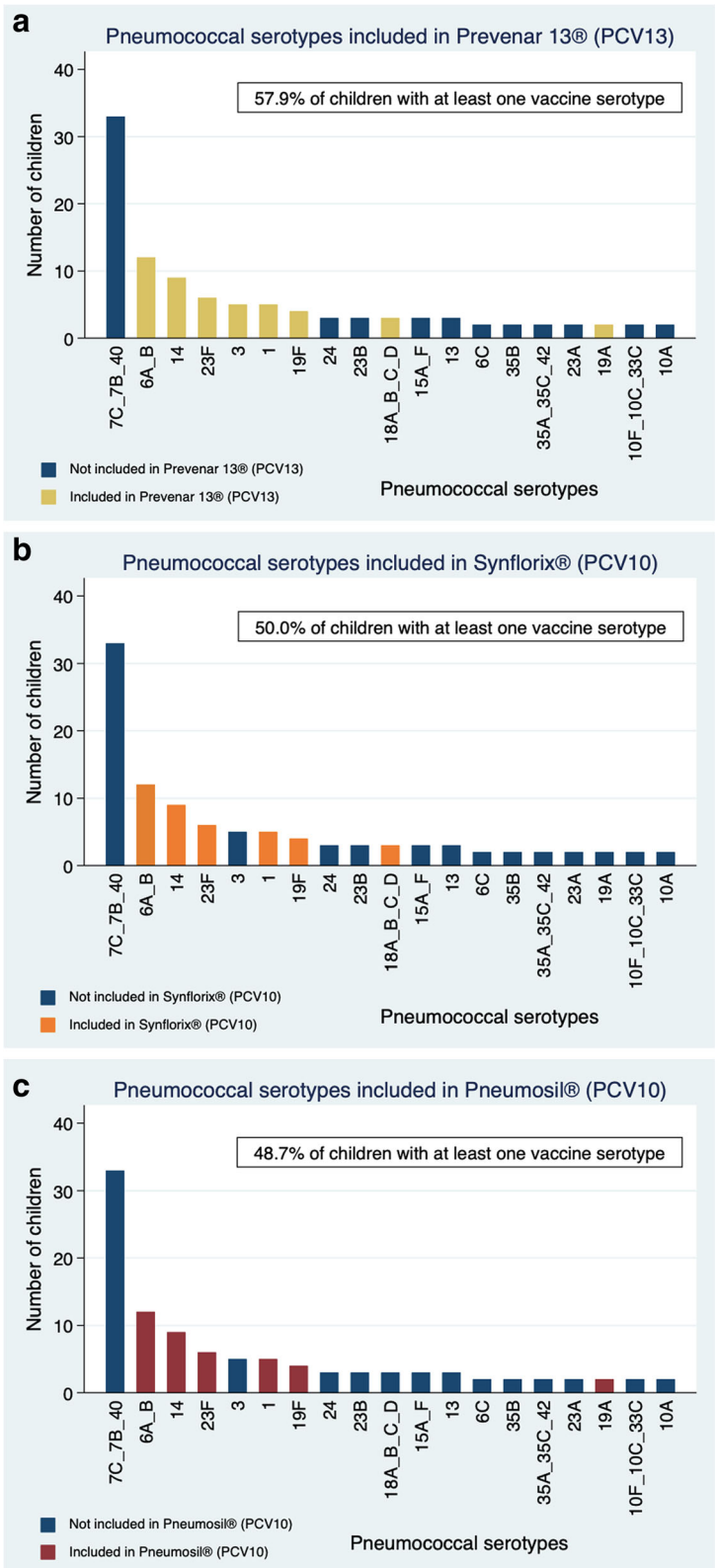

Fig. 3 (See legend on next page.)

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Fig. 3 Bar graphs of pneumococcal serotypes included in WHO pre-qualified PCV. **a**. Pneumococcal serotypes included in Prevenar 13® (PCV13). **b**. Pneumococcal serotypes included in Synflorix® (PCV10). **c**. Pneumococcal serotypes included in Pneumosil® (PCV10). We identified 3 children with serotype 18A/B/C/D, the laboratory technique not being able to differentiate among 18A, 18B, 18C or 18D. However, Prevenar 13® and Synflorix® only include the serotype 18C. While Prevenar 13® include both serotypes 6A and 6B, Synflorix® only include the serotype 6B. We identified 2 children with serotype 6A; 2 children with serotype 6B, and it was not possible to differentiate between 6A and 6B in the remaining 8 cases

decreasing order were 14, 1, 19F, 6B, 5, 6A, 9 V and 23F [49]. However, these were isolated from normally sterile sites such as blood, cerebrospinal fluid or pleural fluid. Data from other neighbouring countries are scarce, except for some data on pneumococcal serotype distribution among healthy children in Nepal [50, 51]. In our study, 19F and 6A/B were identified in 4/76 (5.3%) and 12/76 (15.8%) of the children, respectively. The proportion of the serotypes 14 and 23F, however, was similar to other studies. Serotype 1 has been identified as an important cause of highly invasive pneumococcal disease and is atypically found in carriage studies of healthy children [49, 52]. In our study, serotype 1 was the fifth most common serotype (together with serotype 3) identified, which is in line with findings of PNC in children sick with pneumonia.

PCV13, which was introduced in Bhutan after the end of the study, could potentially prevent the infection by at least one serotype in up to 57.9% of the children recruited in this study. Similar studies including two from India showed incongruences in these estimates, reporting PCV13 coverage ranging between 32 and 84% [7, 41, 42]. However, this is in line with the different serotype distribution we described above, as the most commonly identified serotypes (19F, 6A and 6B) in other studies are covered by PCV13, while the commonly identified serotypes 7B/7C/40 in our study are not.

Bacterial pneumonia secondary to viral respiratory infections such as influenza have been previously well characterized, and *S. pneumoniae* is the most common bacteria involved [53]. The nasopharynx is colonised by many bacteria and viruses and while the interaction between respiratory viruses and *S. pneumoniae* is likely to play a crucial role on the progression to the disease, this is a less well understood area [6, 7]. In the present study, respiratory viruses were detected in similar proportion of children with and without PNC. However, when looking at specific viral infections, children with PNC were less likely to be co-infected with rhinovirus (28.6% versus 47.5%; $p = 0.046$), and there was a trend of higher proportion of co-infection with RSV among colonized children (50.0% versus 32.5%; albeit non statistically significant, $p = 0.075$). Positive association of pneumococcal colonisation with influenza, RSV, adenovirus and rhinovirus have been found by previous studies, but findings have not been consistent [54–56]. Differences between studies might be explained by different pneumococcal serotype distribution as well as different circulation of viruses depending on seasonality and local epidemiology. One of the best understood and well documented synergistic viral-bacterial interaction is that of the influenza virus, identified as a risk factor for the acquisition, colonization and development of pneumonia due to *S. pneumoniae* [53, 55, 57–59]. Conversely in our study, presence of influenza virus was not related with PNC. This might be due to the small number of children with influenza and by the fact that this is a cross-sectional study with collection of NPW specimen at the time of admission, while bacterial superinfection is often sequential, occurring a few days after the viral episode.

This study has a number of limitations. These data and the statistically significant differences identified need to be interpreted with caution, due to the relatively small number of children enrolled. NPW samples were unavailable (not collected or insufficient for testing) for one third of children. However, children with and without NPW findings did not significantly differ in regard to baseline characteristics, evolution and outcomes (analysis not shown), except for severity of pneumonia during admission (99/121 [81.8%] in children with NPW findings versus 65/68 [95.6%] in children without; $p = 0.007$). Our findings relate to children admitted with clinical pneumonia and as such, they do not reflect PNC rate and pneumococcal serotype distribution in healthy children. This is a cross-sectional survey, where we collected NPW once (upon admission or as soon as possible after enrolment). Therefore, we did not pretend to determine whether viral infection precedes pneumococcal colonization or to evaluate their impact in the development of pneumonia. Longitudinal cohort studies would be required to address these questions.

Conclusions

This study provides baseline information on the status of pneumococcal carriage among sick Bhutanese children just before the introduction of the pneumococcal vaccine, which is valuable to monitor its impact. Most common serotypes identified were 7B/7C/40, 6A/B, 14 and 23F, which differs from comparable studies and neighbouring countries. PCV13 has a potential coverage of at least one serotype presented by over half of the children, suggesting a role for the vaccine to reduce the burden associated with *S. pneumoniae*.

Abbreviations

CRP: C-reactive protein; CXR: Chest radiography; ESR: Erythrocyte sedimentation rate; HDU: High dependency unit; Hib: Haemophilus influenza type b; IBD: Invasive Bacterial Disease; IQR: Interquartile range; JDWNRH: Jigme Dorji Wangchuck National Referral Hospital; LMIC: Low- and middle-income countries; NA: Not applicable; NPW: Nasopharyngeal washing; PCV: Pneumococcal conjugated vaccine; PICU: Paediatric intensive care unit; PNC: Pneumococcal nasopharyngeal colonization; RIBhuC: Respiratory Infection in Bhutanese Children; RSV: Respiratory Syncytial Virus; RT-PCR: Real time polymerase chain reaction; SpO2: Peripheral capillary oxygen saturation

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Authors' contributions

SJ, CMA, and QB conceived and designed the study. SJ implemented recruitment process with the contribution of ML, collected and entered data into the database. RS, DH, and CMA performed and supervised the laboratory testing and analysis. SJ, QB and CMA conducted and participated to the analyses and interpretation of data. SJ wrote the first draft of the manuscript. QB and CMA appraised the manuscript and contributed to it by revising the different versions. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during this current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study protocol was approved by the Research Ethics Board of Health, Royal Government of Bhutan Ministry of Health, in Thimphu, Bhutan, on the 17th March 2017 (protocol number PO/2016/086), and by the Comité Ético de Investigación Clínica del Hospital Clínic de Barcelona (clinical research ethics committee from the Hospital Clínic in Barcelona), Spain (HCB/2017/0741). All children were enrolled to the study after their guardians signed a written informed consent.

Consent for publication

Not applicable.

Competing interests

CMA received a Research grant paid to the Sant Joan de Déu Foundation outside this work. The other authors declare that they have no competing interests.

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Publisher's Note

ARTICLE 5

Association of clinical signs, host biomarkers, and aetiology with radiological findings in Bhutanese children hospitalized with pneumonia

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Association of clinical signs, host biomarkers, and aetiology with radiological findings in Bhutanese children hospitalised with pneumonia.

Short title: Clinical signs and biomarkers in radiological pneumonia

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Abstract

Background

Diagnosing pneumonia and identifying those requiring antibiotherapy remain challenging. Chest radiographs (CXR) are often used as the reference standard. We aimed to describe clinical characteristics, host-response biomarkers, and aetiology as well as assess their relationship to CXR findings in children admitted with pneumonia in Thimphu, Bhutan.

Methods

Children between 2-59 months admitted to the Jigme Dorji Wangchuck National Referral Hospital with WHO-defined clinical pneumonia were prospectively enrolled in this study. Blood and nasopharyngeal washing were collected for microbiological analyses. In addition, we measured plasma levels of eleven host-response biomarkers, including markers of endothelial and immune activation. A CXR was taken upon admission and children were classified into radiological endpoint and non-endpoint pneumonia.

Results

Among 149 children with readable CXR, 39 (26.2%) presented with endpoint pneumonia. Identification of respiratory viruses was common, with no significant differences by radiological outcomes. A higher proportion of children with endpoint pneumonia reported symptoms (64.1% versus 38.5%, p=0.007) or presented fever (42.1% versus 21.3%, p=0.045) for ≥5 days prior to admission and fulfilled clinical criteria for WHO severe pneumonia (92.3% versus 75.5%, p=0.033). No single clinical sign was more suggestive of presenting endpoint pneumonia or non-endpoint pneumonia. However, plasma levels of erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and procalcitonin (PCT) were higher among endpoint pneumonia and remained statistically significant after adjusting for observed cofounders. At established thresholds, neutrophilia (42.1% versus 24.6%, p=0.042), high ESR (33.3% versus 11.2%, p=0.005), high CRP (27.8% versus 11.2%,

p=0.021), and high PCT (56.0% versus 25.4%, p=0.007) were also significantly more frequent among children with endpoint pneumonia. None of the other biomarkers demonstrated good discriminatory ability between endpoint and non-endpoint pneumonia.

Conclusions

No clinical sign was suggestive of radiological pneumonia, but children with radiological pneumonia presented higher ESR, CRP and PCT. Markers of endothelial and immune activation had little accuracy for the reliable identification of radiological pneumonia.

Key words: Pneumonia; Children; Bhutan; Respiratory infection; Radiography; Inflammatory markers

Introduction

Pneumonia causes 15.5% of all deaths among children under five years of age worldwide, translating to over 800,000 deaths annually [1,2]. Early identification and treatment of children with pneumonia is fundamental to reduce mortality [3]. While most pneumonia are caused by viruses and are self-limited without antibiotic treatment, it is important to identify those with a bacterial underlying aetiology requiring antibiotics and those likely to evolve to severe disease with potential fatal outcome [4–6]. However, there is no universally accepted gold standard for the diagnosis of pneumonia. Currently, we lack diagnostic tools with high sensitivity and specificity that allow for accurate identification of children that require antibiotics, and at risk of poor prognosis [7]. Initial evaluation of clinical pneumonia cases is of importance to identify those of presumed bacterial aetiology since these cases may become life-threatening in the absence of appropriate antimicrobial treatment. Clinical diagnostic criteria for pneumonia, such as those proposed by World Health Organization (WHO), primarily used in low- and middle-income countries, are highly sensitive, but are not able to discern children requiring antibiotics from those who will present a self-limited pneumonia with unnecessary antibiotics. This leads to an overtreatment of clinical pneumonia cases with antibiotics, with potential implications in the emergence of antimicrobial resistance [8,9]. Although the accuracy of chest radiographs (CXR) for diagnosing pneumonia and its ability to differentiate between bacterial and viral aetiology is imperfect, CXR have been traditionally considered the practical reference standard [10–13]. Radiograph-based standardized endpoints are also commonly used as reference in studies assessing clinical signs and symptoms of pneumonia and in vaccine trials [7,13,14]. More recently, a series of inflammatory host-response biomarkers have been described that may help differentiate between bacterial and viral underlying aetiologies. However, their diagnostic role in childhood pneumonia remains unclear, with conflicting findings between studies [15–17].

A heterogeneity of evidence exists in describing clinical signs and host-response biomarkers associated with radiological findings among children diagnosed with clinical pneumonia. Recent findings suggest that these characteristics might differ according to geographic areas [18]. Indeed, predominant respiratory pathogens, co-infections such as malaria, and other factors such as altitude vary between geographical regions and are likely to contribute to these correlations. There is a paucity of data describing radiological findings in children with clinical pneumonia in Bhutan, a small kingdom in the Himalayas [19]. Therefore, we aimed to describe the radiological findings of children under five years of age admitted with WHO-defined pneumonia in the Respiratory Infections in Bhutanese Children (RIBhuC) study conducted in Thimphu, the capital of Bhutan. We looked at differences in radiological findings by demographic characteristics, aetiology, clinical presentation, host-response biomarkers, evolution and final outcome.

Methods

Setting and participants

The RIBhuC study was conducted prospectively for 12 consecutive months at the Jigme Dorji Wangchuck National Referral Hospital (JDWNRH) in Thimphu, Bhutan, to describe the epidemiology, aetiology and clinico-radiological presentation of WHO-defined pneumonia among children under five years of age. Details of this study have been reported elsewhere [20]. Briefly, the paediatric department at JDWNRH consists of 38 beds: 29 in the paediatric ward, four in the paediatric high dependency unit (HDU), and five in the paediatric intensive care unit (PICU). The pneumococcal conjugated vaccine (PCV) was introduced in the country in January 2019, after the study period.

All children aged 2 to 59 months admitted at JDWNRH with a diagnosis of pneumonia or severe pneumonia according to the WHO definitions were eligible for recruitment [9] (Box 1). Children whose principal reason for admission was a non-respiratory illness or a condition that was not caused by respiratory illness, those admitted in the preceding seven days, and children with evidence of a foreign body in the respiratory tract were excluded. We recruited all eligible children provided parent(s) or caregiver(s) consented on writing to study participation.

Pneumonia:
• History of cough or reported breathing difficulty, AND
• Increased respiratory rate OR chest indrawing.

Severe pneumonia:
• History of cough or reported breathing difficulty
AND at least one of the following:
• Oxygen saturation < 90% or central cyanosis,
• Severe respiratory distress (e.g. grunting, very severe chest indrawing),
• Signs of pneumonia with a general danger sign: inability to breastfeed or drink, lethargy or reduced level of consciousness, convulsions.

Increased respiratory rate is defined according to age as follows:
• ≥ 50 breaths per minute in children aged two months or more and less than 12 months.
• ≥ 40 breaths per minute in children aged 12 months or more and less than 60 months.

Box 1. Case definitions according to WHO for pneumonia, severe pneumonia and increased respiratory rate, used as inclusion criteria [9]**.**

Data collection

On admission, we assigned a study identification number, recorded vital signs and performed a comprehensive physical examination. Demographic and clinical data from the medical records and through family interviews were collected. Blood samples and a nasopharyngeal washing (NPW) specimen were collected upon enrolment or as soon as possible after enrolment. An antero-posterior CXR was performed within 24 hours of admission using either a digital machine (Model IDC DR. 1590x 3C, Eureka) or an analog one (Model KH/HD/STANDIX-31667, Siemens), depending on availability. Recruited children were clinically managed and discharged as per the criteria of the

treating nurses and paediatricians and were followed-up by one study investigator in terms of outcome determination.

Chest radiographs interpretation

We followed WHO criteria for the interpretation of CXR [14]. Readers judged the quality of the film as uninterpretable or interpretable (the latter stratified as suboptimal or adequate). All interpretable films were classified as confirmed 'endpoint pneumonia' (consolidation, pleural effusion, or both on any hemithorax), 'other infiltrates' (all others non-endpoint infiltrates in any hemithorax), or 'normal' (no abnormalities identified). 'Non-endpoint pneumonia' comprised other infiltrates and normal CXR.

Two study paediatricians interpreted the CXR independently. As substantial discordance was found among these two readers, and following our study protocol, a paediatric radiologist read all CXR, which was accepted as final interpretation for analysis. All CXR readers were blinded to clinical and laboratory findings.

Biological sample testing and laboratory methods

Blood cultures were incubated and continuously monitored for growth using the automated blood culture system BacT/ALERT (BioMérieux). Blood was collected in paediatric blood culture bottles (BACT/ALERT PF, BioMérieux) according to manufacturer guidelines. Bacterial isolates from positive blood cultures were identified by colony morphology, growth requirements, and basic biochemical tests according to the standard of care at JDWNRH. Antibiotic susceptibility was determined using disk diffusion using zone size interpretations from the Clinical Laboratory Standard Institute [21].

For investigation of host-response biomarkers, blood samples (2mL, EDTA tube) were centrifuged at 3000 g for three minutes, and the plasma was separated and stored at -80ºC until shipped to Toronto, Canada.

NPW samples were homogenized, aliquoted, stored at -80ºC, and shipped to Barcelona, Spain, for molecular analysis. A multiplex real-time polymerase chain reaction (RT-PCR; QIAStat respiratory panel, Qiagen) was used for identification of respiratory pathogens (17 viral and four atypical bacterial targets) and a RT-PCR for pneumococcal detection (*lytA* gene) [22–24]. We performed capsular typing of *Streptococcus pneumoniae* in all *lytA* positive samples with a fragment analysis multiplex PCR designed to distinguish 40 serotypes [25]. We considered the serotypes 1, 3, 4, 5, 7F, 14, 18C and 19A as highly invasive [26–31].

On site rapid influenza diagnostic tests (Alere BinaxNOW®) were performed as per discretion of the treating clinicians and nurses, independently of the RIBhuC study.

Host-response biomarker assays

Host-response biomarkers were measured blinded to patient clinical and radiological characteristics. White blood cell (WBC) count, platelets, erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP) were analysed at the study centre (JDWNRH). At the reference centre (Sandra Rotman Centre for Global Health in Toronto, Canada), CRP and procalcitonin (PCT) were quantified by enzyme-linked immunosorbent assay (ELISA), and the plasma concentration of six additional endothelial and immune activation biomarkers were measured using a multiplex Luminex platform with reagents from R&D Systems (Minneapolis, MN): interleukin-6 (IL-6), interleukin-8 (IL-8), angiopoietin-2 (Ang-2), soluble fms-like tyrosine kinase-1 (sFLT1), soluble triggering receptor expressed on myeloid cells 1 (sTREM-1), and tumor necrosis factor receptor 1 (TNFR1) [32]. Biomarker concentrations outside of the detection limits were assigned a value of one third below or above the lowest or highest limit in the standard curve, respectively. We refer to CRP-study and CRP-ref for differentiating CRP measured at the study and reference laboratories, respectively.

Data management and statistical analysis

Data were entered into a computerized password-protected database (ODK Aggregate version 1.4.13) with study identification number [33]. Errors in data entry were limited by using pre-defined ranges for every value. Stata™ v.16.0 (StataCorp, College Station, Texas, USA) was used for data analyses [34]. We examined the association between radiological outcomes and a set of variables (demographic and clinical characteristics, and biomarkers) using Chi-square or Fisher exact tests for categorical variables. Mann-Whitney U and Kruskal-Wallis tests were used for non-parametric continuous variables. Univariable logistic regression models were used to estimate odds ratios of radiological outcomes for predictors of clinical characteristics and biomarkers, and multivariable logistic regression models to estimate the degree of association between each biomarker and radiological findings after adjusting for observed confounders. All continuous variables with non-parametric distribution were log transformed for inclusion in logistic regression models. To assess the predictive capability of each biomarker considered, area under the curve (AUC) and other classification performance measures (sensitivity and specificity) were calculated. These calculations were performed based on each univariable logistic regression model and defining the cut-points using the Youden's index method (J=max[sensitivity+specificity-1]). Significance was set at 0.05.

Ethical approval

The study protocol was approved by the Research Ethics Board of Health, Ministry of Health, in Thimphu, Bhutan, on the 17th March 2017 (protocol number PO/2016/086), and by the research ethics committee from the Hospital Clínic in Barcelona, Spain (HCB/2017/0741).

Results

Between 1st July 2017 and 30th June 2018, 189 children meeting the inclusion criteria were recruited [20]. CXR was performed to 94.2% (178/189) of them. CXR images were not available for

external evaluation for 15.7% (28/178) of participants and one film was deemed uninterpretable. Therefore, 149 children were included in the analysis: 39 (26.2%) with endpoint pneumonia, 31 (20.8%) with other infiltrates, and 79 (53.0%) with normal radiological findings (Fig 1). Comparing children with (n=149) and without (n=40) CXR available, we found no differences in term of baseline characteristics (Table S1).

Missing demographic or clinical data were due to a lack of collection for these variables. Blood samples were collected and analysed for WBC (148/149, 99.3%), platelet (146/149, 98.0%), CRP-study (143/149, 96.0%), ESR (131/149, 87.9%), and the remaining biomarkers at the reference centre (96/149, 64.4%).

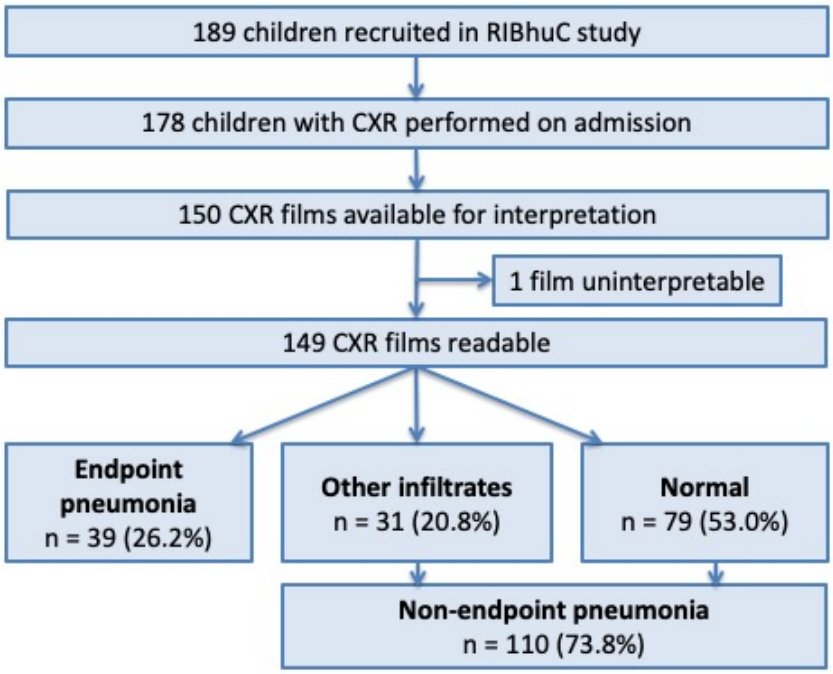

Fig 1. CXR interpretation and findings

Abbreviations: CXR: chest radiograph; RIBhuC: Respiratory infections in Bhutanese children.

Children with radiological endpoint and non-endpoint pneumonia presented similar demographical characteristics in terms of age, gender, vaccine status, and parental education and employment (Table 1). However, there was a higher proportion of children with endpoint pneumonia that had

an access time to health care facilities (proxy measurement of distance to the health system) of 30 minutes or longer (17.1% versus 2.8%, p=0.008). Five children died, one with radiological endpoint pneumonia, and four with non-endpoint pneumonia. A higher proportion of children with endpoint pneumonia required hospitalization for ≥5 days (48.7% versus 27.3%, p=0.016), with no significant differences regarding the need of ventilation, oxygen therapy, or antibiotherapy. No additional differences were observed between the three radiological outcomes (endpoint pneumonia, other infiltrates and normal findings) (Table S2).

Table 1. Baseline characteristics of children with WHO-defined clinical pneumonia for radiological endpoint versus non-endpoint pneumonia

Characteristics	Endpoint pneumonia (N = 39)	Non-endpoint pneumonia (other infiltrates or normal) (N = 110)	Odds Ratio (95% CI)[a]	p-value[a]
Demographic characteristics				
Gender, female	16 (41.0)	49 (44.5)	0.87 (0.41 to 1.82)	0.703
Age in months	16.1 (6.4 to 31.9)	9.9 (6.5 to 24.9)	1.27 (0.85 to 1.89)	0.237
Age category, months				0.364
2 to < 6	9 (23.1)	25 (22.7)	Ref	
6 to < 12	7 (17.9)	35 (31.8)	0.56 (0.18 to 1.69)	
12 to < 24	11 (28.2)	21 (19.1)	1.46 (0.51 to 4.18)	
24 to < 60	12 (30.8)	29 (26.4)	1.15 (0.42 to 3.18)	
Season[b]				0.993
Summer	13 (33.3)	37 (33.6)	Ref	
Fall	12 (30.8)	36 (32.7)	0.95 (0.38 to 2.35)	
Winter	4 (10.3)	10 (9.1)	1.14 (0.30 to 4.26)	
Spring	10 (25.6)	27 (24.6)	1.05 (0.40 to 2.76)	
Vaccine status				0.573
Fully	31 (79.5)	81/108 (75.0)	Ref	
Partially	8 (20.5)	27/108 (25.0)	1.29 (0.53 to 3.15)	
None	0 (0)	0/108 (0)	NA	
Wasting (WAZ ≤ -2 SD)[c]	3 (7.7)	7/109 (6.4)	1.21 (0.30 to 4.95)	0.786
Exposure to tobacco smoke	6/37 (16.2)	13/107 (12.2)	1.40 (0.49 to 3.40)	0.530
Exposure to betel nut (doma)	22/37 (59.5)	73/107 (68.2)	0.68 (0.32 to 1.48)	0.333
Exposure to heater with kerosene	2/34 (5.9)	9/98 (9.2)	0.62 (0.13 to 3.01)	0.552
Known case of HIV infection	0 (0)	0 (0)	NA	NA
Previous admission due to pneumonia	8 (20.5)	25/109 (22.9)	0.87 (0.35 to 2.12)	0.755
Parental education				0.560
Both parents are illiterate	5/36 (13.9)	14/106 (13.2)	Ref	
Only one parent has primary education	6/36 (16.7)	15/106 (14.1)	1.12 (0.28 to 4.51)	
Both parents have primary education	13/36 (36.1)	52/106 (49.1)	0.70 (0.21 to 2.30)	
At least one parent has university education	12/36(33.3)	25/106 (23.6)	1.34 (0.39 to 4.60)	
Parental employment				0.540
Both parents are unemployed	0/36 (0)	1/104 (0.9)	NA	
Only one parent is unemployed	21/36 (58.3)	66/104 (63.5)	Ref	
Both parents are employed	15/36 (41.7)	37/104 (35.6)	0.78 (0.36 to 1.70)	
≥ 6 persons living in the household	11/36 (30.6)	40/107 (37.4)	0.74 (0.33 to 1.66)	0.460
Time to access health care facility ≥ 30 minutes	6/35 (17.1)	3/106 (2.8)	7.10 (1.67 to 30.16)	0.008
Evolution and clinical outcome				

Duration of hospitalization ≥ 5 days	19 (48.7)	30 (27.3)	2.53 (1.19 to 5.39)	0.016
Admission to PICU or HDU or both	13 (33.3)	25 (22.7)	1.70 (0.76 to 3.79)	0.194
Invasive mechanical ventilation[d]	1 (2.6)	5 (4.6)	0.55 (0.06 to 4.88)	0.594
Non-invasive mechanical ventilation[d]	3 (7.7)	8 (7.3)	1.06 (0.27 to 4.22)	0.931
Oxygen therapy	31 (79.5)	81/109 (74.3)	1.34 (0.55 to 3.26)	0.519
Antibiotics during admission	32 (82.1)	76 (69.1)	2.05 (0.82 to 5.09)	0.124
Antibiotics stopped within first 48h	1/32 (3.1)	8/76 (10.5)	0.27 (0.03 to 2.29)	0.232
Fatal outcome	1 (2.6)	4 (3.6)	0.70 (0.08 to 6.44)	0.751
Poor prognosis score[e]	7 (18.0)	14 (12.7)	1.50 (0.56 to 4.04)	0.423

Abbreviations: CI: confidence interval; HDU: high dependency unit; NA: not applicable; PICU: paediatric intensive care unit; SD: standard deviation; WAZ: weight-for-age Z-score.

Variables presented as number (column percentage) or median (interquartile range). N represents total number of children per category unless otherwise specified.

[a]Odds ratios for endpoint pneumonia versus non-endpoint pneumonia using univariable logistic regression. Continuous variables with non-normal distribution were log transformed for logistic regression analyses.

[b]Seasonality was defined according to the Northern hemisphere seasonal patterns.

[c]Nutritional status was based on the WAZ score generated using the 2000 Centers for Disease Control and Prevention Growth Reference [35,36].

[d]Mechanical ventilation support was considered non-invasive when it was delivered through high flow nasal canula oxygen, continuous positive airway pressure (CPAP) or bilevel positive airway pressure (BiPAP), and invasive when positive pressure was delivered through an endotracheal tube. High frequency oscillatory ventilation is currently not used in Bhutan.

[e]Poor prognosis defined by fatal outcome or admission in PICU.

Association of aetiology with radiological findings

Bacteria were isolated by blood culture in six children, 2/31 (6.5%) with endpoint pneumonia and 4/63 (6.4%) with normal CXR (Table 2). Detailed findings are published elsewhere [20]. There were no significant differences in the proportion of nasopharyngeal pneumococcal carriers and highly invasive serotype distribution between children with endpoint and non-endpoint pneumonia. At least one virus was detected in respiratory secretions of most children, and a third of those (30/89, 33.7%) had ≥2 viruses identified. All children with other infiltrates had at least one virus, and half of them had ≥2 viruses identified. Respiratory syncytial virus was the most commonly isolated virus (44.0%), detected in around one-quarter of children with endpoint pneumonia and in half of those with non-endpoint pneumonia (p=0.056). Rhinovirus was isolated in over half of children with other infiltrates and a third of those with endpoint pneumonia (p=0.083). Parainfluenza virus was more frequent in children with endpoint pneumonia compared to those with non-endpoint pneumonia (28.6% versus 9.7%, p=0.023; Table S4). Other viruses were identified in similar proportion between children with different radiological endpoints.

Table 2. Microbiological investigations by radiological findings

Characteristics	All children (N = 149)	Endpoint pneumonia (N = 39)	Other infiltrates (N = 31)	Normal (N = 79)
Number of children tested				
Number of children with blood culture performed	121/149 (81.2)	31/121 (25.6)	27/121 (22.3)	63/121 (52.1)
Number of children with pneumococcal testing in NPW[a]	90/149 (60.4)	26/90 (28.9)	14/90 (15.5)	50/90 (55.6)
Number of children with viral testing in NPW[a]	100/149 (67.1)	28/100 (28.0)	17/100 (17.0)	55/100 (55.0)
Bacterial findings				
Non-contaminated positive bacterial blood culture	6/121 (5.0)	2/31 (6.5)	0/27 (0)	4/63 (6.4)
S. pneumoniae isolated by blood culture	1/6 (16.7)	1/2 (50.0)	0/0 (0)	0/4 (0)
S. pneumoniae nasal carriage (positive RT-PCR in NPW)	67/105 (61.1)	20/30 (66.7)	7/18 (38.9)	40/57 (70.2)
Highly invasive *S. pneumoniae* (among NPW positive samples)	22/67 (32.8)	9/20 (45.0)	2/7 (28.6)	11/40 (27.5)
Most common *S. pneumonia* serotypes identified				
7B/7C/40	28/67 (41.8)	7/20 (35.0)	3/7 (42.9)	18/40 (45.0)
6A/6B	10/67 (14.9)	1/20 (5.0)*	3/7 (42.9)	6/40 (15.0)
14	9/67 (13.4)	4/20 (20.0)	2/7 (28.6)	3/40 (7.5)
Viral findings				
Positive flu rapid test in pharyngeal swab	9/30 (30.0)	4/11 (36.4)	2/5 (40.0)	3/14 (21.4)
Positive for any virus in NPW	89/100 (89.0)	24/28 (85.7)	17/17 (100)	48/55 (87.3)
Positive for ≥ 2 viruses	30/89 (33.7)	10/24 (41.7)	8/17 (47.1)	12/48 (25.0)
Positive for Respiratory Syncytial Virus	44/100 (44.0)	8/28 (28.6)	9/17 (52.9)	27/55 (49.1)
Positive for Rhinovirus	36/100 (36.0)	9/28 (32.1)	10/17 (58.8)	17/55 (30.9)
Positive for Influenza A or B virus	13/100 (13.0)	5/28 (17.9)[b]	2/17 (11.8)[c]	6/55 (10.9)[d]
Positive for Parainfluenza virus 1, 2, 3, or 4	15/100 (15.0)	8/28 (28.6)[#]	2/17 (11.8)	5/55 (9.1)
Positive for Adenovirus	8/100 (8.0)	2/28 (7.1)	3/17 (17.7)	3/55 (5.5)
Positive for Bocavirus	6/100 (6.0)	2/28 (7.1)	1/17 (5.9)	3/55 (5.5)
Positive for Human Metapneumovirus	3/100 (3.0)	3/28 (10.7)	0/17 (0)	0/55 (0)
Positive for Coronavirus-229E, HKU1, NL63, or OC43	2/100 (2.0)	1/28 (3.6)[e]	0/17 (0)	1/55 (1.8)[f]

Abbreviations: CI: confidence interval; NA: not applicable; NPW: nasopharyngeal washing; OR: odd ratio; RT-PCR: real-time polymerase chain reaction.
Variables presented as n/N (column percentage).
*$p < 0.05$ when comparing the proportions between endpoint pneumonia and other infiltrates, using univariable logistic regression (Table S3).
[#]$p < 0.05$ when comparing the proportions between endpoint pneumonia and non-endpoint pneumonia, using univariable logistic regression (Table S4).
[a]Viral analysis was first performed in NPW samples. For some children, no NPW was left for pneumococcal analysis after viral analysis, explaining the lower number of children with pneumococcal results as compared to viral results.
[b]Four children with endpoint pneumonia were positive for influenza A virus, and one child for both influenza A and B virus.
[c]Two children with other infiltrates were positive for influenza A virus.
[d]Five children with normal radiological findings were positive for influenza A virus, and one child for both influenza A and B virus.
[e]Coronavirus-OC43 was identified in one child with endpoint pneumonia.
[f]Coronavirus-NL63 was identified in one child with normal radiological findings.

Association of clinical characteristics with radiological findings

A high proportion of children presented with clinical signs usually considered more indicative of radiological consolidation (endpoint pneumonia) as a proxy for bacterial pneumonia, including

hypoxemia (79/108, 73.1%) or crackles (63/108, 58.3%), in spite of having CXR which did not confirm the pneumonia endpoint. Similar proportions of children with and without radiological pneumonia presented with increased work of breathing including lower and severe chest wall indrawing, and nasal flaring.

A higher proportion of children with endpoint pneumonia were symptomatic for at least five days prior to admission (64.1% versus 38.5%, p=0.007), had fever for at least five days (42.1% versus 21.3%, p=0.045), and presented with WHO severe pneumonia (92.3% versus 75.5%, p=0.033) (Table 3). No single clinical sign could differentiate between radiological outcomes. Hypoxemia was significantly less frequent in children with radiological normal findings compared to those with endpoint pneumonia or other infiltrates (Table S5).

Table 3. Association of clinical characteristics with radiological endpoint pneumonia in children with WHO-defined clinical pneumonia

Characteristics	Endpoint pneumonia (N = 39)	Non-endpoint pneumonia (other infiltrates or normal) (N = 110)	Odds Ratio (95% CI)[a]	p-value[a]
Current episode				
Reported duration of illness prior to admission ≥ 5 days	25 (64.1)	42/109 (38.5)	2.85 (1.33 to 6.09)	0.007
Reported duration of fever prior to admission				0.045
No fever	4/38 (10.5)	20/108 (18.5)	Ref	
< 5 days	18/38 (47.4)	65/108 (60.2)	1.38 (0.42 to 4.57)	
≥ 5 days	16/38 (42.1)	23/108 (21.3)	3.48 (0.99 to 12.13)	
Started on antibiotics prior to admission	11 (28.2)	23/109 (21.1)	1.47 (0.64 to 3.39)	0.367
Any danger sign[b]	9 (23.1)	17 (14.5)	1.64 (0.66 to 4.06)	0.284
Severe pneumonia on admission	36 (92.3)	83 (75.5)	3.90 (1.11 to 13.70)	0.033
Clinical characteristics				
Increased respiratory rate[c]	19/38 (50.0)	56/106 (52.8)	0.89 (0.43 to 1.87)	0.765
Hypoxemia (SpO2 < 90%)[d]	32 (82.1)	79/108 (73.1)	1.68 (0.67 to 4.22)	0.271
Fever (≥ 37.5ºC, axillary)	19 (48.7)	43/108 (39.8)	1.43 (0.69 to 3.00)	0.336
High fever (> 39ºC, axillary)	3 (7.7)	4/108 (3.7)	2.17 (0.46 to 10.15)	0.326
Lower chest wall indrawing	23 (59.0)	61/106 (57.6)	1.06 (0.50 to 2.23)	0.877
Severe chest indrawing[e]	5 (12.8)	11/108 (10.2)	1.30 (0.42 to 4.00)	0.651
Nasal flaring	8 (20.5)	21/106 (19.8)	1.04 (0.41 to 2.60)	0.925
Grunting	4 (10.3)	4/108 (3.7)	2.97 (0.71 to 12.51)	0.138
Head nodding	0/38 (0)	0/108 (0)	NA	NA
Prolonged expiration	6/38 (15.8)	20/105 (19.05)	0.80 (0.29 to 2.16)	0.656
Crackles	25 (64.1)	63/108 (58.3)	1.28 (0.60 to 2.72)	0.529
Ronchi	16 (41.0)	51/108 (47.2)	0.78 (0.37 to 1.63)	0.506
Wheezing	8 (20.5)	31/105 (29.5)	0.62 (0.25 to 1.49)	0.282

Abbreviations: CI: confidence interval; NA: not applicable
Variables presented as number (column percentage). N represents total number of children per category unless otherwise specified.
[a]Odds ratios for endpoint pneumonia versus non-endpoint pneumonia using univariable logistic regression.
[b]Danger signs as per WHO definition: inability to breastfeed or drink, lethargy or reduced level of consciousness, convulsions.
[c]Increased respiratory rate according to age is defined as >50breaths per minute in children aged 2 to 12 months and >40 breaths per minute in children aged ≥ 12 months.
[d]Peripheral capillary oxygen saturation was measured in room air using Mindray VS-800 Vital Sign Monitor or Biolight BLT M800 Handheld pulse oximeter, and hypoxemia was defined as oxygen saturation in room air under 90% [37].
[e]Severe chest indrawing was defined as supraclavicular and/or suprasternal indrawing.

Association of host-response biomarker levels with radiological findings

Children with endpoint pneumonia presented higher levels of ESR (p=0.008), CRP-study (p=0.007), and PCT (p=0.003) (Table 4; Fig 2). After adjusting for demographic and clinical variables, levels of ESR, CRP-study and PCT remained significantly higher among children with endpoint pneumonia (Table 5). IL-6 and TNFR1 levels were higher in children with endpoint pneumonia but they did not reach statistical significance. When analysing biomarkers as dichotomous variables (high versus normal) using thresholds widely used in clinical practice, we found that neutrophilia (42.1% versus 24.6%, p=0.042), ESR≥50mm (33.3% versus 11.2%, p=0.005), CRP-study>4mg/dL (27.8% versus 11.2%, p=0.021) and PCT≥250pg/mL (56.0% versus 25.4%, p=0.007) were more frequent among children with endpoint pneumonia.

ESR, CRP-study and PCT were significantly higher in children with endpoint pneumonia compared to those with normal radiological findings, and PCT and TNFR1 were also higher in children with endpoint pneumonia compared to those presenting other infiltrates. Children with other infiltrates presented higher levels of CRP-study and IL-8 compared to those with normal radiological findings (Table S7; Fig S1).

We further explored the performance of the biomarkers that showed significant association with radiological findings for identifying endpoint pneumonia, by analysing the AUC (Fig 3). Although none of the biomarkers presented good discriminatory ability between endpoint and non-endpoint

pneumonia, PCT presented the best overall discriminatory ability with 72% (95% CI 50.6 to 87.9) sensitivity and 66.2% (95% CI 54.0 to 77.0) specificity.

Table 4. Association of host response biomarkers with radiological endpoint pneumonia in children with WHO-defined clinical pneumonia

Host-response biomarkers	Endpoint pneumonia (N = 39)	Non-endpoint pneumonia (other infiltrates or normal) (N = 110)	Odds Ratio (95% CI)[a]	p-value[a]
Median (interquartile range) [b]				
WBC (x10^9/L)	11.38 (7.72 to 17.80)	13.14 (9.87 to 16.70)	0.74 (0.36 to 1.52)	0.413
Platelets (x10^9/L)	366 (298 to 411)	376 (299 to 452)	1.00 (1.00 to 1.00)	0.808
ESR (mm)	30 (12 to 60)	12 (6 to 30)	1.67 (1.14 to 2.43)	0.008
CRP-study (mg/dL)	2.1 (1.4 to 4.3)	1.1 (0.4 to 2.9)	1.74 (1.16 to 2.60)	0.007
CRP-ref (mg/dL)	2.1 (0.7 to 12.2)	1.4 (0.6 to 4.3)	1.30 (0.94 to 1.78)	0.108
PCT (pg/mL)	452.8 (46.6 to 2153.2)	46.6 (46.6 to 253.8)	1.51 (1.15 to 1.99)	0.003
IL-6 (pg/mL)	6.6 (2.7 to 24.7)	3.6 (0.7 to 10.5)	1.31 (0.98 to 1.76)	0.068
IL-8 (pg/mL)	16.2 (7.9 to 47.6)	20.9 (7.5 to 37.4)	0.94 (0.70 to 1.28)	0.706
Ang-2 (pg/mL)	2397 (1469 to 3521)	2142 (1243 to 4758)	0.93 (0.55 to 1.58)	0.796
sFLT1 (pg/mL)	155 (121 to 190)	164 (112 to 220)	0.67 (0.31 to 1.46)	0.315
sTREM-1 (pg/mL)	151 (107 to 217)	108 (76 to 172)	1.40 (0.80 to 2.44)	0.239
TNFR1 (pg/mL)	1674 (1543 to 2564)	1487 (1095 to 1979)	2.43 (0.97 to 6.08)	0.059
At established thresholds, n/N (%)				
Leucocytosis[c]	16/38 (42.1)	44 (40.0)	1.09 (0.52 to 2.31)	0.820
Leucopenia (< 5x10^9 WBC/L)	3/38 (7.9)	3 (2.7)	3.06 (0.59 to 15.84)	0.183
Neutrophilia (≥ 70% of WBC)	16/38 (42.1)	27 (24.6)	2.23 (1.03 to 4.86)	0.042
Thrombocytosis (> 450x10^9 platelets/L)	7/37 (18.9)	28/109 (25.7)	0.68 (0.27 to 1.71)	0.407
Thrombocytopenia (< 150x10^9 platelets/L)	1/37 (2.7)	1/109 (0.92)	3.0 (0.18 to 49.20)	0.441
High ESR (≥ 50 mm)	11/33 (33.3)	11/98 (11.2)	3.95 (1.52 to 10.30)	0.005
High CRP-study (> 4 mg/dL)	10/36 (27.8)	12/107 (11.2)	3.04 (1.18 to 7.83)	0.021
High CRP-ref (> 4 mg/dL)	20/25 (80.0)	55/71 (77.5)	1.16 (0.38 to 3.59)	0.792
High PCT (≥ 250 pg/mL)	14/25 (56.0)	18/71 (25.4)	3.75 (1.44 to 9.73)	0.007

Abbreviations: Ang-2: angiopoietin-2; CI: confidence interval; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; IL6: interleukin-6; IL8: interleukin-8; PCT: procalcitonin; sFLT1: soluble fms-like tyrosine kinase-1; sTREM-1: soluble triggering receptor expressed on myeloid cells 1; TNFR1: tumor necrosis factor receptor 1; WBC: white blood cells. Variables presented as number (column percentage) or median (interquartile range). N represents total number of children per category unless otherwise specified.

[a]Odds ratios for endpoint pneumonia versus non-endpoint pneumonia using univariable logistic regression.

[b]All biomarkers except platelets are non-normally distributed and were log transformed for univariable logistic regression.

[b]Leucocytosis was defined as white blood cells greater than 15 x 10^9 cells/L for children aged between 2 and 11 months and greater than 13 x 10^9 cells/L for children aged between 12 and 59 months.

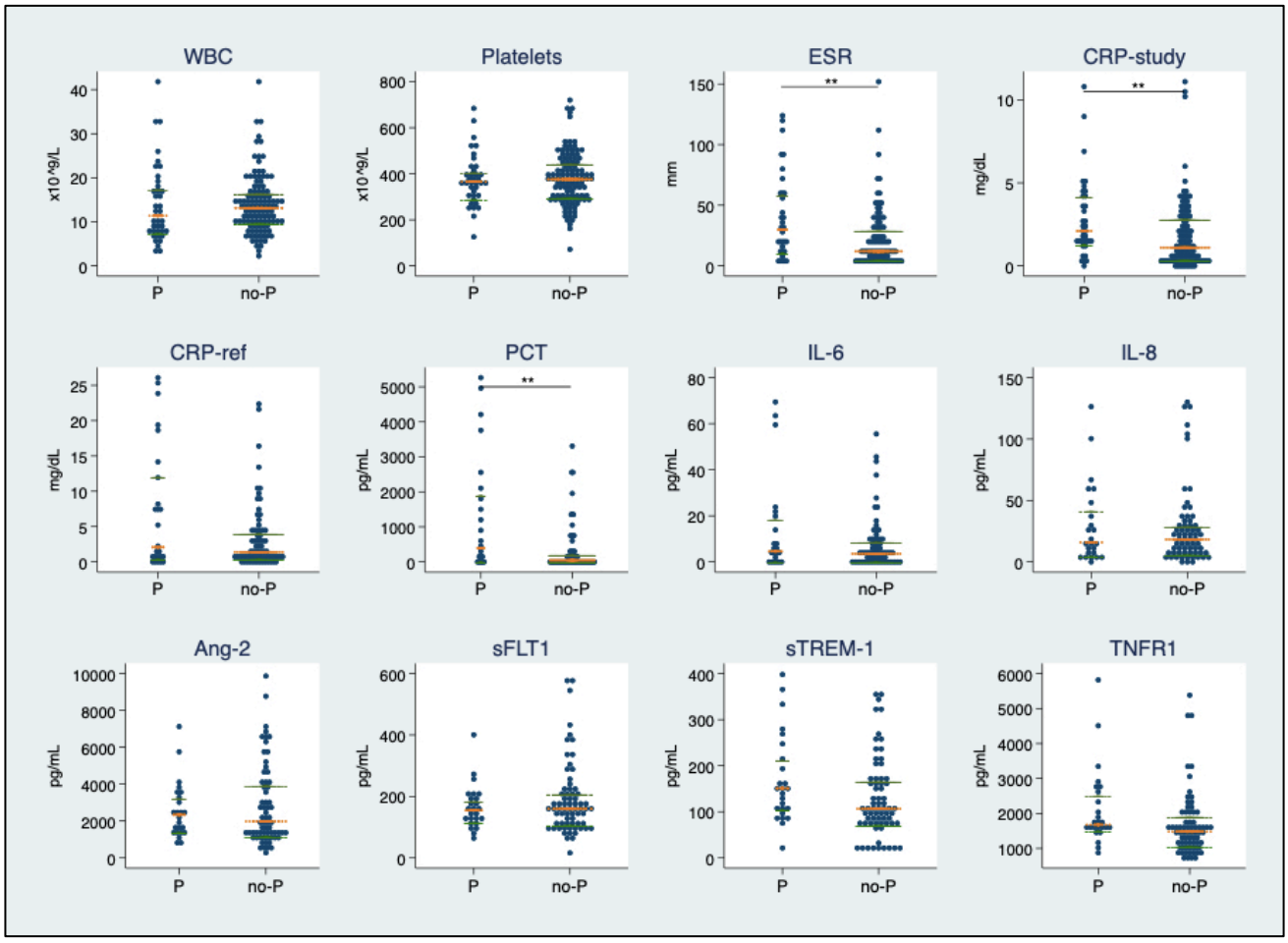

Fig 2. Host biomarkers levels according to radiological findings (endpoint pneumonia versus non-endpoint pneumonia)

Abbreviations: Ang-2: angiopoietin-2; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; IL-6: interleukin-6; IL-8: interleukin-8; no-P: non-endpoint pneumonia; P: endpoint pneumonia; PCT: procalcitonin; sFLT1: soluble fms-like tyrosine kinase-1; sTREM-1: soluble triggering receptor expressed on myeloid cells 1; TNFR1: tumor necrosis factor receptor 1; WBC: white blood cells.

* shows p<0.05 (none) and ** shows p<0.01 for comparisons of biomarker levels between children with endpoint pneumonia and non-endpoint pneumonia using univariable logistic regression. The orange dot lines show the median, the green dot lines show p25 and p75.

Extreme values were removed for better interpretation of the dot plots on the y-scale (Table S6).

Table 5. Adjusted associations for host-response biomarkers with radiological endpoint pneumonia in children with WHO-defined clinical pneumonia

Host-response biomarkers[a]	aOR (95% CI)[b]	p-value
WBC (x10^9/L)	0.63 (0.28 to 1.43)	0.270
Platelets (x10^9/L)	0.99 (0.99 to 1.00)	0.970
ESR (mm)	1.69 (1.09 to 2.62)	0.020
CRP-study (mg/dL)	2.01 (1.25 to 3.21)	0.004
CRP-ref (mg/dL)	1.43 (0.95 to 2.17)	0.090
PCT (pg/mL)	1.77 (1.23 to 2.56)	0.002
IL-6 (pg/mL)	1.41 (0.97 to 2.04)	0.070
IL-8 (pg/mL)	0.88 (0.59 to 1.30)	0.514
Ang-2 (pg/mL)	0.87 (0.33 to 2.27)	0.774
sFLT1 (pg/mL)	0.43 (0.16 to 1.13)	0.088
sTREM-1 (pg/mL)	1.54 (0.68 to 3.49)	0.303
TNFR1 (pg/mL)	3.14 (0.70 to 14.08)	0.135

Abbreviations: Ang-2: angiopoietin-2; aOR: adjusted odd ratio; CI: confidence interval; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; IL6: interleukin-6; IL8: interleukin-8; PCT: procalcitonin; sFLT1: soluble fms-like tyrosine kinase-1; sTREM-1: soluble triggering receptor expressed on myeloid cells 1; TNFR1: tumor necrosis factor receptor 1; WBC: white blood cells.
[a]All biomarkers except platelets are non-normally distributed and were log transformed for logistic regression analyses.
[b]Adjusted by age, sex, time to access health care facilities, duration of fever prior to admission, and severity at admission according to WHO clinical criteria.

Biomarker	AUC (95% CI)	Cut-point	Sensitivity (95% CI)	Specificity (95% CI)
ESR	0.66 (0.55 to 0.77)	>53 mm	33.3 (18.0 to 51.8)	92.9 (85.8 to 97.1)
CRP-study	0.66 (0.55 to 0.76)	>1.25 mg/dL	80.6 (64.0 to 91.8)	53.3 (43.4 to 63.0)
PCT	0.69 (0.56 to 0.81)	>171.96 pg/mL	72.0 (50.6 to 87.9)	66.2 (54.0 to 77.0)

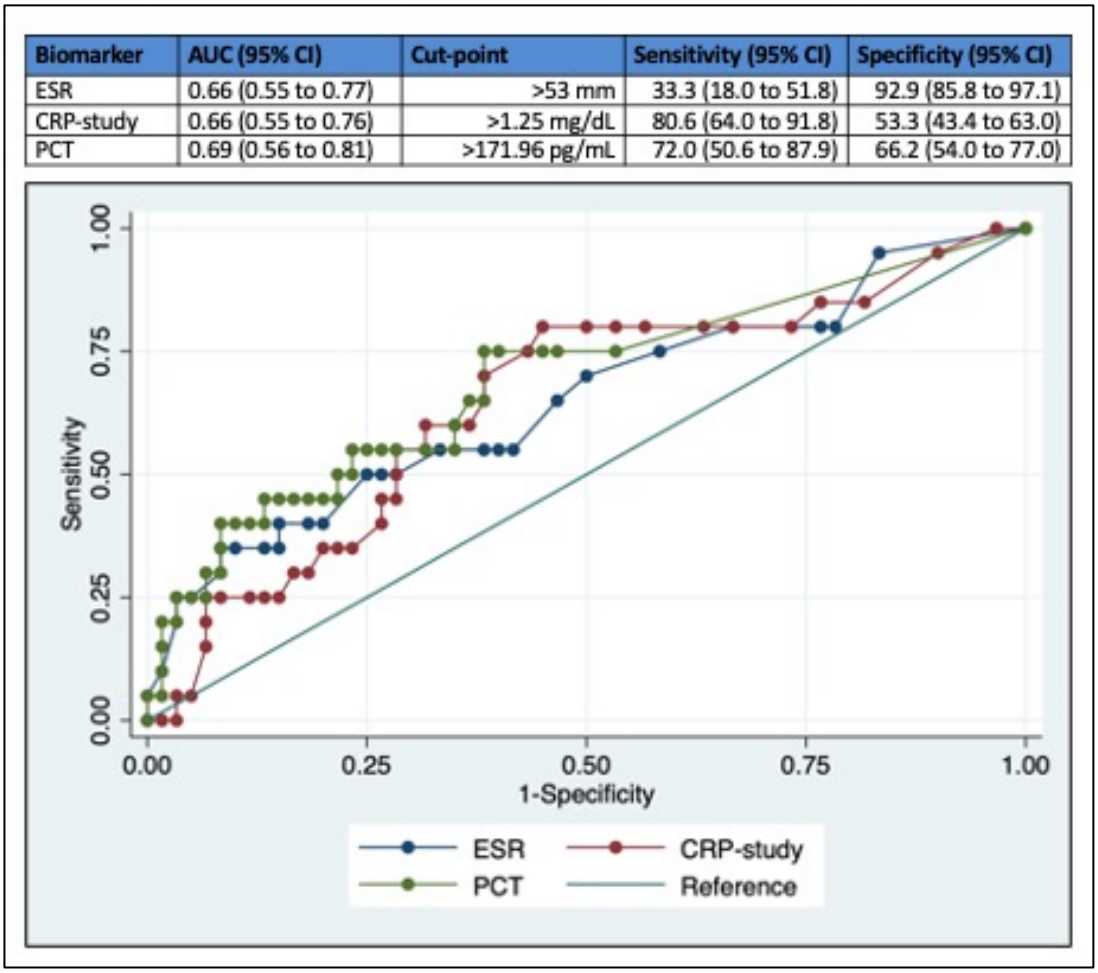

Fig 3. Performance of ESR, CRP and PCT for identifying radiological endpoint pneumonia among children with WHO-defined clinical pneumonia

Abbreviations: AUC: area under the curve; CI: confidence interval; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; PCT: procalcitonin.

Discussion

The following study entailed a comprehensive description of radiological findings among children under five years of age admitted with clinical WHO-defined pneumonia. Over half of the children presented a normal CXR and a quarter of them showed radiological endpoint pneumonia. This is

comparable to the findings of the recently conducted multicentre study that used the same criteria for CXR classification [18].

Radiological endpoint pneumonia has been associated with higher mortality, which might reflect the association between radiological endpoint pneumonia and bacterial aetiology, together with the tendency of bacterial pneumonia being more severe than viral ones [18]. This was not the case in our study, where mortality was similar among children with and without radiological endpoint pneumonia. This is likely to be explained by access to prompt diagnosis and treatment with appropriate antibiotics, and questions the association of radiological findings with bacterial aetiology and overall prognosis.

CXR are still used as reference standard for the diagnosis of pneumonia in clinical practice and for investigation purposes. However, CXR involves radiation exposure, are not always available, and require maintenance of the radiograph and expertise for interpretation of the film. Despite simple criteria for radiological classification [14], identification of consolidation and other infiltrates is not always straightforward. This is reflected in intra- and inter-observer variability in the interpretation of CXR, even among radiologists [13,38–40]. In addition to the above challenges, CXR remains an imperfect diagnosis tool to discriminate between bacterial and viral aetiology. It is widely accepted that consolidation is the radiographic finding most frequently associated with bacterial aetiology, and this is the basis of our analysis, where we used radiological endpoint pneumonia as a proxy for bacterial pneumonia [13,41]. However, in this study, 85.7% of children with radiological endpoint pneumonia (presumably of bacterial origin) presented with at least one virus in their nasopharynx, and 41.7% of those had ≥2 viruses identified, similar to children with other radiological outcomes. We found no association for any single virus with radiological findings, except parainfluenza virus which was more frequent among children with radiological endpoint pneumonia. Nonetheless,

these results need to be interpreted with caution, due to the small number of children identified with such virus (n=15). Furthermore, identification of respiratory virus in the nasopharynx of children with clinical pneumonia requires careful interpretation. Distinction between nasopharyngeal carriage and causative agent is difficult [4], and respiratory virus detection does not exclude a bacterial infection [4,42]. There is a growing evidence showing an overlap of viral and bacterial aetiology in respiratory infections, and the probable important interaction between them in the pathogenesis of pneumonia [42–44].

The association between clinical signs and radiological findings has been assessed to identify children with pneumonia that need antibiotics. Increased respiratory rate, hypoxemia, crackles, fever on admission, and duration of illness were found to be associated with endpoint pneumonia, indicative of bacterial pneumonia [13]. Other studies found that no single clinical finding is sufficient to predict radiological pneumonia [3]. These contradictory findings might be in part due to differences in the definition of clinical pneumonia and the interpretation methods and classification of CXR. In our study, days of fever and severity of pneumonia were associated with endpoint pneumonia. However, increased respiratory rate and hypoxemia (the two backbone criteria of the WHO definition for clinical pneumonia) were present in similar proportions of children with and without endpoint pneumonia, despite hypoxemia occurring significantly more often in children with endpoint pneumonia or with other infiltrates than with normal CXR. Other single clinical characteristics such as crackles and fever on admission were not associated with endpoint pneumonia either. These findings suggest that a high proportion of children presenting with clinical signs usually considered more indicative of bacterial pneumonia, such as hypoxemia (73.1%) or crackles (58.3%), have radiological evidence of non-endpoint pneumonia. Therefore, a proportion of children with radiological non-endpoint pneumonia truly have pneumonia, supporting the notion that standardized definitions of radiological pneumonia have low predictive

value for clinical management and decision on antibiotic needs [18]. These findings reflect the goals of the WHO clinical and radiological definitions for pneumonia. Criteria for clinical pneumonia intend to be highly sensitive to capture all children that would benefit from antibiotics. In contrast, criteria for radiological pneumonia were developed to increase specificity for bacterial pneumonia for epidemiological purposes, and thus has limited use for clinical management of individual patient antibiotic needs [45].

Despite clinical similarities between radiological outcomes in our study, CRP, PCT, and ESR, were significantly higher among children with endpoint pneumonia. The association between CRP and PCT and radiological endpoint pneumonia (as a proxy for bacterial pneumonia) or microbiologically confirmed bacterial pneumonia has been reported in previous studies [16,46,55,47–54], but results are not as clear for ESR [48,56,57].

The other biomarkers investigated in this study were not associated with radiological outcomes, although IL-6 was higher in children with endpoint pneumonia – but not statistically significant. The association of this pro-inflammatory cytokine with bacterial pneumonia has previously been evidenced [58,59]. However, findings from other studies are contradictory for IL-6 as well as for the remaining biomarkers involved in the acute inflammation phase – IL-8, Ang-2, sFLT1, sTREM-1 and TNFR1 [15,56]. Variations in findings between studies may be due to differences in study methods, biomarker kinetics during infection, and cohort demographics [15].

A point-of-care biomarker fulfilling the WHO ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable to ends users) criteria to identify bacterial aetiology, would help decision-making to start or discontinue antibiotics in children with clinical pneumonia [60,61]. Measurement of biomarkers for guiding antibiotherapy in patients with acute respiratory infections at the point-of-care has shown to reduce antibiotic use, without

negative influence on clinical outcome [62,63]. This has also been evidenced for PCT-guided antibiotherapy in children with pneumonia in high-income countries [47,64–66]. In our study, PCT was the biomarker with most promising results to identify radiological endpoint pneumonia as a proxy for bacterial aetiology. This is in accordance with previous findings showing that PCT has a better diagnosis performance for bacterial pneumonia compared to CRP, WBC, and ESR, although there is no consensus on precise cut-off to be used [47,48,50,56]. However, PCT is currently not available in Bhutan. Findings of this study could encourage policymakers in Bhutan to contemplate incorporating the measurement of PCT in clinical practice. This has the potential to improve decisions about antibiotic needs in children with respiratory symptoms, leading to better clinical outcomes and reducing antibiotic overuse. Rural and remote areas where laboratory facilities are of difficult access are likely to benefit of its readiness as a PCT point-of-care diagnostic tool. However, clinical efficacy and cost-effectiveness studies are required to estimate its potential impact in the Bhutanese setting.

In addition, and prior to implementation of any point-of-care diagnostic tool, an important question remains unanswered regarding care of childhood pneumonia. Which kind of marker would best assist clinicians in decision making: etiological markers or prognostic ones? It is possible that the lack of single clinical signs or biomarkers, or simple clinical algorithm that clearly discern bacterial from viral pneumonia is explained by the common mixed aetiology [16]. The combination of several biomarkers – or biomarker signature – derived from different pathophysiological pathways, associated or not with clinical signs, seems to provide a better performance in the differentiation of bacterial from viral pneumonia [16,46,56,67,68]. However, a biomarker able to identify children at risk of severe disease that would benefit from prioritization of care from those (the majority) with a self-limited disease without antibiotics, is likely to present major benefits [5,69,70]. We encourage further investigation to help identify a biomarker with such characteristics, guiding clinical care for

children with pneumonia to improve clinical outcome and reduce the unacceptable high mortality associated to this disease.

This study has several limitations, including the lack of a gold standard for the diagnosis of pneumonia and for discerning bacterial from viral aetiology. Consequently, by using radiological endpoint pneumonia as a proxy for bacterial aetiology, the associations we found between the selected variables (clinical characteristics and biomarkers) and radiological findings may not reflect the associations between those variables and true pneumonia. Our study was not designed to assess the predictive diagnostic value of clinical characteristics or biomarkers. Therefore, due to the relatively small sample size, this study was underpowered to rule in or rule out biomarkers to detect children with antibiotics needs.

It remains very challenging to identify children with pneumonia that require antibiotics, by contemplating clinical, laboratory and radiological characteristics. Conclusions regarding single clinical signs and biomarkers are conflicting, and further investigation is required to validate biomarker signatures capable of accurately identifying bacterial pneumonia and overall prognosis.

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Supplementary material

Table S1. Demographic and clinical characteristics, evolution and clinical outcome of children with and without CXR findings available

Characteristics	No CXR available (N = 40)	CXR available (N = 149)	p-value[a]
Demographic characteristics			
Gender, female	15 (37.5)	65 (43.6)	0.486
Age in months	9.9 (4.0 to 23.3)	11.4 (6.5 to 26.9)	0.271
Age category, months			0.609
2 to < 6	12 (30.0)	34 (22.8)	
6 to < 12	13 (32.5)	42 (28.2)	
12 to < 24	6 (15.0)	32 (21.5)	
24 to < 60	9 (22.5)	41 (27.5)	
Season[b]			0.543
Summer	9 (22.5)	50 (33.6)	
Fall	14 (35.0)	48 (32.2)	
Winter	5 (12.5)	14 (9.4)	
Spring	12 (30.0)	37 (24.8)	
Vaccine status			0.664
Fully	31/39 (79.5)	112/147 (76.2)	
Partially	8/39 (20.5)	35/147 (23.8)	
None	0/39 (0)	0/147 (0)	
Wasting (WAZ ≤ -2 SD)[c]	7/39 (18.0)	10/148 (6.8)	0.054
Exposure to tobacco smoke	2/35 (5.7)	19/144 (13.2)	0.378
Exposure to betel nut (doma)	20/35 (57.1)	95/144 (66.0)	0.328
Exposure to heater with kerosene	3/31 (9.7)	11/132 (8.3)	0.731
Known case of HIV infection	0/39 (0)	0 (0)	NA
Previous admission due to pneumonia	10/38 (26.3)	33/148 (22.3)	0.600
Parental education			0.667
Both parents are illiterate	7/36 (19.4)	19/142 (13.4)	
Only one parent has primary education	5/36 (13.9)	21/142 (14.8)	
Both parents have primary education	13/36 (36.1)	65/142 (45.8)	
At least one parent has university education	11/36 (30.6)	37/142 (26.1)	
Parental employment			0.283
Both parents are unemployed	1/34 (2.9)	1/140 (0.7)	
Only one parent is unemployed	18/34 (52.9)	87/140 (62.1)	
Both parents are employed	15/34 (44.1)	52/140 (37.1)	
≥ 6 persons living in the household	11/36 (30.6)	51/143 (35.7)	0.565
Time to access health care facility ≥ 30 minutes	2/35 (5.7)	9/141 (6.4)	1.000
Current episode			
Reported duration of illness prior to admission ≥ 5 days	19 (47.5)	67/148 (45.3)	0.802
Reported duration of fever prior to admission			0.608
No fever	5/38 (13.2)	24/146 (16.4)	
< 5 days	25/38 (65.8)	83/146 (56.9)	
≥ 5 days	8/38 (21.1)	39/146 (26.7)	
Started on antibiotics prior to admission	9/39 (23.1)	34/148 (23.0)	0.989
Severe pneumonia on admission	31 (77.5)	119 (79.9)	0.743
Evolution and clinical outcome			
Duration of hospitalization ≥ 5 days	15 (37.5)	49 (32.9)	0.584
Admission to PICU or HDU or both	14 (35.0)	38 (25.5)	0.232
Invasive mechanical ventilation[d]	1 (2.5)	6 (4.0)	1.000
Non-invasive mechanical ventilation[d]	2 (5.0)	11 (7.4)	0.739
Invasive or non-invasive mechanical ventilation	3 (7.5)	16 (10.7)	0.769
Oxygen therapy	30 (75.0)	112/148 (75.7)	0.930
Antibiotics during admission	28 (70.0)	108 (72.5)	0.756
Antibiotics stopped within first 48h	1/28 (3.6)	9/108 (8.3)	0.687
Fatal outcome	1 (2.5)	5 (3.4)	0.300
Poor prognosis score[e]	9 (22.5)	21 (14.1)	0.196

Abbreviations: CXR: chest radiography; HDU: high dependency unit; PICU: paediatric intensive care unit; SD: standard deviation; WAZ: weight-for-age Z score.

Variables presented as number (column percentage) or median (interquartile range). N represents total number of children per category unless otherwise specified.

[a]We examined the association between availability of radiological findings and the selected variables using Chi-square or Fisher exact tests (for categorical variables) and Mann-Whitney U test (for non-parametric continuous variables).

[b]Seasonality was defined according to the Northern hemisphere seasonal patterns.

[c]Nutritional status was based on the WAZ score, generated using the 2000 Centers for Disease Control and Prevention Growth Reference [1,2].

[d]Mechanical ventilation support was considered non-invasive when it was delivered through high flow nasal canula oxygen, continuous positive airway pressure (CPAP) or bilevel positive airway pressure (BiPAP), and invasive when positive pressure was delivered through an endotracheal tube. High frequency oscillatory ventilation is currently not used in Bhutan.

[e]Poor prognosis defined by fatal outcome or admission in PICU

Table S2. Baseline characteristics of children with WHO-defined clinical pneumonia by radiological outcome

Characteristics	Endpoint pneumonia (N = 39)	Other infiltrates (N = 31)	Normal (N = 79)	p-value[a]
Demographic characteristics				
Gender, female	16 (41.0)	13 (41.9)	36 (45.6)	0.876
Age in months	16.1 (6.4 to 31.9)	11.4 (7.0 to 29.3)	9.6 (5.5 to 24.0)	0.190
Age category, months				0.186
2 to <6	9 (23.1)	3 (9.7)	22 (27.9)	
6 to <12	7 (17.9)	13 (41.9)	22 (27.9)	
12 to <24	11 (28.2)	5 (16.1)	16 (20.3)	
24 to <60	12 (30.8)	10 (32.3)	19 (24.1)	
Season[b]				0.999
Summer	13 (33.3)	11 (35.5)	26 (32.9)	
Fall	12 (30.8)	9 (29.0)	27 (34.2)	
Winter	4 (10.3)	3 (9.7)	7 (8.9)	
Spring	10 (25.6)	8 (25.8)	19 (24)	
Vaccine status				0.180
Fully	31 (79.5)	19/30 (63.3)	62/78 (79.5)	
Partially	8 (20.5)	11/30 (36.7)	16/78 (20.5)	
None	0 (0)	0/30 (0)	0/78 (0)	
Wasting (WAZ ≤ -2 SD)[c]	3 (7.7)	1 (3.2)	6/78 (7.7)	0.825
Exposure to tobacco smoke	6/37 (16.2)	4/30 (13.3)	9/77 (11.7)	0.799
Exposure to betel nut (doma)	22/37 (59.5)	19/30 (63.3)	54/77 (70.1)	0.500
Exposure to heater with kerosene	2/34 (5.9)	2/29 (6.9)	7/69 (10.1)	0.725
Known case of HIV infection	0 (0)	0 (0)	0 (0)	NA
Previous admission due to pneumonia	8 (20.5)	7 (22.6)	18/78 (23.1)	0.951
Parental education				0.606
Both parents are illiterate	5/36 (13.9)	2/29 (6.9)	12/77 (15.6)	
Only one parent has primary education	6/36 (16.7)	3/29 (10.3)	12/77 (15.6)	
Both parents have primary education	13/36 (36.1)	15/29 (51.7)	37/77 (48.1)	
At least one parent has university education	12/36(33.3)	9/29 (31.0)	16/77 (20.8)	
Parental employment				0.849
Both parents are unemployed	0/36 (0)	0/30 (0)	1/74 (1.4)	
Only one parent is unemployed	21/36 (58.3)	18/30 (60.0)	48/74 (64.9)	
Both parents are employed	15/36 (41.7)	12/30 (40.0)	25/74 (33.8)	
≥ 6 persons living in the household	11/36 (30.6)	14/29 (48.3)	26/78 (33.3)	0.272
Time to access health care facility ≥ 30 minutes	6/35 (17.1)	0/30 (0)	3/76 (3.9)	0.014
Evolution and clinical outcome				
Duration of hospitalization ≥ 5 days	19 (48.7)	8 (25.8)	22 (27.9)	0.049
Admission to PICU or HDU or both	13 (33.3)	9 (29.0)	16 (20.3)	0.271
Invasive mechanical ventilation[d]	1 (2.6)	3 (9.7)	2 (2.5)	0.190
Non-invasive mechanical ventilation[d]	3 (7.7)	3 (9.7)	5 (6.3)	0.842
Oxygen therapy	31 (79.5)	27 (87.1)	54/78 (69.2)	0.119
Antibiotics during admission	32 (82.1)	23 (67.1)	53 (67.1)	0.224
Antibiotics stopped within first 48h	1/32 (3.1)	0/23 (0)	8/53 (15.1)	0.062
Fatal outcome	1 (2.6)	3 (9.7)	1 (1.3)	0.085
Poor prognosis score[e]	7 (18.0)	6 (19.4)	8 (10.1)	0.331

Abbreviations: HDU: high dependency unit; NA: not applicable; PICU: paediatric intensive care unit; SD: standard deviation; WAZ: weight-for-age Z-score.

Variables presented as number (column percentage) or median (interquartile range). N represents total number of children per category unless otherwise specified.

[a]We examined the association between radiological findings and the selected variables using Chi-square or Fisher exact tests (for categorical variables) and Kruskal-Wallis test (for non-parametric continuous variables).

[b]Seasonality was defined according to the Northern hemisphere seasonal patterns.

[c]Nutritional status was based on the WAZ score, generated using the 2000 Centers for Disease Control and Prevention Growth Reference [1,2].

[d]Mechanical ventilation support was considered non-invasive when it was delivered through high flow nasal canula oxygen, continuous positive airway pressure (CPAP) or bilevel positive airway pressure (BiPAP), and invasive when positive pressure was delivered through an endotracheal tube. High frequency oscillatory ventilation is currently not used in Bhutan.

[e]Poor prognosis defined by fatal outcome or admission in PICU.

Table S3. Microbiological findings between children with radiological endpoint pneumonia and other infiltrates

	Endpoint pneumonia (N = 39)	Other infiltrates (N = 31)	Odds Ratio (95% CI)[a]	p-value[a]
Bacterial findings				
Non-contaminated positive bacterial blood culture	2/31 (6.5)	0/27 (0)	NA	NA
S. Pneumoniae isolated by blood culture	1/2 (50.0)	0/0 (0)	NA	NA
S. pneumoniae nasal carriage (positive RT-PCR in NPW)	20/30 (66.7)	7/18 (38.9)	0.32 (0.09 to 1.07)	0.065
Highly invasive *S. pneumoniae* (among NPW positive samples)	9/20 (45.0)	2/7 (28.6)	0.49 (0.08 to 3.15)	0.451
Most common *S. pneumonia* serotypes identified				
7B/7C/40	7/20 (35.0)	3/7 (42.9)	1.39 (0.24 to 8.07)	0.712
6A/6B	1/20 (5.0)	3/7 (42.9)	14.3 (1.16 to 174.80)	0.038
14	4/20 (20.0)	2/7 (28.6)	1.6 (0.22 to 11.50)	0.640
Viral findings				
Positive flu rapid test in pharyngeal swab	4/11 (36.4)	2/5 (40.0)	1.17 (0.13 to 10.22)	0.889
Positive for any virus in NPW	24/28 (85.7)	17/17 (100)	NA	NA
Positive for ≥ 2 virus	10/24 (41.7)	8/17 (47.1)	1.24 (0.36 to 4.35)	0.732
Positive for RSV	8/28 (28.6)	9/17 (52.9)	2.81 (0.80 to 9.88)	0.102
Positive for Rhinovirus	9/28 (32.1)	10/17 (58.8)	3.02 (0.86 to 10.52)	0.083
Positive for Influenza A or B virus	5/28 (17.9)[b]	2/17 (11.8)[c]	0.61 (0.11 to 3.58)	0.587
Positive for Parainfluenza virus 1, 2, 3, or 4	8/28 (28.6)	2/17 (11.8)	0.33 (0.06 to 1.80)	0.202
Positive for Adenovirus	2/28 (7.1)	3/17 (17.7)	2.79 (0.42 to 18.69)	0.291
Positive for Bocavirus	2/28 (7.1)	1/17 (5.9)	0.81 (0.07 to 9.70)	0.870
Positive for Human Metapneumovirus	3/28 (10.7)	0/17 (0)	NA	NA
Positive for Coronavirus-229E, HKU1, NL63, or OC43	1/28 (3.6)[d]	0/17 (0)	NA	NA

Abbreviations: CI: confidence interval; NA: not applicable; NPW: nasopharyngeal washing; RT-PCR: real-time polymerase chain reaction.

Variables presented as n/N (column percentage).

[a]Odds ratios for endpoint pneumonia versus other infiltrates using univariable logistic regression.

[b]Four children with endpoint pneumonia were positive for influenza A virus, and one child for both influenza A and B virus.

[c]Two children with other infiltrates were positive for influenza A virus.

[d]Coronavirus-OC43 was identified in one child with endpoint pneumonia.

Table S4. Microbiological investigations between children with and without radiological endpoint pneumonia

	Endpoint pneumonia (N = 39)	Non-endpoint pneumonia (other infiltrates or normal) (N = 110)	Odds Ratio (95% CI)[a]	p-value[a]
Bacterial findings				
Non-contaminated positive bacterial blood culture	2/31 (6.5)	4/90 (4.4)	1.48 (0.26 to 8.52)	0.659
S. Pneumoniae isolated by blood culture	1/2 (50.0)	0/4 (0)	NA	NA
S. pneumoniae nasal carriage (positive RT-PCR in NPW)	20/30 (66.7)	47/75 (62.7)	1.19 (0.49 to 2.91)	0.700
Highly invasive *S. pneumoniae* (among NPW positive samples)	9/20 (45.0)	13/47 (27.7)	2.14 (0.72 to 6.35)	0.171
Most common *S. pneumonia* serotypes identified				
7B/7C/40	7/20 (35.0)	21/47 (44.7)	0.67 (0.23 to 1.97)	0.463
6A/6B	1/20 (5.0)	9/47 (19.2)	0.22 (0.03 to 1.89)	0.168
14	4/20 (20.0)	5/47 (10.6)	2.10 (0.50 to 8.82)	0.311
Viral findings				
Positive flu rapid test in pharyngeal swab	4/11 (36.4)	5/19 (26.3)	1.60 (0.32 to 7.90)	0.564
Positive for any virus in NPW	24/28 (85.7)	65/72 (90.3)	0.65 (0.17 to 2.41)	0.515
Positive for ≥ 2 virus	10/24 (41.7)	20/65 (30.8)	1.61 (0.61 to 4.23)	0.336
Positive for RSV	8/28 (28.6)	36/72 (50.0)	0.40 (0.16 to 1.03)	0.056
Positive for Rhinovirus	9/28 (32.1)	27/72 (37.5)	0.79 (0.31 to 1.99)	0.617
Positive for Influenza A or B virus	5/28 (17.9)[b]	8/72 (11.1)[c]	1.74 (0.52 to 5.86)	0.372
Positive for Parainfluenza virus 1, 2, 3, or 4	8/28 (28.6)	7/72 (9.7)	3.71 (1.20 to 11.51)	0.023
Positive for Adenovirus	2/28 (7.1)	6/72 (8.3)	0.85 (0.16 to 4.47)	0.844
Positive for Bocavirus	2/28 (7.1)	4/72 (5.6)	1.31 (0.23 to 7.57)	0.765
Positive for Human Metapneumovirus	3/28 (10.7)	0/72 (0)	NA	NA
Positive for Coronavirus-229E, HKU1, NL63, or OC43	1/28 (3.6)[d]	1/72 (1.4)[e]	2.63 (0.16 to 43.55)	0.500

Abbreviations: CI: confidence interval; NA: not applicable; NPW: nasopharyngeal washing; RT-PCR: real-time polymerase chain reaction.

Variables presented as n/N (column percentage).

[a]Odds ratios for endpoint pneumonia versus non-endpoint pneumonia using univariable logistic regression.

[b]Four children with endpoint pneumonia were positive for influenza A virus, and one child for both influenza A and B virus.

[c]Seven children with non-endpoint pneumonia were positive for influenza A virus, and one child for both influenza A and B virus.

[d]Coronavirus-OC43 was identified in one child with endpoint pneumonia.

[e]Coronavirus-NL63 was identified in one child with normal radiological findings.

Table S5. Association of clinical characteristics with radiological outcome in children with WHO-defined clinical pneumonia

Characteristics	Endpoint pneumonia (N = 39)	Other infiltrates (N = 31)	Normal (N = 79)	p-value[a]
Current episode				
Reported duration of illness prior to admission ≥ 5 days	25 (64.1)	13 (41.9)	29/78 (37.2)	0.020
Reported duration of fever prior to admission				0.162
No fever	4/38 (10.5)	6 (19.3)	14/77 (18.2)	
< 5 days	18/38 (47.4)	18 (58.1)	47/77 (61.0)	
≥ 5 days	16/38 (42.1)	7 (22.6)	16/77(20.8)	
Started on antibiotics prior to admission	11 (28.2)	7 (22.6)	16/78 (20.5)	0.646
Any danger sign[b]	9 (23.1)	7 (22.6)	10 (12.7)	0.261
Severe pneumonia on admission	36 (92.3)	28 (90.3)	55 (69.6)	0.004
Clinical characteristics				
Increased respiratory rate[c]	19/38 (50.0)	14/30 (46.7)	42/76 (55.3)	0.695
Hypoxemia (SpO2 < 90%)[d]	32 (82.1)	27/30 (90.0)	52/78 (66.7)	0.022
Fever (≥ 37.5ºC, axillary)	19 (48.7)	13/30 (43.3)	30/78 (38.5)	0.565
High fever (>39ºC, axillary)	3 (7.7)	3/30 (10.0)	1/78 (1.3)	0.068
Lower chest wall indrawing	23 (59.0)	22/30 (73.3)	39/76 (51.3)	0.116
Severe chest indrawing[e]	5 (12.8)	5/30 (16.7)	6/78 (7.7)	0.335
Nasal flaring	8 (20.5)	8/30 (26.7)	13/76 (17.1)	0.539
Grunting	4 (10.3)	0/30 (0)	4/74 (5.1)	0.258
Head nodding	0/38 (0)	0/30 (0)	0/78 (0)	NA
Prolonged expiration	6/38 (15.8)	5 (16.1)	15/74 (20.3)	0.798
Crackles	25 (64.1)	20/30 (66.7)	43/78 (55.1)	0.450
Ronchi	16 (41.0)	16/30 (53.3)	35/78 (44.9)	0.586
Wheezing	8 (20.5)	9/30 (30.0)	22/75 (29.3)	0.556

Abbreviations: NA: not applicable.

Variables presented as number (column percentage). N represents total number of children per category unless otherwise specified.

[a]We examined the association between radiological findings and the selected variables using Chi-square or Fisher exact test (for categorical variables) and Kruskal-Wallis test (for non-parametric continuous variables).

[e]Danger signs as per WHO definition: inability to breastfeed or drink, lethargy or reduced level of consciousness, convulsions.

[c]Increased respiratory rate according to age is defined as >50 breaths per minute in children aged 2 to 12 months and >40 breaths per minute in children aged ≥ 12 months.

[d]Peripheral capillary oxygen saturation was measured in room air using Mindray VS-800 Vital Sign Monitor or Biolight BLT M800 Handheld pulse oximeter, and hypoxemia was defined as oxygen saturation in room air under 90% [3].

[e]Severe chest indrawing was defined as supraclavicular and/or suprasternal indrawing.

Table S6. Host-response biomarkers according to radiological findings (endpoint pneumonia versus non-endpoint pneumonia)

Host-response biomarkers		Endpoint pneumonia	Non-endpoint pneumonia (other infiltrates or normal)
WBC (x10^9/L)	Median (IQR)	11.38 (7.72 to 17.80)	13.14 (9.87 to 16.70)
	Range (min, max)	3.3 to 42.35	2.7 to 41.28
	Extreme values[a]	None	None
Platelets (x10^9/L)	Median (IQR)	366 (298 to 411)	376 (299 to 452)
	Range (min, max)	133 to 682	75 to 713
	Extreme values[a]	None	None
ESR (mm)	Median (IQR)	30 (12 to 60)	12 (6 to 30)
	Range (min, max)	2 to 122	2 to 150
	Extreme values[a]	None	None
CRP-study (mg/dL)	Median (IQR)	2.1 (1.4 to 4.3)	1.1 (0.4 to 2.9)
	Range (min, max)	0 to 10.7	0 to 11
	Extreme values[a]	None	None
CRP-ref (mg/dL)	Median (IQR)	2.1 (0.7 to 12.2)	1.4 (0.6 to 4.3)
	Range (min, max)	0.2 to 26.2	0.1 to 22.1
	Extreme values[a]	None	None
PCT (pg/mL)	Median (IQR)	452.8 (46.6 to 2153.2)	46.6 (46.6 to 253.8)
	Range (min, max)	46.6 to 22,634.6	46.6 to 18,711.364
	Extreme values >10,000[a]	22,634.6	10,717.2 18,711.4
IL-6 (pg/mL)	Median (IQR)	6.6 (2.7 to 24.7)	3.6 (0.7 to 10.5)
	Range (min, max)	0.7 to 350.9	0.7 to 135.8
	Extreme values >100[a]	103.6 145.3 350.9	111.7 135.8
IL-8 (pg/mL)	Median (IQR)	16.2 (7.9 to 47.6)	20.9 (7.5 to 37.4)
	Range (min, max)	0.7 to 660.1	0.7 to 2794.6
	Extreme values >150[a]	660.1	169.5 304.7 485.6 638.8 2794.6
Ang-2 (pg/mL)	Median (IQR)	2397 (1469 to 3521)	2142 (1243 to 4758)
	Range (min, max)	736 to 10,964	338 to 44,229
	Extreme values >10,000[a]	10,964	11,674 12,689 15,700 27,359 44,229
sFLT1 (pg/mL)	Median (IQR)	155 (121 to 190)	164 (112 to 220)
	Range (min, max)	65 to 402	21 to 4857
	Extreme values >1000[a]	None	1715 4857
sTREM-1 (pg/mL)	Median (IQR)	151 (107 to 217)	108 (76 to 172)
	Range (min, max)	23 to 399	23 to 2522
	Extreme values >500[a]	None	702 1107 2522
TNFR1 (pg/mL)	Median (IQR)	1674 (1543 to 2564)	1487 (1095 to 1979)
	Range (min, max)	907 to 5775	681 to 10,635
	Extreme values >10,000[a]	None	10,635

Abbreviations: Ang-2: angiopoietin-2; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; IL6: interleukin-6; IL8: interleukin-8; IQR: interquartile range; PCT: procalcitonin; sFLT1: soluble fms-like tyrosine kinase-1; sTREM-1: soluble triggering receptor expressed on myeloid cells 1; TNFR1: tumor necrosis factor receptor 1; WBC: white blood cells.

[a]Extreme values that were removed from dotplots in Figure 2.

Table S7. Association of host response biomarkers with radiological outcome in children with WHO-defined clinical pneumonia

Host-response biomarkers	Endpoint pneumonia (N = 39)	Other infiltrates (N = 31)	Normal (N = 79)	p-value[a]
n/N (percentage)				
Leucocytosis[b]	16/38 (42.1)	13 (41.9)	31 (39.2)	0.942
Leucopenia (< $5x10^9$ WBC/L)	3/38 (7.9)	2 (6.5)	3/38 (7.9)	0.122
Neutrophilia (≥ 70% of WBC)	16/38 (42.1)	8 (25.8)	19 (24.1)	0.119
Thrombocytosis (> $450x10^9$ platelets/L)	7/37 (18.9)	9 (29.0)	19/78 (24.4)	0.619
Thrombocytopenia (< $150x10^9$ platelets/L)	1/37 (2.7)	0 (0)	1/78 (1.3)	0.716
High ESR (≥ 50mm)	11/33 (33.3)	4/26 (15.4)	7/72 (9.7)	0.011
High CRP-study (> 4 mg/dL)	10/36 (27.8)	5 (16.1)	7/76 (9.2)	0.039
High CRP-ref (> 4 mg/dL)	20/25 (80.0)	14/20 (70.0)	41/51 (80.4)	0.613
High PCT (≥ 250 pg/mL)	14/25 (56.0)	4/20 (20.0)	14/51 (27.5)	0.017
Median levels (interquartile range)				
WBC ($x10^9$/L)	11.38 (7.72 to 17.80)	13.92 (8.80 to 17.70)	13 (9.89 to 16.50)	0.713
Platelets ($x10^9$/L)	366 (298 to 411)	372 (287 to 469)	378 (303 to 446)	0.925
ESR (mm)	30 (12 to 60)	20 (10 to 40)	11 (4 to 30)	0.010
CRP-study (mg/dL)	2.1 (1.4 to 4.3)	1.4 (0.4 to 3.2)	1.1 (0.4 to 2.65)	0.013
CRP-ref (mg/dL)	2.1 (0.7 to 12.2)	1.2 (0.4 to 8.8)	1.4 (0.6 to 3.4)	0.243
PCT (pg/mL)	452.8 (46.6 to 2153.2)	46.6 (46.6 to 202.7)	46.6 (46.6 to 309.1)	0.015
IL-6 (pg/mL)	6.6 (2.7 to 24.7)	4.1 (1.0 to 9.2)	3.1 (0.7 to 10.5)	0.389
IL-8 (pg/mL)	16.2 (7.9 to 47.6)	25.7 (16.2 to 82.1)	16.6 (7.1 to 33.3)	0.168
Ang-2 (pg/mL)	2397 (1469 to 3521)	1665 (1248 to 3114)	2259 (1243 to 5808)	0.375
sFLT1 (pg/mL)	155 (121 to 190)	164 (102 to 237)	164 (116 to 220)	0.708
sTREM-1 (pg/mL)	151 (107 to 217)	98 (73 to 219)	112 (80 to 171)	0.291
TNFR1 (pg/mL)	1674 (1543 to 2564)	1426 (938 to 1601)	1506 (1131 to 2078)	0.037

Abbreviations: Ang-2: angiopoietin-2; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; IL6: interleukin-6; IL8: interleukin-8IRR: increased respiratory rate; PCT: procalcitonin; sFLT1: soluble fms-like tyrosine kinase-1; sTREM-1: soluble triggering receptor expressed on myeloid cells 1; TNFR1: tumor necrosis factor receptor 1; WBC: white blood cells.

Variables presented as number (column percentage) or median (interquartile range). N represents total number of children per category unless otherwise specified.

[a]We examined the association between radiological findings and the selected variables using Chi-square or Fisher exact test (for categorical variables) and Kruskal-Wallis test (for non-parametric continuous variables).

[b]Leucocytosis was defined as white blood cells greater than 15 x 10^9 cells/L for children aged between 2 and 11 months and greater than 13 x 10^9 cells/L for children aged between 12 and 59 months.

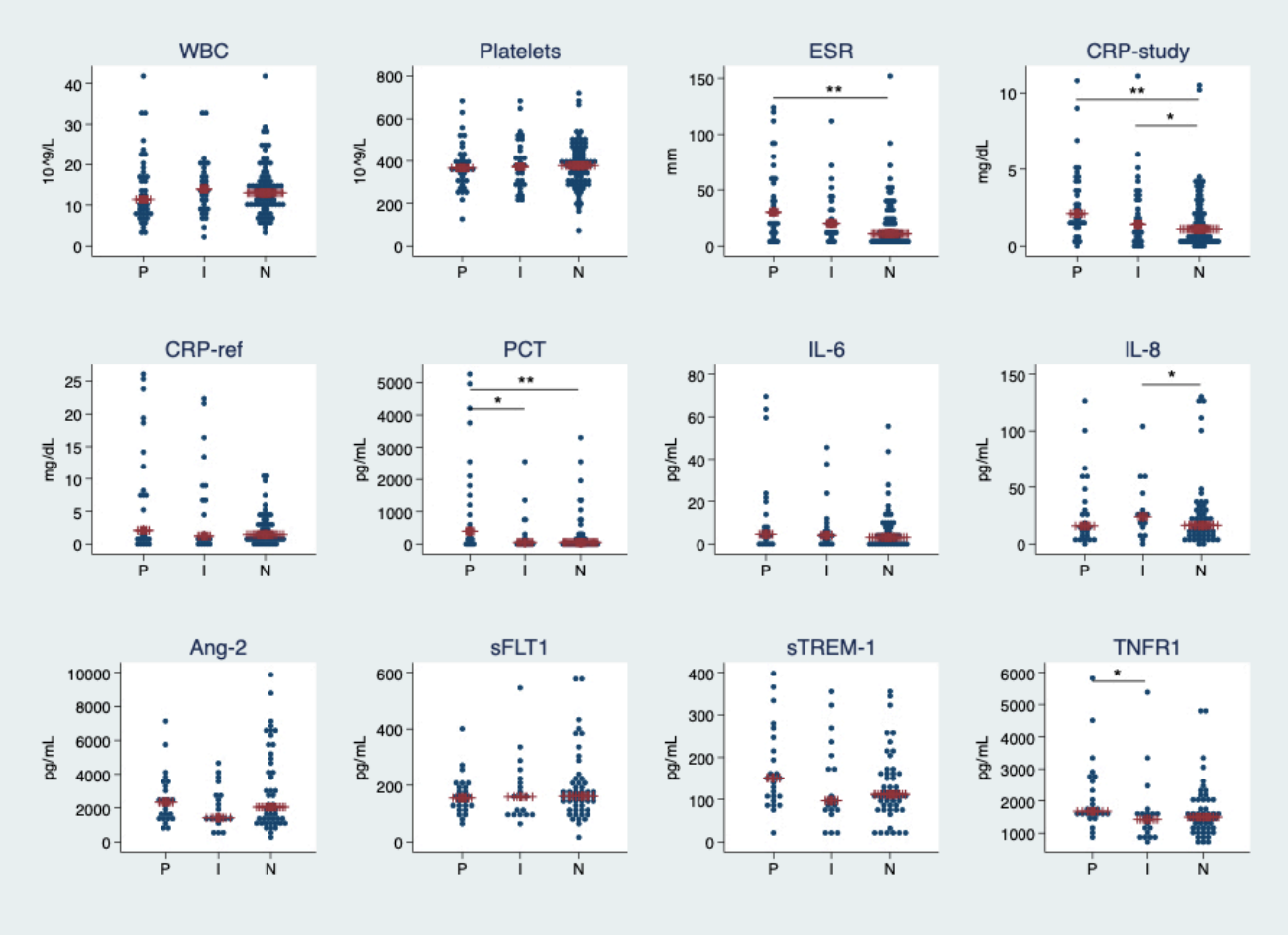

Fig S1. Host biomarkers levels according to radiological findings (endpoint pneumonia versus non-endpoint pneumonia)

Abbreviations: Ang-2: angiopoietin-2; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; I: other infiltrates; IL-6: interleukin-6; IL-8: interleukin-8; N: normal radiological finding; P: endpoint pneumonia; PCT: procalcitonin; sFLT1: soluble fms-like tyrosine kinase-1; sTREM-1: soluble triggering receptor expressed on myeloid cells 1; TNFR1: tumor necrosis factor receptor 1; WBC: white blood cells.

* shows $p<0.05$ and ** shows $p<0.01$ for comparisons of biomarker levels using univariable logistic regression. The orange plus lines show the median.

Extreme values were removed for better interpretation of the dot plots on the y-scale (see details in Supplementary table 4).

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ARTICLE 6

Performance of host-response biomarkers versus standard clinical and laboratory parameters to risk-stratify children with clinical pneumonia in Bhutan

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Abstract

Pneumonia is the leading cause of death among children under five years of age, however there is no simple triage tool to identify children at risk of progressing to severe and fatal disease. Such a tool could assist for early referral and prioritization of care to improve outcomes and would enhance allocation of scarce resources. We assessed the performance of inflammatory and endothelial activation markers in addition to clinical signs or scoring scales to risk-stratify children hospitalized with pneumonia at the national referral hospital of Bhutan and predict their outcome. Of 118 children, 23 evolved to a poor prognosis, defined as either mortality or the requirement of >5 days of oxygen therapy. IL-8, sTREM-1, sTNFR1, Angpt-2, and sFlt-1 were significantly associated with poor prognosis, whereas white blood cells, platelets, ESR, and CRP were not. sTNFR1 levels upon admission had good predictive accuracy to identify children ≥12 months with pneumonia at risk of poor prognosis and performed better than clinical scores. Our findings confirm that immune and endothelial activation markers have the potential to inform risk-stratification and clinical decision-making in children with pneumonia; however, further external validation is needed.

Background

Pneumonia is the leading infectious cause of preventable deaths among children under five years of age [1]. Every year, up to 226 million children in this age group suffer from pneumonia [2]. While most children will have self-limited disease, a minority of them will progress to severe disease and fatal outcome [3]. Early recognition of children with severe pneumonia enables more aggressive referral and treatment, leading to reduced mortality [4]. Therefore, there is a need for early identification of children at risk of progressing to severe disease starting at the first contact with the healthcare system. At a primary health care level, a simple triage tool that would discriminate children at risk of severe pneumonia from those with self-limited disease could assist decision-making for early referral to a superior healthcare level, particularly in resource-limited settings. In addition, the identification of high-risk children will aid prioritization of care in busy healthcare centres and guide rational allocation of scarce resources.

Clinical signs and simple laboratory testing have been combined to generate clinical severity scores to improve early detection of children with fever or respiratory symptoms at risk of poor outcomes [5–7]. However, most of these severity scores involve the measurement of vital signs (e.g., temperature, respiratory rate, or blood pressure), the assessment of clinical signs (e.g., recognising chest retractions), or the interpretation of laboratory parameters that require trained healthcare workers. Furthermore, risk scores are validated and routinely used in adults with pneumonia, but none have been widely implemented for childhood pneumonia [7]. Therefore, the unresolved need for a simple severity assessment for children with respiratory symptoms may require an innovative approach to current clinical strategies [8].

Specific biomarkers of host response including immune and endothelial pathways, have been previously identified to be activated in the pathogenesis of severe infections, irrespective of their underlying aetiology ("pathogen-agnostic") [4,9]. The quantification of such biomarkers may enable risk stratification and guide clinical decision-making regarding the need for early triage, referral, hospitalization, and admission to intensive care units [10]. Quantifying these markers at clinical presentation has been shown to be useful in predicting severity and outcomes in adults and children with life-threatening infections, including pneumonia, severe malaria, COVID-19, haemorrhagic fevers, or sepsis [11–18]. However, they have not been widely evaluated in low- and middle-income countries, and their prognostic utility in childhood pneumonia has not been validated vis-à-vis standard risk-stratification clinical algorithms [10,16].

The Respiratory Infections in Bhutanese Children (RIBhuC) study recruited Bhutanese children aged 2 to 59 months hospitalized with clinical pneumonia. Hereby, we assessed the performance of

inflammatory, immune, and endothelial activation markers alone or in addition to clinical signs or scoring scales to risk-stratify children hospitalized with pneumonia and predict their outcome.

Methods

Study design

The RIBhuC study was prospectively conducted during 12 consecutive months at the Jigme Dorji Wangchuck National Referral Hospital (JDWNRH) in Thimphu, Bhutan [19]. Briefly, the paediatric department at JDWNRH consists of 38 beds, including five in the paediatric intensive care unit. All children aged 2 to 59 months admitted at JDWNRH and fulfilling the World Health Organization (WHO) criteria for pneumonia or severe pneumonia were recruited [20]. Pneumonia was defined as history of cough or reported breathing difficulty and increased respiratory rate (≥ 50 breaths per minute in children aged 2-11 months or ≥ 40 breaths per minute in children aged 12-59 months) or chest retractions (subcostal and/or intercostal retractions defined as lower chest retractions and supraclavicular and/or suprasternal retractions defined as upper chest retractions). Severe pneumonia was defined as history of cough or reported breathing difficulty, and at least one of the following: oxygen saturation < 90%, central cyanosis, severe respiratory distress, or any danger sign (inability to breastfeed or drink, lethargy or reduced level of consciousness, convulsions). We excluded children when the principal reason for admission was a non-respiratory illness or a condition that was not caused by respiratory illness, those admitted in the previous seven days in order to exclude hospital-acquired infection, and children with evidence of a foreign body in the respiratory tract.

For all eligible patients whose parents provided written consent for study participation, we collected demographic and clinical data, biological samples, and a chest radiography on admission. Radiographical endpoints were defined as per WHO radiological criteria [21]. The study protocol was approved by the Research Ethics Board of Health, Ministry of Health, in Thimphu, Bhutan (PO/2016/086) and by the research ethics committee from the Hospital Clínic in Barcelona, Spain (HCB/2017/0741). All methods were performed in accordance with the relevant guidelines and regulations.

Clinical scoring scales and outcomes

We used three simple clinical scoring scales developed for predicting disease severity and mortality in low-resource settings (Table 1). Clinical parameters were assessed upon admission. The Respiratory Index of Severity in Children (RISC) score was developed among children 0-24 months hospitalized with respiratory infections [22]. The RISC-Malawi is a modified version, which was developed among children <59 months hospitalized with WHO-defined pneumonia [5]. The Lambaréné

Organ Dysfunction Score (LODS) was developed among children with severe malaria for identifying those needing referral or close monitoring [23]. Although LODS was not specifically developed for pneumonia, it is a promising prognostic tool used in childhood diseases other than malaria [6].
The primary outcome was prognosis, defined as "good" if the child survived and did not require supplemental oxygen or only received oxygen therapy for ≤5 days, and "poor" if the child died or received oxygen for >5 days. The usual time of duration of hypoxaemia is 2 to 5 days, therefore we considered longer duration as poor prognosis [24,25]. Secondary outcomes were death, duration of hospital stay, admission to the paediatric intensive care unit, and WHO-defined severe pneumonia. Clinical decisions such as weaning oxygen and time of discharge were taken by any treating paediatrician working at JDWNRH, unaware of the study outcomes for analysis, and therefore at low risk of introducing performance bias.

Table 1. Clinical scoring scales

RISC[a] score		RISC-Malawi score		LODS	
Severity of respiratory signs					
SpO2 ≤90%	3 points	-2 ≤ WAZ < -3 SD[b]	3 points	Prostration[c]	1 point
OR		WAZ ≤ -3 SD[b]	7 points	Blantyre coma score <3	1 point
Chest indrawing	2 points	SpO2 90-92%	2 points	Deep breathing[d]	1 point
Wheezing	-2 points	SpO2 <90%	7 points		
Refusal to feed	1 point	Wheezing	-2 points		
Growth standards		Unconscious at exam	8 points		
WAZ ≤ -3 SD	2 points	Female gender	1 point		
-2 ≤ WAZ < -3 SD	1 point				

Abbreviations: LODS: Lambaréné Organ Dysfunction Score; RISC: respiratory index of severity in children; SD: standard deviations; WAZ: weight-for-age Z-score.
[a]For non-HIV infected children.
[b]Moderate and severe malnutrition were originally assessed with middle-upper arm circumference (MUAC). We substituted these measurements by using WAZ as we did not collect MUAC in our study.
[c]Prostration was defined by not being able to breastfeed, sit, stand, or walk, depending on the age of the child.
[d]Deep breathing is also known as Kussmaul's respiration or 'acidotic' breathing.

Laboratory testing

Blood samples were collected from each participant at time of enrolment and were processed following local standard care [19]. For measurement of immune and endothelial activation markers, blood (2mL) was collected in EDTA tube and centrifugated (3000 g for three minutes). Plasma was separated and stored at -80ºC until shipment to the University of Toronto, Canada, for analyte testing. Plasma concentration of interleukin-6 (IL-6), interleukin-8 (IL-8), soluble triggering receptor expressed on myeloid cells 1 (sTREM-1), soluble tumour necrosis factor receptor 1 (sTNFR1), angiopoietin-2 (Angpt-2), soluble fms-like tyrosine kinase-1 (sFlt1), and procalcitonin (PCT) were quantified using a multiplex Luminex platform with reagents from R&D Systems (Minneapolis, MN)

as described [26]. C-reactive protein (CRP) was quantified by enzyme-linked immunosorbent assay. Biomarker concentrations outside of the detection limits were assigned a value of one third below or above the lowest or highest limit in the standard curve, respectively.

Erythrocyte sedimentation rate (ESR), and CRP were measured at the study site (JDWNRH). We refer to CRP-study and CRP-ref for differentiating CRP analyzed at the study and reference laboratories, respectively. Biomarkers were measured blinded to children clinical characteristics and outcome.

Data management and statistical analysis

Data were entered into a computerized password-protected database (ODK Aggregate version 1.4.13) with study identification number [27]. The statistical associations were assessed using Chi-square, Fisher exact, and Mann-Whitney U tests, as appropriate. Univariable logistic regression models were used to estimate odds ratios of biomarker levels as predictors of prognosis, and multivariable logistic regression models to estimate the degree of association after adjusting for observed confounders. All continuous variables with non-parametric distribution were log transformed for inclusion in logistic regression models. Area under the receiver operating characteristics (AUROC) curve and other performance characteristics (sensitivity, specificity, and likelihood ratios) were calculated to assess the predictive capability, based on each univariable logistic regression model and using cut-off points defined with the Youden's index method (J=max[sensitivity+specificity-1]). AUROCs were compared using the algorithm suggested by DeLong *et al* (1988) [28]. Classification and regression tree analyses were performed to create simple algorithms based on risk-stratification. We established the settings of a minimum of 5 cases for parent node and 1 for child node, and maximum levels for tree depth of 2. We assigned the cost of misclassifying poor prognosis as 10 times greater than the cost of misclassifying good prognosis, as a helpful triage tool requires high sensitivity [13,17]. We performed subgroup analysis by age groups, as age is a potentially relevant cofounder for clinical signs and biomarker levels. Data analyses and figures were performed with Stata™ v.16.0 (StataCorp, College Station, Texas, USA), SPSS Statistics version 23, and RStudio [29,30]. Significance was set at 0.05.

Results

Of 189 children with clinical pneumonia recruited to the RIBhuC study, 118 (62.4%) had biomarker quantification and were included in the analysis (Supplementary Fig. S1). Our study did not perform additional blood draws outside of clinical care, and therefore children that did not have blood collected at first presentation did not have biomarker analysis performed. The characteristics of children included and excluded from the analysis are summarized in Supplementary Table S1. Except for hypoxemia, which was more common among children included in the analysis, there were no significant differences between children included and excluded from the analysis.

Association of demographic characteristics, clinical signs, and scoring scales with prognosis

Of the 118 children included, 23 evolved to a poor prognosis, defined as either the requirement of >5 days of oxygen therapy (n=20) or mortality (n=3). Tables 2 and 3 present demographic, clinical, radiological, and laboratory findings collected upon admission, according to prognosis. A lower proportion of females evolved to a poor prognosis. Parental education, employment, and access to care were not associated with prognosis. Nearly half of the children with a poor prognosis presented with a normal chest radiograph, while one-quarter of children with a good prognosis presented radiological endpoint pneumonia. A positive blood culture was not associated with prognosis. Hypoxemia, high fever, lower chest retractions, upper chest retractions, nasal flaring, rhonchi, and prostration at presentation were all associated with poor prognosis, while auscultation of wheezing was associated with good prognosis. The oxygen saturation upon admission was significantly lower among children with poor prognosis. An elevated score in any of the four clinical scoring scales (WHO, RISC, RISC-Malawi, and LODS) was associated with poor prognosis.

Table 2. Demographic characteristics of participants according to prognosis

Characteristics	Good prognosis (N = 95)	Poor prognosis (N = 23)	p-value[a]
Infants (<12 months)	47 (49.5)	15 (65.2)	0.175
Gender, female	48 (50.5)	6 (26.1)	0.035
Immunization status			0.379
Fully	72/92 (78.3)	16 (69.6)	
Partially	20/92 (21.7)	7 (30.4)	
None	0/92 (0)	0 (0)	
Wasting (WAZ ≤ -2SD)[b]	7/94 (7.5)	4 (17.4)	0.223
Known case of HIV infection	0 (0)	0 (0)	NA
Exposure to tobacco smoke	14 (15.2)	2 (9.1)	0.733
Exposure to betel nut (doma)	58/92 (63.0)	16/22 (72.7)	0.393
Exposure to heater with kerosene	8/83 (9.6)	0 (0)	0.354
Parental education			0.665
Both parents are illiterate	15/92 (16.3)	6/22 (27.3)	
Only one parent has primary education	12/92 (13.0)	3/22 (13.6)	
Both parents have primary education	41/92 (44.6)	9/22 (40.9)	
At least one parent has university education	24/92 (26.1)	4/22 (18.2)	
Both parents unemployed			0.340
Both parents are unemployed	1/89 (1.1)	1/20 (5.0)	
Only one parent is employed	56/89 (62.9)	14/20 (70.0)	
Both parents are employed	32/89 (36.0)	5/20 (25.0)	
Time to access health care facility > 30 minutes	6/92 (6.5)	2/21 (9.5)	0.640

Abbreviations: NA: not applicable; SD: standard deviations; WAZ: weight-for-age Z-score.

[a]Comparison of proportions using the chi-square or fisher tests.

[b]Nutritional status was based on the WAZ score generated using the 2000 Centers for Disease Control and Prevention Growth Reference [31,32].

Table 3. Clinical characteristics of participants according to prognosis

Characteristics	Good prognosis (N = 95)	Poor prognosis (N = 23)	p-value[a]
Clinical history for current illness			
Reported duration of illness prior to admission ≥ 5 days	44 (46.3)	12 (52.2)	0.614
Reported duration of fever prior to admission			0.647
No fever	15/94 (16.0)	5/22 (22.7)	
< 5 days	55/94 (58.5)	11/22 (50.0)	
≥ 5 days	24/94 (25.5)	6/22 (27.3)	
Referred from another healthcare centre	10 (10.5)	4 (17.4)	0.470
Started on antibiotics prior to admission	20/93 (21.5)	5 (21.7)	0.981
Clinical characteristics at admission			
Capillary refill > 3 seconds	3 (3.2)	1 (4.4)	1.000
Tachycardia for age[b]	27/94 (28.7)	6 (26.1)	0.801
Increased respiratory rate[c]	48 (50.5)	14/22 (63.6)	0.267
SpO2 (median, IQR)[d]	86 (80 to 90)	77 (70 to 84)	0.003
Hypoxemia (SpO2<90%)	71 (74.7)	23 (100)	0.004
Fever (≥37.5ºC, axillar)	41 (43.2)	8 (34.8)	0.465
High fever (>39ºC, axillar)	2 (2.1)	3 (13.0)	0.050
Lower chest retractions[e]	44/94 (46.8)	19 (82.6)	0.002
Upper chest retractions[e]	8/94 (8.5)	6 (26.1)	0.031
Nasal flaring	17/94 (18.1)	9 (39.1)	0.030
Grunting	4 (4.2)	3 (13.0)	0.133
Crackles	52/94 (55.3)	16 (69.6)	0.215
Ronchi	43/94 (45.7)	16 (69.6)	0.041
Wheezing	29/91 (31.9)	3 (13.0)	0.073
Alteration consciousness	3 (3.2)	1 (4.4)	1.000
Prostration	11 (11.6)	8 (34.8)	0.012
Seizure	0 (0)	0 (0)	NA
Clinical scoring scales at admission			
Severe WHO pneumonia	72 (75.8)	23 (100)	0.006
RISC score (median, IQR)	3 (1 to 3)	4 (3 to 5)	<0.0001
RISC-Malawi score (median, IQR)	6 (3 to 8)	7 (7 to 8)	0.0033
LODS (median, IQR)	0 (0 to 0)	0 (0 to 1)	0.0066
Radiological findings			
Endpoint pneumonia	19/79 (24.1)	6/17 (35.3)	0.682
Other infiltrates	17/79 (21.5)	3/17 (17.6)	
Normal	43/79 (54.4)	8/17 (47.1)	
Laboratory findings at admission			
Anaemia (Haemoglobin < 11 g/dL)	28 (29.5)	14 (60.9)	0.005
Leucocytosis[f]	34 (35.8)	7 (30.4)	0.628
Thrombocytosis (> 450x10^9 platelets/L)	23/93 (24.7)	7 (30.4)	0.576
High ESR (≥ 50 mm)	13/86 (15.1)	5/22 (22.7)	0.521
High CRP-study (> 4 mg/dL)	15/91 (16.5)	3 (13.0)	1.000
High CRP-ref (> 4 mg/dL)	74 (77.9)	16 (69.6)	0.399
High PCT (≥ 250 pg/mL)	26 (27.4)	11 (47.8)	0.058
Non-contaminated positive bacterial blood culture	5/80 (6.3)	2/20 (10.0)	0.625
Hospital management			
Antibiotic therapy	64 (67.4)	20 (87.0)	0.063
Oxygen therapy	66 (69.5)	23 (100)	0.002
Hospital stay ≥ 7 days	7 (7.4)	15 (65.2)	<0.001

Abbreviations: CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; IQR: interquartile range; LODS: Lambaréné Organ Dysfunction Score; NA: not applicable; PCT: procalcitonin; RISC: respiratory index of severity in children; WHO: World Health Organization.

[a]Comparison of proportions using the chi-square or fisher tests.

[b]Tachycardia was defined as heart rate > 160/minute for infants <12 months, and > 150/minute for children ≥ 12 months of age [6].

[c]Increased respiratory rate was defined as >50breaths per minute in children aged 2 to 12 months and >40 breaths per minute in children aged ≥ 12 months.

[d]Peripheral capillary oxygen saturation was measured in room air using Mindray VS-800 Vital Sign Monitor or Biolight BLT M800 Handheld pulse oximeter. Eight children (2 with good prognosis and 6 with poor prognosis including two children with fatal outcome) had SpO2 <90% and were put on oxygen before or at arrival, with missing exact SpO2 value.
[e]Lower chest retractions were defined as subcostal and/or lower intercostal retractions, and upper chest retractions were defined as supraclavicular and/or suprasternal retractions.
[f]Leucocytosis was defined as white blood cells greater than 15 x 10^9 cells/L for children aged between 2 and 11 months and greater than 13 x 10^9 cells/L for children aged between 12 and 59 months.

Association of host-response biomarkers with prognosis

Overall, results of the routinely-ordered laboratory testing, including white blood cells (WBC) (>15x10^9/L for children 2-11 months and >13x10^9/L for children 12-59 months), platelets (>450x10^9/L), ESR (≥50 mm), PCT (≥250 pg/mL), and CRP (>4 mg/dL), were not associated with prognosis when evaluated using common clinical thresholds (Table 3). Similar results were observed when these laboratory parameters were assessed as continuous variables, with the exception of PCT, which was significantly associated with prognosis (Fig. 1 and Supplementary Table S2). In contrast, plasma levels of all the immune and endothelial activation factors, except for IL-6, were significantly higher at presentation in children evolving to poor prognosis (Fig. 1). Differences remained statistically significant after adjusting for selected potential confounding factors for sTREM-1, sTNFR1, Angpt-2, and sFlt1 (Supplementary Fig. S2).

Furthermore, we evaluated the association of biomarker levels with additional severity and prognosis outcomes (Supplementary Table S3). High levels of IL-8, sTREM-1, sTNFR1, and sFlt1 were associated with fatal outcome and hospitalization ≥7 days, and Angpt-2 with hospitalization ≥7 days too. All inflammatory markers but none of the endothelial activation factors were associated with admission to the paediatric intensive care unit. No biomarkers were associated with WHO-defined severe pneumonia. Finally, WBC, platelets, ESR, CRP, and PCT were not associated with any of these secondary outcomes.

Performance of clinical characteristics, scoring scales, and biomarkers at predicting poor prognosis

Of single clinical characteristics, oxygen saturation (AUROC 0.73, 95% confidence interval [CI] 0.60 to 0.86) and lower chest retractions (AUROC 0.68, 95%CI 0.58 to 0.77) on admission displayed the best predictive accuracy for prognosis (Fig.2). Of the clinical scoring scales, RISC presented the best predictive performance (AUROC 0.75, 95%CI 0.66 to 0.85) but was not significantly better than other single clinical characteristics at predicting prognosis (P >0.05).

The best host-response biomarkers for predicting poor prognosis were sFlt1 (AUROC 0.71, 95%CI 0.60 to 0.82), sTNFR1 (AUROC 0.69, 95%CI 0.55 to 0.82), and sTREM-1 (AUROC 0.68, 95%CI 0.55 to 0.82) (Fig.2). These three immune and endothelial activation markers performed significantly better than the commonly used inflammatory markers WBC, ESR, and CRP, but no better than IL-6, IL-8,

Angpt-2, or PCT (AUROC between 0.58 and 0.65) (Supplementary Table S4). Supplementary Table S5 summarizes additional performance characteristics of clinical scoring scales and biomarkers.

Top performing biomarkers improve the prognostic performance of clinical characteristics

We assessed the performance of combinations of the best performing clinical signs, scales, and biomarkers. The addition of either sFlt1, sTNFR1, or sTREM-1 significantly improved the prognostic performance of lower chest retractions or the RISC score, but these combinations did not perform better than sFlt1, sTNFR1, or sTREM-1 alone (Table 4). Taking into consideration that RISC is a clinical scoring scale that includes the assessment of chest indrawing, we concluded that sFlt1, sTNFR1, or sTREM-1 combined with assessment of lower chest retractions was the most parsimonious prognostic model.

Table 4. Performance of clinical parameters associated with top predicting biomarkers sFlt1, sTNFR1, or sTREM-1

AUROC				
	Clinical parameter	**+ sFlt1**	**+ sTNFR1**	**+ sTREM-1**
Oxygen saturation	0.73 (0.60 to 0.86)	0.78	0.79	0.77
Lower chest retractions	0.68 (0.58 to 0.77)	0.79*	0.81*	0.80*
RISC score	0.75 (0.66 to 0.85)	0.82*	0.83*	0.82[a]
sFlt1	0.71 (0.60 to 0.82)	-	0.73	0.75
sTNFR1	0.69 (0.55 to 0.82)	-	-	0.73
sTREM1	0.68 (0.55 to 0.82)	-	-	-

Abbreviations: AUROC: area under the receiver operating characteristics; RISC: respiratory index of severity in children; sFlt1: soluble fms-like tyrosine kinase-1; sTNFR1: soluble tumour necrosis factor receptor 1; sTREM-1: soluble triggering receptor expressed on myeloid cells 1.
Differences in AUROCs were assessed using the algorithm suggested by DeLong *et al* (1988) [28].
*p<0.05 for comparison of AUROC of the clinical parameter alone versus AUROC of the combination of the clinical parameter with either sFlt1, sTNFR1, or sTREM-1.
[a]p=0.0509 comparing AUROCs of the RISC score versus RISC score with sTREM-1.

Prognosis performance of clinical characteristics, scoring scales, and biomarkers differ by age groups

We investigated the performance of biomarkers by age groups since inflammatory response varies by age [33,34]. We performed subgroup analyses among infants (<12 months) and older children (≥12 months) (Table 5). Except for increased respiratory rate, the performance of all the clinical characteristics and scoring scales was higher in infants compared to older children, although these findings were not statistically significant. Conversely, platelets, ESR, CRP-study, CRP-ref, PCT, and all the immune and endothelial activation markers except Angpt-2 performed better at predicting poor outcome in children ≥12 months compared to infants, although statistically significant only for PCT and sTNFR1.

The RISC score in infants (AUROC 0.80, 95%CI 0.69 to 0.92) performed significantly better than any of the biomarkers except for the two endothelial activation markers (Angpt-2 and sFlt1) in predicting poor prognosis. In older children, the RISC score (AUROC 0.71, 95%CI 0.54 to 0.87) performance was similar to all biomarkers except sTNFR1 (AUROC 0.90, 95%CI 0.80 to 1.00; p=0.0557). Indeed, in children ≥12 months, sTNFR1, PCT, and IL-6, presented good performance at predicting prognosis, with AUROC >0.80. Biomarker levels by age group are reported in Supplementary Table S2.

Table 5. Performance of clinical characteristics and biomarkers for identifying children at risk of poor prognosis

	AUROC (95% CI)		
	All	**<12 mo**	**≥12mo**
Clinical characteristics			
Increased respiratory rate	0.57 (0.45 to 0.68)	0.55 (0.39 to 0.70)	0.64 (0.49 to 0.78)
Oxygen saturation	0.73 (0.60 to 0.86)	0.79 (0.65 to 0.92)	0.62 (0.36 to 0.88)
Lower chest retractions	0.68 (0.58 to 0.77)	0.74 (0.65 to 0.84)	0.57 (0.37 to 0.76)
Upper chest retractions	0.59 (0.49 to 0.68)	0.60 (0.48 to 0.72)	0.57 (0.41 to 0.74)
Clinical scoring scales			
WHO severity score	0.62 (0.58 to 0.66)	0.68 (0.61 to 0.75)	0.56 (0.52 to 0.61)
RISC score	0.75 (0.66 to 0.85)	0.80 (0.69 to 0.92)	0.71 (0.54 to 0.87)
RISC-Malawi score	0.69 (0.59 to 0.80)*	0.75 (0.63 to 0.87)	0.67 (0.46 to 0.89)
LODS	0.62 (0.51 to 0.72)**	0.64 (0.50 to 0.77)**	0.57 (0.41 to 0.74)
Acute phase proteins and inflammatory markers			
WBC	0.48 (0.34 to 0.63)**	0.65 (0.46 to 0.83)**	0.64 (0.40 to 0.87)
Platelets	0.56 (0.43 to 0.70)**	0.58 (0.42 to 0.75)**	0.74 (0.52 to 0.96)
ESR	0.51 (0.35 to 0.67)**	0.63 (0.44 to 0.83)**	0.71 (0.48 to 0.95)
CRP-study	0.50 (0.36 to 0.63)**	0.53 (0.36 to 0.71)**	0.59 (0.39 to 0.79)**
CRP-ref	0.51 (0.37 to 0.65)**	0.59 (0.41 to 0.76)**	0.71 (0.50 to 0.92)
PCT	0.60 (0.46 to 0.74)* ##	0.54 (0.38 to 0.70)**	0.84 (0.66 to 1.00)
Immune activation factors			
IL-6	0.58 (0.43 to 0.73)** #	0.57 (0.37 to 0.76)**	0.81 (0.66 to 0.96)
IL-8	0.62 (0.49 to 0.75)	0.57 (0.40 to 0.74)**	0.66 (0.46 to 0.87)
sTREM-1	0.68 (0.55 to 0.82)	0.60 (0.40 to 0.79)*	0.75 (0.53 to 0.97)
sTNFR1	0.69 (0.55 to 0.82)##	0.50 (0.30 to 0.69)**	0.90 (0.80 to 1.00)*
Endothelial activation factors			
Angpt-2	0.65 (0.51 to 0.80)	0.69 (0.51 to 0.87)	0.60 (0.33 to 0.86)
sFlt1	0.71 (0.60 to 0.82)	0.67 (0.51 to 0.83)	0.73 (0.55 to 0.92)

Abbreviations: Angpt-2: angiopoietin-2; AUROC: area under the receiver operating characteristics; CI: confidence interval; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; IL-6: interleukin-6; IL-8: interleukin-8; LODS: Lambaréné Organ Dysfunction Score; NA: not applicable; PCT: procalcitonin; RISC: respiratory index of severity in children; sFlt1: soluble fms-like tyrosine kinase-1; sTNFR1: soluble tumour necrosis factor receptor 1; sTREM-1: soluble triggering receptor expressed on myeloid cells 1; WBC: white blood cells; WHO: World Health Organization.

Differences in AUROCs were assessed using the algorithm suggested by DeLong *et al* (1988) [28].

*p<0.10 and **p<0.05 for comparison of AUROCs of the RISC score versus each of the other scoring scales and biomarkers.

#p<0.10 and ##p<0.05 for comparison of AUROCs between age groups for each biomarker.

sTNFR1-based algorithms predict poor prognosis in children ≥12 months with pneumonia

As sTNFR1 demonstrated excellent prognostic accuracy for children ≥12 months with pneumonia, we examined this marker with age-related top performing clinical characteristics to generate simple algorithms for risk-stratification in community and hospital settings. sTNFR1 was found to predict outcome significantly better than increased respiratory rate, lower chest retractions, and the WHO severity criteria ($p<0.05$), but not significantly better than SpO2 or the RISC score (Supplementary Table S6).

We performed classification and regression tree analysis to identify optimal cut-off points. We forced the clinical variable to be included in the model first for clinical relevance. The combination of increased respiratory rate or lower chest retractions with sTNFR1 led to a higher but not significantly better performance than sTNFR1 alone ($p=0.1414$ and $p=0.9454$, respectively). With similarity in their overall performance (AUROC 0.95 and 0.94, respectively), the algorithm of increased respiratory rate and sTNFR1 exhibited a higher specificity (95.8%) and positive likelihood ratio (17.86), while the algorithm of low chest retractions and sTNFR1 exhibited a higher sensitivity (87.5%) and lower negative likelihood ratio (0.14) (Fig. 3).

Alone, sTNFR1 presented a sensitivity of 75.0% and specificity of 93.8%, and the positive and negative likelihood ratios were 12.1 and 0.27, respectively (Fig. 3). Finally, the performance of sTNFR1 alone was not significantly inferior to the performance of any combinations of clinical sign and biomarker that we assessed.

Discussion

Prognostic tools that enable the early identification of children with pneumonia that will progress to severe and potentially fatal disease are currently lacking. Early risk-stratification of children with respiratory symptoms could improve triage, early referral, and prioritization of care, and improve outcomes. In the following study, we assessed potential prognostic factors in children hospitalized with WHO-defined pneumonia, including clinical characteristics and a wide range of host-response biomarkers.

We found that several clinical signs upon admission were associated with poor prognosis, while other typical clinical indicators of pneumonia such as increased respiratory rate or grunting, were not. Overall, there was a trend towards higher prognostic performance of clinical signs among infants compared to older children. These findings need to be interpreted with caution given the small sample size, especially because reasons explaining why clinical signs could perform better at predicting prognosis in infants are uncertain. Nevertheless, and in agreement with previous studies,

the clinical signs evaluated demonstrated poor prognostic performance that would not support clinical decision-making in the field [10,14,35,36]. In addition, the detection of clinical signs depends on the health worker ability to correctly assess them, leading to applicability limitations due to interobserver variability and the need of trained health workers [35,37].

To improve prognostic performance of single clinical signs, several scoring scales have been developed, combining clinical signs, risk factors, and simple laboratory testing. LODS was initially developed for the risk assessment of children with malaria but was then found to yield good discrimination to predict in-hospital mortality (AUROC 0.86) among febrile Ugandan children aged 2-59 months with no malaria [6,23]. In the Bhutanese cohort, LODS was associated with poor prognosis but showed a low sensitivity and low prognostic performance (AUROC 0.62). Each of the three components of LODS (coma, prostration, and deep breathing) is indicative of severe disease and therefore may have limited utility in the early stages of severe disease [8]. The RISC score was developed specifically for children with respiratory infections and include clinical signs, several of which may have greater utility in early identification of severe pneumonia [22]. RISC demonstrated superior performance to other scoring systems in our cohort, especially in infants. The high sensitivity of RISC (91.3%) is an essential characteristic for a community-based triage tool. However, the RISC score is difficult to determine in low resource settings as it requires anthropometric measurement to assess weight-for-age, a pulse oximeter, the ability to recognize chest indrawing, and auscultation for wheezing. The WHO severity criteria are widely used and rely on their high sensitivity to detect most cases for antibiotic therapy and hospital management [20]. We observed similar findings in our cohort, where all the children progressing to poor prognosis were classified as severe pneumonia, and no child with poor prognosis was missed according to the WHO criteria. In conclusion, we found that clinical scoring scales were significantly associated with poor prognosis and presented high sensitivity at established cut-off points, but specificity was low, leading to a high number of false-positive cases. In addition, they rely on clinical signs, which does not solve the problem of subjectivity and interobserver variability in their assessment.

Biomarker concentrations can be measured in the blood, with the benefits of objectivity, accuracy, and reproducibility. WBC, platelets, ESR, and CRP are commonly used in clinical practice as aetiological and prognostic markers. However, studies have consistently shown that these biomarkers are poor prognostic predictors for childhood pneumonia [22,35,38]. In previous studies, levels of immune and endothelial activation markers were associated with disease severity and fatal outcome in life-threatening infections, including pneumonia, sepsis, severe malaria, haemorrhagic fevers, or COVID-19 [11–18,39,40]. In the Bhutanese cohort, IL-8, sTNFR1, sTREM-1, Angpt-2, and sFlt1

were all significantly associated with poor prognosis despite the moderately small size of the cohort and few children with fatal outcome.

Similarly, the majority of immune and endothelial markers were good prognostic predictors among children ≥12 months with pneumonia. In this age group, sTNFR1 exhibited the highest AUROC (0.90, 95% CI 0.80 to 1.00) and performed significantly better than clinical parameters and severity scoring systems in predicting poor outcomes. These findings suggest that simple sTNFR1-based algorithms for pneumonia management may represent a strategy to improve care and outcome in children, particularly in resource-limited settings. Selection of which biomarker-based model to apply clinically will depend on the primary goal of the triage tool. For example, highly sensitive algorithms with associated low negative likelihood ratio, such as the one based on chest retractions and sTNFR1, perform well to correctly classify children at low risk of evolving to poor prognosis. These children could be sent home confidently, while those classified at high risk might require close monitoring to ensure early detection of deterioration of the child. On the other hand, algorithms aiming for higher specificity with associated higher positive likelihood ratio, such as the one based on increased respiratory rate and sTNFR1, perform better at correctly classifying children at risk of poor prognosis and as such, could assist care prioritization decisions. This approach is also useful in the context of the COVID-19 pandemic in any setting, to help improve rationale allocation of resources and decision on patient triage in overburdened hospitals.

In contrast, the prognostic performance of immune and endothelial markers was relatively poor among infants, which is likely the result of an immature immune response during infancy [41,42]. The production of IL-6 and TNF among other cytokines, for example, increases within the first three years of life [41]. Our findings suggest that larger studies are needed to elucidate the accuracy of prognostic performance of biomarkers between age groups [33,34]. Angpt-2 demonstrated superior performance in infants compared to older children. Angpt-2 is a key mediator of inflammation and pulmonary vascular permeability. When Angpt-2 binds the Tie2 receptor and antagonizes the binding of Angpt-1, it results in endothelial activation and microvascular leak [14,43]. The reason why Angpt-2 performed better in infants in our cohort, which is opposite of what we expected, may be due to developmental differences by age and/or by our small sample size. Elevated plasma levels of Angpt-2 are reported to be predictive of disease severity and fatal outcome in young infants with sepsis, but also in older children and adults with pneumonia, acute respiratory distress syndrome, sepsis, and acute febrile illness [13,14,39,43–46]. In a cohort of Ugandan children hospitalized with pneumonia, elevated Angpt-2 was also associated with longer duration of oxygen therapy and longer hospital stay [39].

This study has several limitations. The number of cases was not large enough to perform an adequately powered analysis of differences between the prognostic accuracy of clinical signs or biomarkers among age groups. As there were only three deaths in the cohort, we used a composite primary outcome, which limits direct comparison with other studies using mortality as the primary outcome. However, we assessed mortality alone as a secondary outcome to make such comparison possible. Despite the small sample size in this study, our findings point to the importance of considering age-dependent differences in biomarker concentrations, especially in paediatric populations. However, we did not include other factors known to impact circulating biomarker concentrations such as duration of illness, prior administration of antibiotics, malnutrition, and other comorbidities, which are important considerations in biomarker discovery and validation studies [47–49]. Since pneumonia progresses rapidly, increases or decreases between two measurements of the same biomarker over time (dynamic monitoring) might further help in the risk-stratification of children with this disease [50]. Also, since excess mortality can be observed up to three months after discharge from severe infections, we encourage assessing outcomes including post-discharge mortality to avoid missing late events [51,52]. Further research is needed to elucidate the best biomarker or combination of biomarkers and which cut-off points to use for risk-stratification of children with pneumonia.

Nonetheless, our study confirms that immune and endothelial activation markers have the potential to become objective risk-stratification tools of children with pneumonia. A biomarker point-of-care tool alone or integrated into a simple clinical algorithm is likely to enhance clinical decision-making (such as triage and prioritization of care) and improve outcomes, in addition to optimizing resource allocation, especially in low- and middle-income countries, where mortality associated with childhood pneumonia is greatest.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

SJ, QB, MRG, and KCK conceptualized the work. SJ, TL, and KD collected the data. MN and RS performed the laboratory investigations. SJ run the analyses and drafted the main text. QB, MRG, KCK, and MN substantively revised the manuscript. MRG and MN prepared figures 1-3 and S2. All authors reviewed the manuscript.

Additional information

The authors declare no competing interests.

Figures

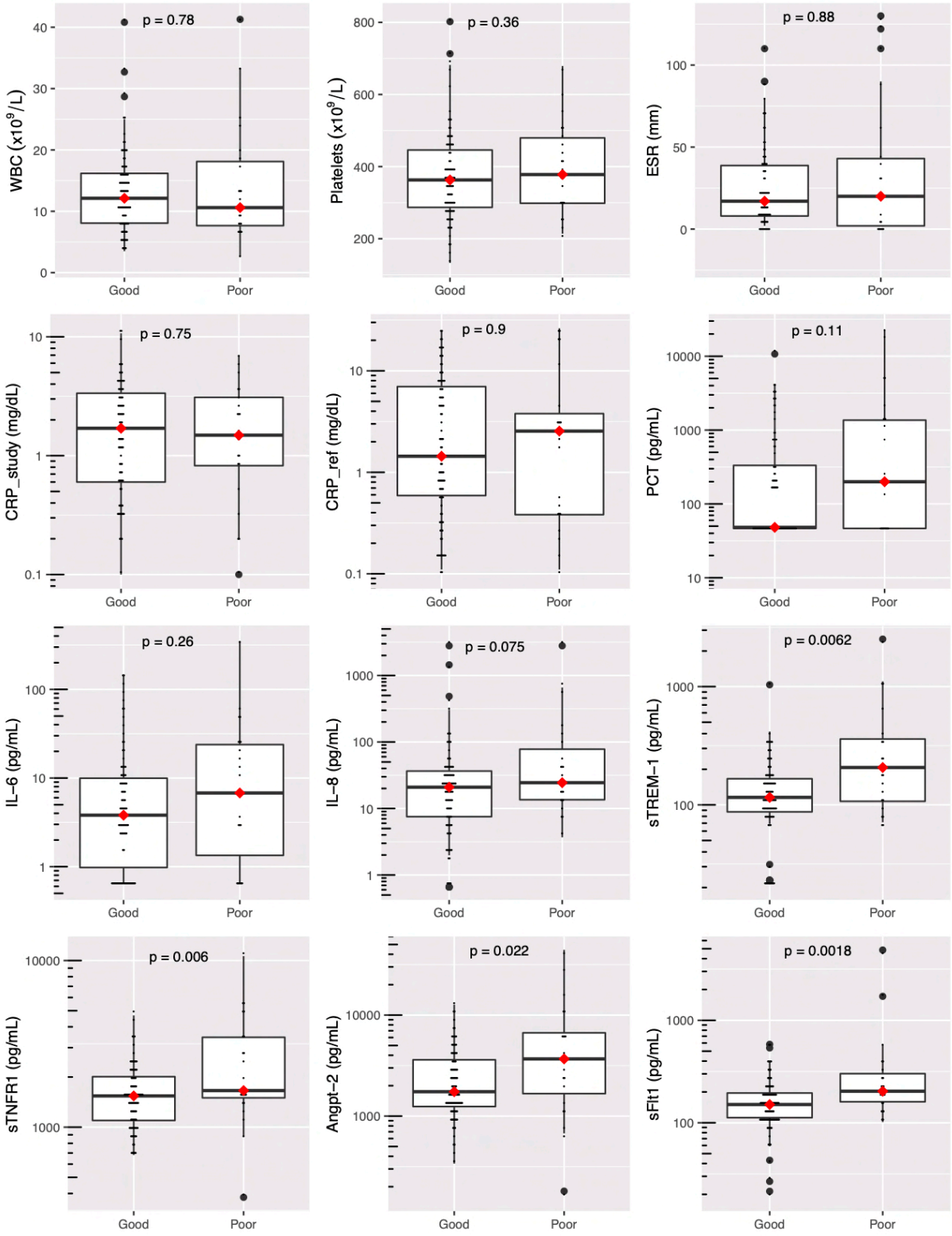


Figure 1. Performance of host-biomarkers levels according to prognosis.
Levels of each biomarker is summarized graphically through the median (red dot) and interquartile range (lower and upper side of the box). Good prognosis was defined as survival and no requirement of oxygen or oxygen therapy for ≤5 days, while poor prognosis was defined as death and/or oxygen therapy for >5 days. Statistical significance of differences between good and poor outcome for each

biomarker level was calculated using the Mann-Whitney U tests, with p-value shown at the top of each biomarker comparison.

Abbreviations: Angpt-2: angiopoietin-2; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; IL6: interleukin-6; IL8: interleukin-8; PCT: procalcitonin; sFlt1: soluble fms-like tyrosine kinase-1; sTNFR1: soluble tumour necrosis factor receptor 1; sTREM-1: soluble triggering receptor expressed on myeloid cells 1; WBC: white blood cells.

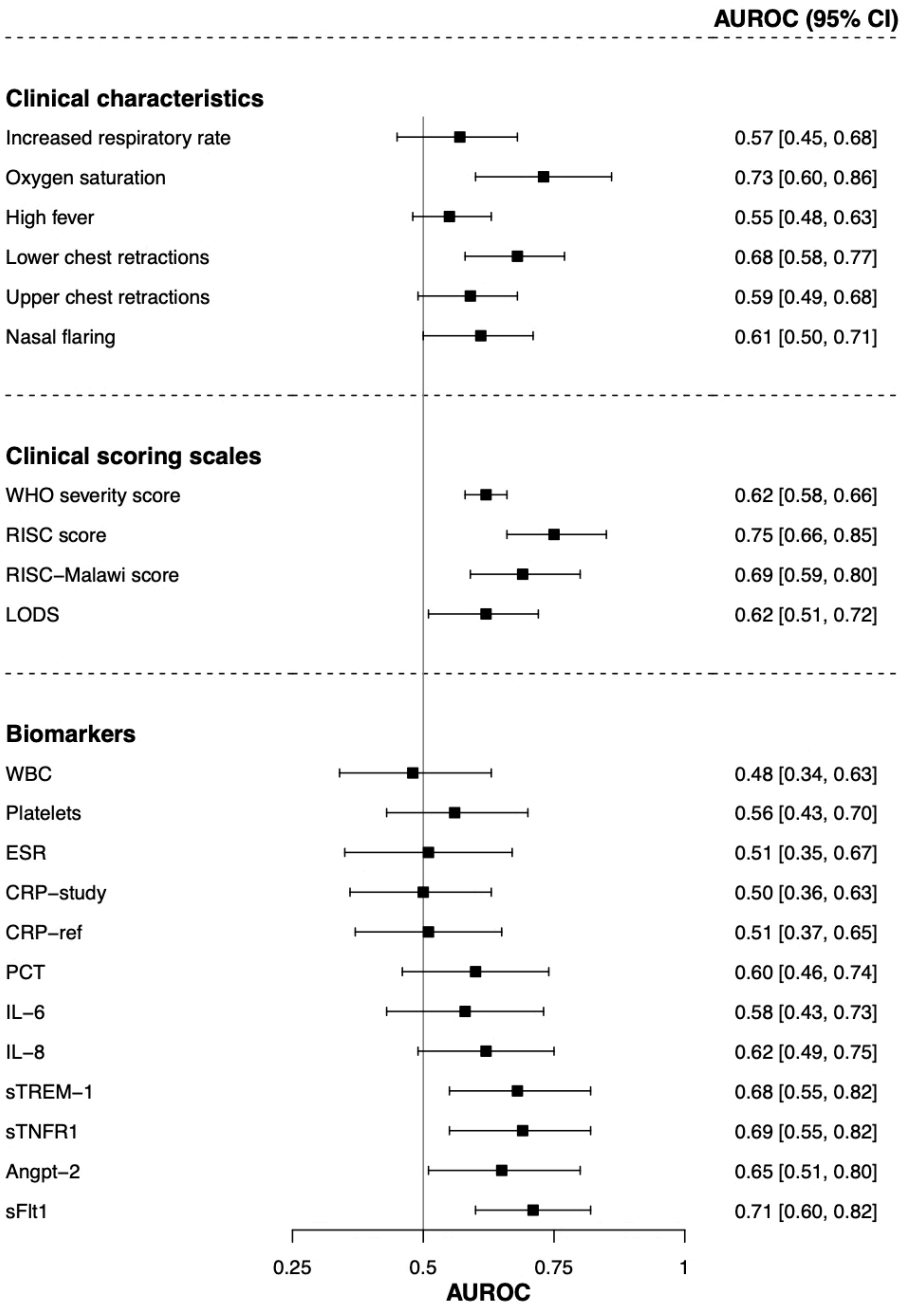
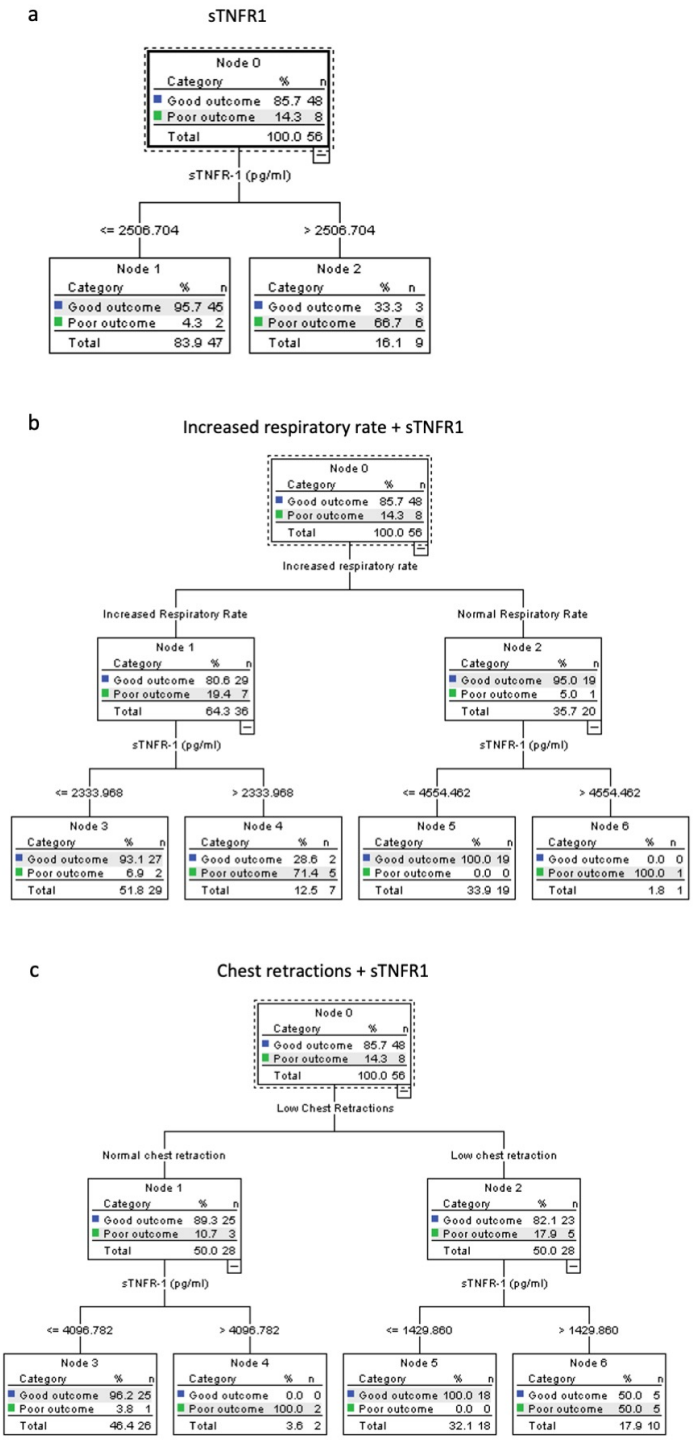


Figure 2. Prognostic accuracy of clinical characteristics, scoring scales and host-response biomarkers in children with pneumonia.

Nonparametric ROC curves were generated. AUROC was plotted for each variable to illustrate its ability to discriminate between good and poor prognosis. For each variable, AUROC value with the 95% confidence interval in parenthesis are displayed to the right of its plot.

Abbreviations: Angpt-2: angiopoietin-2; AUROC: area under the receiver operating characteristics; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; IL6: interleukin-6; IL8: interleukin-8; LODS: Lambaréné Organ Dysfunction Score; PCT: procalcitonin; RISC: respiratory index of severity in children; sFlt1: soluble fms-like tyrosine kinase-1; sTNFR1: soluble tumour necrosis factor receptor 1; sTREM-1: soluble triggering receptor expressed on myeloid cells 1; WBC: white blood cells; WHO: World health Organization.



	Sensitivity	Specificity	+ LR	- LR
sTNFR1	75.0%	93.8%	12.1	0.27
Increased respiratory rate + sTNFR1	75.0%	95.8%	17.86	0.26
Chest retractions + sTNFR1	87.5%	89.6%	8.41	0.14

Figure 3. Classification and regression tree analysis algorithms to predict poor outcome in children ≥12 months with pneumonia.

The algorithms were generated for sTNFR1 **(a)**, increased respiratory rate and sTNFR1 **(b)**, and chest retractions and sTNFR1 **(c)**. Good prognosis was defined as survival and no requirement of oxygen or oxygen therapy for ≤5 days; poor prognosis was defined as death and/or oxygen therapy for >5 days.

For all models, the cost of misclassifying a child with poor prognosis was designated as 10 times the cost of misclassifying a child with good prognosis. Classification and regression tree analysis selected the optimal cut-off points. We forced the clinical variable to be included in the model first for clinical relevance. The performance of each of the three algorithms are presented in the table below them.

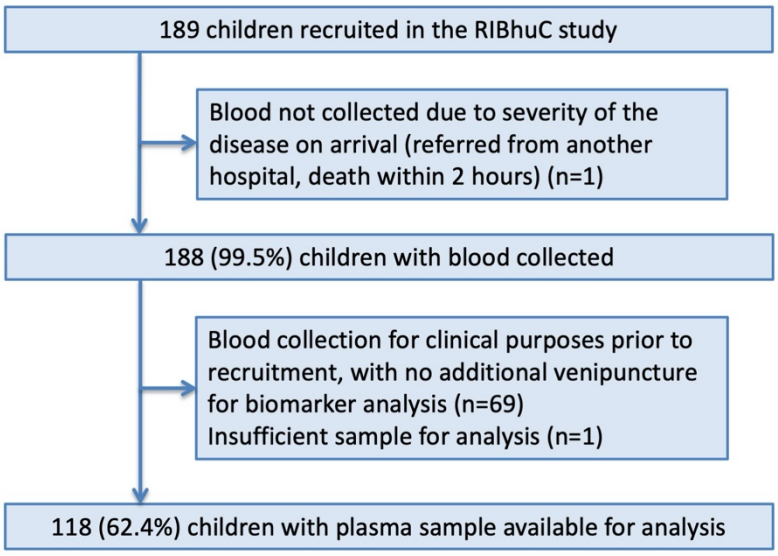
Abbreviations: LR: likelihood ratio; sTNFR1: soluble tumour necrosis factor receptor 1.

Performance of host-response biomarkers versus standard clinical and laboratory parameters to risk-stratify children with clinical pneumonia in Bhutan

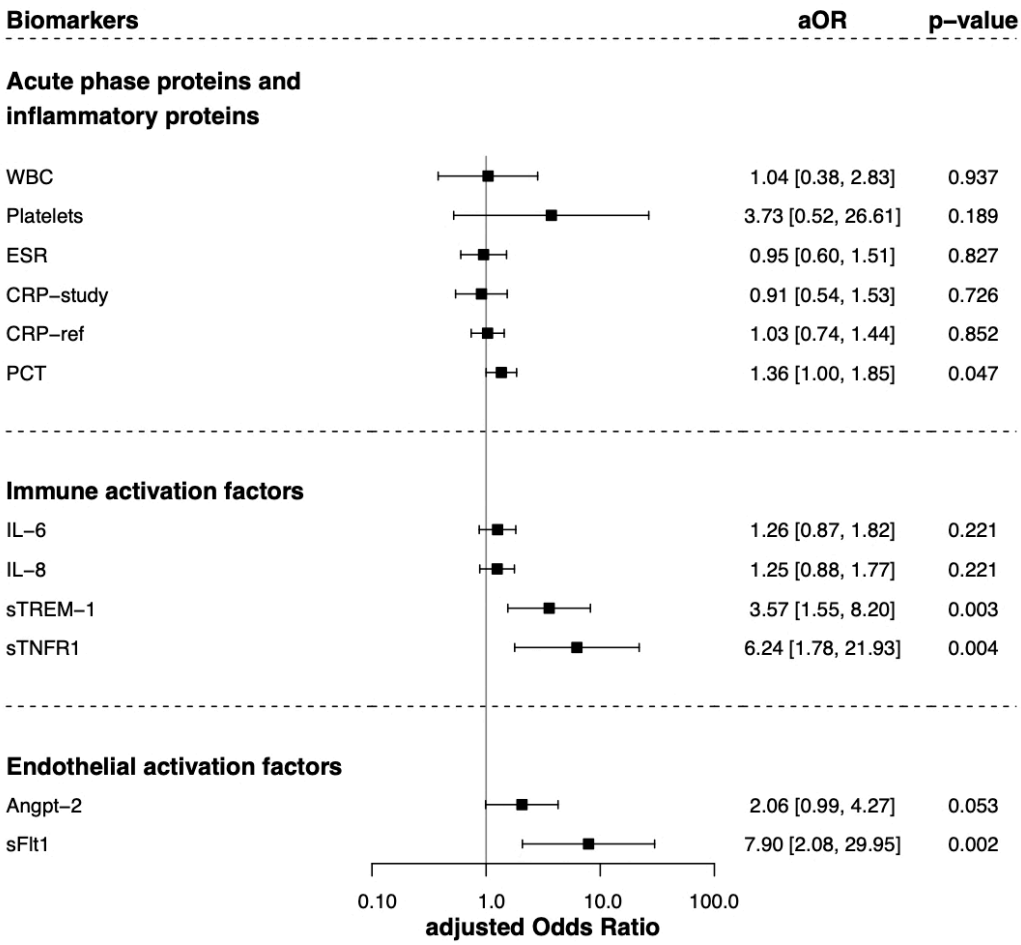
Supplementary material

Authors

Sophie Jullien, Melissa Richard-Greenblatt, Michelle Ngai, Tenzin Lhadon, Ragunath Sharma, Kumbu Dema, Kevin C. Kain, Quique Bassat



Supplementary Figure S1. Flowchart of children recruited in RIBhuC with biomarkers findings available for inclusion in this study.



Supplementary Figure S2. Adjusted associations for different biomarkers with their capacity to risk-stratify children with pneumonia

All variables were continuous with non-normal distribution and were log transformed for logistic regression analyses. Variables were adjusted by sex, age, high fever, lower and upper chest wall retractions, nasal flaring and rhonchi.
Abbreviations: Angpt-2: angiopoietin-2; aOR: adjusted Odds Ratio; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; IL6: interleukin-6; IL8: interleukin-8; PCT: procalcitonin; sFlt1: soluble fms-like tyrosine kinase-1; sTNFR1: soluble tumour necrosis factor receptor 1; sTREM-1: soluble triggering receptor expressed on myeloid cells 1; WBC: white blood cells.

Supplementary Table S1. Baseline characteristics and comparison between children with available (included) and unavailable (excluded) biomarker results.

Characteristics	Children excluded (no biomarker findings) (N = 71)	Children included in analysis (N = 118)	p-value[a]
Demographic characteristics			
Gender, female	26 (36.6)	54 (45.8)	0.218
Infants (<12 months)	39 (54.9)	62 (52.5)	0.750
Immunization status			0.882
Fully	55 (77.5)	88/115 (76.5)	
Partially	16 (22.5)	27/115 (23.5)	
None	0 (0)	0/115 (0)	
Wasting (WAZ ≤ -2SD)[b]	6/70 (8.6)	11/117 (9.4)	0.848
Known case of HIV infection	0 (0)	0 (0)	NA
Exposure to tobacco smoke	5/65 (7.7)	16/114 (14.0)	0.205
Exposure to betel nut (doma)	41/65 (63.1)	74/114 (64.9)	0.805
Exposure to heater with kerosene	6/59 (10.2)	8/104 (7.7)	0.588
Parental education			0.231
Both parents are illiterate	5/64 (7.8)	21/114 (18.4)	
Only one parent has primary education	11/64 (17.2)	15/114 (13.2)	
Both parents have primary education	28/64 (43.8)	50/114 (43.9)	
At least one parent has university education	20/64 (31.2)	28/114 (24.5)	
Both parents unemployed			0.212
Both parents are unemployed	0/65 (0)	2/109 (1.8)	
Only one parent is employed	35/65 (53.9)	70/109 (64.2)	
Both parents are employed	30/65 (46.1)	37/109 (33.9)	
Time to access health care facility > 30 minutes	3/63 (4.8)	8/113 (7.1)	0.748
Clinical history for current illness			
Reported duration of illness prior to admission ≥ 5 days	30/70 (42.9)	56 (47.5)	0.540
Reported duration of fever prior to admission			0.733
No fever	9/68 (13.2)	20/116 (17.2)	
< 5 days	42/68 (61.8)	66/116 (56.9)	
≥ 5 days	17/68 (25.0)	30/116 (25.9)	
Referred from another healthcare centre	13 (18.3)	14 (11.9)	0.220
Started on antibiotics prior to admission	18 (25.4)	25/116 (21.6)	0.549
Clinical characteristics at admission			
Capillary refill > 3 seconds	3/70 (4.3)	4 (3.4)	0.713
Tachycardia for age[c]	24/69 (34.8)	33 (28/2)	0.347
Increased respiratory rate[d]	30/67 (44.8)	62/117	0.284
SpO2 (median, IQR)[e]	86 (81 to 91)	84.5 (79 to 88)	0.096
Hypoxemia (SpO2<90%)	46/69 (66.7)	94 (79.7)	0.048
Fever (≥37.5ºC, axillar)	28/69 (40.6)	49 (41.5)	0.899
High fever (>39ºC, axillar)	4/69 (5.8)	5 (4.2)	0.728
Lower chest wall retractions[f]	39/68 (57.4)	63/117 (53.9)	0.644
Upper chest retractions[f]	8/69 (11.6)	14/117 (12.0)	0.940
Nasal flaring	13/68 (19.1)	26/117 (22.2)	0.618
Grunting	3/69 (4.4)	7 (5.9)	0.747
Crackles	40/70 (57.1)	68/117 (58.1)	0.896
Ronchi	26/70 (37.1)	59/117 (50.4)	0.077
Wheezing	15/70 (21.4)	32/114 (28.1)	0.316
Alteration consciousness	4/70 (5.7)	4 (3.4)	0.473
Prostration	18 (25.4)	19 (16.1)	0.133
Seizure	2 (2.8)	0 (0)	0.140
Clinical scoring scales at admission			
Severe WHO pneumonia	55 (77.5)	95 (80.5)	0.617
RISC (median, IQR)	3 (1 to 3)	3 (1 to 3)	0.268
RISC-Malawi (median, IQR)	7 (2 to 8)	7 (5 to 8)	0.3791
LODS	0 (0 to 1)	0 (0 to 0)	0.1128
Laboratory tests and biomarkers at admission			
Non-contaminated positive bacterial blood culture	1/48 (2.1)	7/100 (7.0)	0.438

Anaemia (Haemoglobin < 11 g/dL)	25/69 (36.2)	42 (35.6)	0.930
Radiological findings and hospital management			
Radiological findings			0.999
Endpoint pneumonia	14/53 (26.4)	25/96 (26.0)	
Other infiltrates	11/53 (20.8)	20/96 (20.8)	
Normal	28/53 (52.8)	51/96 (53.1)	
Antibiotics during admission	52 (73.2)	84 (71.2)	0.761
Oxygen therapy during admission	53/70 (75.7)	89 (75.4)	0.964
Oxygen therapy > 5 days	8 (11.3)	20 (17.0)	0.287
Hospital stay ≥ 7 days	9 (12.7)	22 (18.6)	0.283
Fatal outcome	3 (4.2)	3 (2.5)	0.674

Abbreviations: IQR: interquartile range; LODS: Lambaréné Organ Dysfunction Score; NA: not applicable; RISC: respiratory index of severity in children; SD: standard deviations; WAZ: weight-for-age Z-score; WHO: World Health Organization.

[a]Comparison of proportions using the chi-square or fisher tests.

[b]Nutritional status was based on the WAZ score generated using the 2000 Centers for Disease Control and Prevention Growth Reference.

[c]Tachycardia was defined as heart rate > 160/minute for infants <12 months, and > 150/minute for children ≥ 12 months of age.

[d]Increased respiratory rate was defined as >50breaths per minute in children aged 2 to 12 months and >40 breaths per minute in children aged ≥ 12 months.

[e]Peripheral capillary oxygen saturation was measured in room air using Mindray VS-800 Vital Sign Monitor or Biolight BLT M800 Handheld pulse oximeter

[f]Lower chest retractions were defined as subcostal and/or lower intercostal retractions, and upper chest retractions were defined as supraclavicular and/or suprasternal retractions.

Supplementary Table S2. Levels of host-response biomarkers levels by prognostic outcome.

Biomarkers	Subgroups	Good N = 95 for all N1 = 47 for <12 months N2 = 48 for ≥12 months	Poor N = 23 for all N1 = 15 for <12 months N2 = 8 for ≥12 months	Odd ratio (95% CI)	p-value[a]
WBC	All	12.1 (7.9 to 16.3)	10.6 (7.6 to 19.0)	1.02 (0.42 to 2.48)	0.965
	<12 months	13.6 (10.1 to 16.6)	9.9 (7.6 to 13.9)	0.53 (0.17 to 1.68)	0.281
	≥12 months	11.2 (7.2 to 15.6)	15.1 (9.6 to 22.0)	2.54 (0.61 to 10.48)	0.199
Platelets	All	363 (287 to 446)	378 (293 to 493)	1.90 (0.45 to 8.09)	0.385
	<12 months	389 (301 to 470)	362 (293 to 409)	0.36 (0.05 to 2.61)	0.311
	≥12 months	322 (264 to 394)	463 (351 to 537)	18.77 (1.25 to 281.46)	0.034
ESR	All	17 (8 to 40)	20 (2 to 44)	0.94 (0.64 to 1.37)	0.734
	<12 months	11 (6 to 34)	4 (2 to 30)	0.69 (0.41 to 1.16)	0.159
	≥12 months	19 (9 to 40)	45 (20 to 100)	2.05 (0.87 to 4.84)	0.100
CRP-study	All	1.4 (0.4 to 3.2)	1.3 (0.5 to 3.2)	0.91 (0.59 to 1.41)	0.682
	<12 months	1.2 (0.4 to 2.7)	1.0 (0.2 to 2.8)	0.79 (0.45 to 1.39)	0.420
	≥12 months	2.1 (0.6 to 3.5)	2.2 (1.2 to 4.2)	1.37 (0.61 to 3.09)	0.442
CRP-ref	All	1.4 (0.6 to 7.1)	2.5 (0.4 to 4.3)	1.03 (0.77 to 1.37)	0.855
	<12 months	1.3 (0.4 to 5.4)	0.6 (0.3 to 3.2)	0.83 (0.56 to 1.23)	0.355
	≥12 months	1.8 (0.6 to 7.6)	7.8 (2.2 to 20.6)	1.66 (0.93 to 2.94)	0.085
PCT	All	48.1 (46.6 to 332.4)	199.6 (46.6 to 1378.2)	1.31 (1.02 to 1.70)	0.036
	<12 months	48.1 (46.6 to 309.1)	46.6 (46.6 to 716.4)	0.95 (0.63 to 1.43)	0.813
	≥12 months	46.6 (46.6 to 392.6)	3144.6 (757.1 to 12,004.8)	2.04 (1.28 to 3.27)	0.003
IL-6	All	3.8 (0.7 to 10.2)	6.8 (0.7 to 24.7)	1.23 (0.91 to 1.65)	0.178
	<12 months	4.6 (2.4 to 13.02)	3.1 (0.7 to 23.0)	0.87 (0.58 to 1.30)	0.501
	≥12 months	3.0 (0.7 to 9.0)	16.0 (8.6 to 44.0)	2.09 (1.20 to 3.64)	0.010
IL-8	All	20.9 (7.5 to 37.4)	24.4 (13.1 to 100.2)	1.41 (1.04 to 1.90)	0.027
	<12 months	25.5 (9.7 to 43.6)	24.4 (13.1 to 60.1)	1.26 (0.85 to 1.85)	0.250
	≥12 months	15.8 (5.2 to 28.1)	23.5 (13.3 to 134.8)	1.60 (0.96 to 2.66)	0.069
sTREM-1	All	116 (86 to 166)	207 (107 to 365)	3.01 (1.54 to 5.89)	0.001
	<12 months	130 (106 to 184)	171 (107 to 356)	2.71 (1.03 to 7.16)	0.044
	≥12 months	97 (73 to 153)	227 (141 to 382)	3.27 (1.24 to 8.65)	0.017
sTNFR1	All	1540 (1096 to 2051)	1658 (1458 to 3575)	4.19 (1.63 to 10.75)	0.003
	<12 months	1627 (1404 to 2168)	1602 (1285 to 3351)	1.23 (0.37 to 4.14)	0.737
	≥12 months	1200 (918 to 1590)	2792 (2106 to 5114)	33.68 (3.78 to 299.86)	0.002
Angpt-2	All	1744 (1243 to 3697)	3683 (1428 to 6722)	2.00 (1.18 to 3.39)	0.010
	<12 months	2991 (1674 to 4905)	5860 (2991 to 11,674)	2.04 (0.99 to 4.18)	0.053
	≥12 months	1282 (1031 to 1831)	1853 (975 to 3040)	1.90 (0.50 to 7.26)	0.349
sFlt1	All	151 (112 to 198)	203 (153 to 335)	4.00 (1.62 to 9.69)	0.003
	<12 months	166 (134 to 228)	214 (168 to 391)	3.14 (1.13 to 8.75)	0.029
	≥12 months	123 (99 to 171)	176 (137 to 241)	6.67 (1.09 to 40.7)	0.040

Abbreviations: Angpt-2: angiopoietin-2; CI: confidence interval; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; IL-6: interleukin-6; IL-8: interleukin-8; PCT: procalcitonin; sFlt1: soluble fms-like tyrosine kinase-1; sTNFR1: soluble tumour necrosis factor receptor 1; sTREM-1: soluble triggering receptor expressed on myeloid cells 1; WBC: white blood cells.

Biomarker concentrations reported as median (interquartile range) as pg/mL except WBC and platelets ($x10^9$/L), ESR (mm), and CRP (mg/dL).

[a]Odd ratios for children evolving to good (survival and duration of oxygen therapy ≤ 5 days) versus poor prognosis (death or duration of oxygen therapy > 5 days) using univariable logistic regression. All variables were continuous with non-normal distribution and were log transformed for logistic regression analyses.

Supplementary Table S3. Association of biomarkers with secondary outcomes

Biomarkers	Survival vs death[a]		Short hospital stay vs long hospital stay		No PICU vs PICU		WHO severe vs non-severe pneumonia	
	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value
Acute phase proteins and inflammatory markers								
WBC	0.31 (0.03; 2.78)	0.298	1.75 (0.66; 4.63)	0.260	1.05 (0.40; 2.81)	0.915	0.91 (0.37; 2.21)	0.832
Platelets	4.84 (0.12; 198.45)	0.405	2.23 (0.49; 10.19)	0.303	4.37 (0.76; 25.00)	0.098	0.90 (0.22; 3.73)	0.882
ESR	0.86 (0.28; 2.66)	0.792	0.80 (0.54; 1.18)	0.257	1.15 (0.75; 1.77)	0.521	1.08 (0.74; 1.60)	0.683
CRP-study	0.46 (0.15; 1.34)	0.153	1.23 (0.76; 1.99)	0.396	1.29 (0.78; 2.12)	0.323	1.01 (0.64; 1.58)	0.978
CRP-ref	1.22 (0.58; 2.58)	0.604	1.04 (0.77; 1.40)	0.794	1.40 (0.99; 1.97)	0.054	1.03 (0.77; 1.38)	0.833
PCT	0.93 (0.45; 1.92)	0.845	1.15 (0.88; 1.49)	0.298	1.04 (0.78; 1.40)	0.775	1.06 (0.80; 1.40)	0.689
Immune factors								
IL-6	1.97 (0.93; 4.22)	0.079	1.24 (0.92; 1.68)	0.165	1.41 (1.01; 1.96)	0.042	0.85 (0.63; 1.14)	0.272
IL-8	2.07 (1.12; 3.85)	0.021	1.42 (1.04; 1.95)	0.027	1.61 (1.15; 2.25)	0.006	0.95 (0.70; 1.27)	0.715
sTREM-1	7.31 (1.81; 29.55)	0.005	2.99 (1.44; 6.20)	0.003	3.77 (1.76; 8.04)	0.001	1.26 (0.72; 2.20)	0.427
sTNFR1	146.2 (2.28; 9372)	0.019	3.41 (1.22; 9.53)	0.019	3.71 (1.39; 9.89)	0.009	0.71 (0.30; 1.69)	0.439
Endothelial activation factors								
Angpt-2	2.40 (0.77; 7.47)	0.131	2.04 (1.17; 3.58)	0.012	1.68 (0.97; 2.93)	0.065	0.90 (0.54; 1.49)	0.674
sFlt1	3.61 (1.21; 10.77)	0.021	2.01 (0.94; 4.31)	0.014	1.68 (0.83; 3.41)	0.148	1.63 (0.74; 3.56)	0.223

Abbreviations: Angpt-2: angiopoietin-2; CI: confidence interval; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; IL-6: interleukin-6; IL-8: interleukin-8; OR: odd ratio; PCT: procalcitonin; sFlt1: soluble fms-like tyrosine kinase-1; sTNFR1: soluble tumour necrosis factor receptor 1; sTREM-1: soluble triggering receptor expressed on myeloid cells 1; WBC: white blood cells.

All variables were continuous with non-normal distribution and were log transformed for logistic regression analyses.

[a]There were 3 deaths in total, 2 infants <12 months, and 1 child ≥12 month. We did not present biomarker levels by age group for this outcome as we judged it to be statistically inappropriate due to the small sample size. However, we decided to present the biomarker levels for this outcome despite the low sample size, as this is a common outcome used, for potential comparison.

Supplementary Table S4. Comparison of performance among biomarkers

Biomarkers	AUROC	Comparison of biomarker AUROCs (p-value)					
		sFlt1	Angpt-2	sTNFR1	sTREM-1	IL-8	IL-6
WBC	0.48	0.0199	0.0988	0.0247	0.0047	0.1879	0.3443
Platelets	0.56	0.0680	0.3580	0.1633	0.1921	0.5301	0.8319
ESR	0.51	0.0483	0.2360	0.0432	0.0559	0.2948	0.3710
CRP-study	0.50	0.0051	0.1085	0.0627	0.0470	0.1951	0.4544
CRP-ref	0.51	0.0444	0.2133	0.0214	0.0200	0.2585	0.4045
PCT	0.60	0.2091	0.6487	0.3213	0.3547	0.8334	0.7577
IL-6	0.58	0.0757	0.4901	0.1357	0.1502	0.5719	
IL-8	0.62	0.1813	0.7429	0.3740	0.4189		
sTREM-1	0.68	0.7484	0.7739	0.9906			
sTNFR1	0.69	0.7863	0.7352				
Angpt-2	0.65	0.5018					
sFlt1	0.71						

Abbreviations: Angpt-2: angiopoietin-2; AUROC: area under the receiver operating characteristics; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; IL-6: interleukin-6; IL-8: interleukin-8; PCT: procalcitonin; sFlt1: soluble fms-like tyrosine kinase-1; sTNFR1: soluble tumour necrosis factor receptor 1; sTREM-1: soluble triggering receptor expressed on myeloid cells 1; WBC: white blood cells.
Comparison of AUROCs using the algorithm suggested by DeLong *et al* (1988).

Supplementary Table S5. Performance characteristics of clinical scoring scales and biomarkers

	Cut-off point	Sensitivity (95% CI)	Specificity (95% CI)	Positive LR	Negative LR
Clinical scoring scales					
RISC	>1	91.3 (72.0 to 98.9)	46.3 (36.0 to 56.8)	1.70	0.19
RISC-Malawi	>6	87.0 (66.4 to 97.2)	52.6 (42.1 to 63.0)	1.84	0.25
LODS	>0	34.8 (16.4 to 57.3)	88.4 (80.2 to 94.1)	3.00	0.74
Biomarkers					
WBC	>23.2	17.4 (5.0 to 38.8)	94.7 (88.1 to 98.3)	3.28	0.87
Platelets	>402.5	47.8 (26.8 to 69.4)	69.9 (59.5 to 79.0)	1.59	0.75
ESR	>85	18.2 (5.2 to 40.3)	96.5 (90.1 to 99.3)	5.20	0.85
CRP-study	>0.75	73.9 (52.0 to 89.8)	36.3 (26.4 to 47.0)	1.16	0.72
CRP-ref	>1.67	60.9 (38.5 to 80.3)	54.7 (44.2 to 65.0)	1.34	0.71
PCT	>1133.4	39.1 (19.7 to 61.5)	86.3 (77.7 to 92.5)	2.85	0.71
IL-6	>16.6	39.1 (19.7 to 61.5)	85.3 (76.5 to 91.7)	2.66	0.71
IL-8	>37.6	43.5 (23.2 to 65.5)	76.8 (67.1 to 84.9)	1.88	0.74
sTREM-1	>168	60.9 (38.5 to 80.3)	75.8 (65.9 to 84.0)	2.52	0.52
sTNFR1	>2569	43.5 (23.2 to 65.5)	91.6 (84.1 to 96.3)	5.18	0.62
Angpt-2	>2942	60.9 (38.5 to 80.3)	70.5 (60.3 to 79.4)	2.06	0.55
sFlt1	>166	73.9 (51.6 to 89.8)	62.1 (51.6 to 71.9)	1.95	0.42

Abbreviations: Angpt-2: angiopoietin-2; CI: confidence interval; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; IL6: interleukin-6; IL8: interleukin-8; LODS: Lambaréné Organ Dysfunction Score; LR: likelihood ratio; PCT: procalcitonin; RISC: respiratory index of severity in children; sFlt1: soluble fms-like tyrosine kinase-1; sTNFR1: soluble tumour necrosis factor receptor 1; sTREM-1: soluble triggering receptor expressed on myeloid cells 1; WBC: white blood cells.

All biomarkers reported in pg/mL except WBC and platelets ($x10^9$/L), ESR (mm), and CRP (mg/dL).

Cut points were calculated using the Youden index.

Supplementary Table S6. Performance characteristics of clinical signs, biomarkers, and combinations of them in children ≥12 months

	AUROC	Sensitivity	Specificity	Positive LR	Negative LR
Single clinical signs					
IRR	0.64**	87.5	39.6	1.45	0.32
SpO2 (<71%)	0.62*	28.6	100	Infinity	0.71
Lower chest retractions	0.57**	62.5	51.1	1.28	0.73
Clinical scoring scales					
WHO severity	0.56**	100	12.5	1.14	0
RISC score (>3)	0.71*	37.5	85.4	2.57	0.73
Biomarkers					
sTNFR1 (>2506.7)	0.90	75.0	93.8	12.1	0.27
Combinations					
IRR + sTNFR1	0.95	75.0	95.8	17.86	0.26
SpO2 + sTNFR1	0.89	62.5	100	Infinity	0.38
Low chest retractions + sTNFR1	0.94	87.5	89.6	8.41	0.14
WHO severity + sTNFR1	0.90	75.0	93.8	12.1	0.27
WHO severity + PCT	0.85	50.0	100	Infinity	0.50
RISC + sTNFR1	0.93	50.0	100	Infinity	0.50
RISC + sTREM1	0.84	75.0	85.4	5.14	0.29

Abbreviations: AUROC: area under the receiver operating characteristics; IRR: increased respiratory rate; PCT: procalcitonin; RISC: respiratory index of severity in children; SpO2: oxygen saturation; sTNFR1: soluble tumour necrosis factor receptor 1; sTREM-1: soluble triggering receptor expressed on myeloid cells 1; WHO: World Health Organization. *p<0.10 and **p<0.05 for comparison of AUROC of clinical signs, scores, or combinations versus AUROC of sTNFR1 using the algorithm suggested by DeLong *et al* (1988).

Summary of results and discussion

Article 1. The challenges of combining clinical work with research in Bhutan: a changing status quo

The first article of this thesis illustrates how essential reliable data are to inform evidence-based policies and practices. On the one hand, data obtained from work conducted in other countries can be used when applicable to the setting and the population. On the other hand, reliable local data are essential to monitor progress made at a national level, to determine the burden of diseases and identify their characteristics, and to better describe local problems so that tailored solutions can be offered. Local health research capacity in LMICs is critical for addressing health challenges locally and globally, and this is not without associated major challenges. This article commented on the health research capacity in Bhutan. While considerable progress has been made in this field in the last few years, the main challenges identified to prevent the expansion of local research in Bhutan were: the lack of awareness that research is needed; the lack of motivation, methodological knowledge, analytical skills, and critical appraisal; funding; and priority for clinical work, which represents a huge workload.

Article 2. Pneumonia in Bhutanese children: what we know and what we need to know.

This systematic review was conducted to summarize all that we know around childhood pneumonia in Bhutan and to consequently identify knowledge gaps in this area. We included studies and documents that reported data on the burden of ARI, the aetiology, risk factors, clinical and radiological characteristics, prognosis, national surveillance systems in place, and national preventive strategies. We searched PubMed, ScienceDirect, and Google scholar, as well as WHO, UNICEF, Bhutan's Ministry of Health and other local databases for relevant reports up to 3rd December 2018. We included 44 documents (10 studies and 34 reports) for qualitative synthesis.

Burden of ARI in Bhutan

According to national estimates, this review showed that ARI, including pneumonia, are a significant national public health concern in Bhutan. The incidence of pneumonia in Bhutan has exhibited a decreasing trend, from 1479 cases per 10,000 children under 5

years of age in 2008 to 809 in 2017. However, ARI are still responsible for a high proportion of hospitalizations and outpatient visits. From the available data, we found that children under five years of age were the most-affected group. This age group presented the highest influenza-associated hospitalization rates of 182 (95% CI: 153 to 210) and 532 (95% CI: 473 to 591) per 100,000 persons in 2015 and 2016, respectively. In 2016 ARI were responsible for 15% of under-five deaths and 27% post-neonatal deaths. Since 2015, the WHO has ranked pneumonia as the leading individual cause of under-five mortality worldwide, and Bhutan is not an exception. However, we found scarce data characterizing this predominant illness in Bhutan.

National surveillance systems and aetiology for ARI

Most of the available data reporting the aetiology of ARI in Bhutan were focused on influenza virus and were mainly collected through a national surveillance system. An influenza surveillance system among outpatients and hospitalized patients was developed in Bhutan in 2012. Every week, respiratory samples are collected at selected sentinel sites and are tested by RT-PCR for several types of influenza virus. For those samples negative to any type of influenza, there is a further attempt to identify RSV and human metapneumovirus. From mid-2018, identification of further viruses also included adenovirus and parainfluenza viruses 1, 2, and 3. The weekly reports of 2018 showed that RSV was identified in a considerable number of cases, while human metapneumovirus, adenovirus, and parainfluenza viruses were isolated in only a few cases.

This relatively new surveillance system has made great efforts since 2012. However, an external evaluation of the SARI surveillance platform in 2015 and 2016 identified significant underreporting of SARI cases, consequently questioning the national data ensuing from this surveillance system. In addition, the identification of RSV, human metapneumovirus, adenovirus, and parainfluenza viruses only in samples negative for influenza virus leads to underreporting of pathogens and failure to adequately describe or identify mixed viral infections, commonly occurring in ARI.

Beyond the virology data reported from the national surveillance system, we did not find any studies that described in detail the viral aetiology of ARI in children, despite the importance that viruses are known to play in the aetiology of ARI in this age group. Data on the bacterial aetiology of ARI were also very limited.

Clinical and radiological characteristics, prognosis, and management of children with ARI

We did not find any study describing the clinical or radiological characteristics, prognosis, and management of Bhutanese children suffering from community-acquired pneumonia or ARI.

National preventive strategies

Activities for ARI control were initiated in 1987. The activities that aim to reduce mortality associated with childhood pneumonia in Bhutan are mainly the implementation of IMCI for management of sick children and vaccination.

A health economics analysis was conducted to determine the cost-utility of PCV compared to no vaccination in Bhutan. The authors suggest that PCV13 and PCV10 could prevent 30 and 18 deaths respectively in the vaccinated population and concluded that both PCVs would be cost-effective in Bhutan, thus recommending their inclusion in the immunization programme. PCV13 was introduced to the EPI in January 2019.

However, we did not find comprehensive data describing the circulating pneumococcal serotypes in the Bhutanese population before PCV introduction, and very limited data describing the burden of pneumococcal diseases in Bhutanese children.

Article 3. Pneumonia in children admitted to the national referral hospital in Bhutan: a prospective cohort study

The third article reports the main finding of the RIBhuC study, among children between 2 and 59 months of age with clinical pneumonia admitted at JDWNRH over 12 consecutive months. The article describes the burden of childhood pneumonia in terms of the number of children hospitalized and mortality, the demographic and clinical characteristics, and the aetiological profile of the children recruited to this study.

Burden of childhood pneumonia at JDWNRH

- Between 1 July 2017 and 30 June 2018, 1591 children were admitted to the paediatric department of JDWNRH, of whom 286 (18.0%) were children aged 2–59 months with respiratory symptoms. Among them, 189 (66.1%) fulfilled the WHO definition for pneumonia and were recruited to the RIBhuC study, which corresponds

to 11.9% of all children admitted to the paediatric department during 12 consecutive months. This figure is likely to be an underestimate as some cases may have been missed or may not have fulfilled the inclusion criteria of the study. This highlights the high burden that pneumonia represents for the healthcare services in the country. Furthermore, children hospitalized with pneumonia represent only a small proportion of the pneumonia burden, as many more pneumonia cases are managed in the emergency or outpatient departments without requiring hospitalization.

- Six children died, leading to a case fatality rate of 3.2%. Although similar to other studies from LMICs, this mortality rate is high for Bhutan despite the country offering free healthcare services. The six children who died were referred from healthcare centres far from Thimphu. They reached the study centre in critical condition, and two children died within the first 24 h of admission. This suggests that there was probably a delay in recognition of children with severe disease, leading to delayed referral with subsequent advanced disease and worse outcome.
- Children were hospitalized for a median of four days (interquartile range 2 to 6). Thirty children required PICU admission, with a median stay of 72 h (interquartile range 24 to 96). Three-quarters of the children were placed on oxygen therapy; half for at least three days. Most children (72.0%) received antibiotics during admission.

Demographic characteristics

- The median age was 10.8 months. Over half of the children were infants. This highlights that infants are particularly vulnerable and prone to hospitalization due to severe pneumonia.
- There was no known or suspected case of HIV infection, which is consistent with the very low number of children infected with HIV in the country.
- Most children (75.7%) were adequately immunized according to age (the remaining children were partially immunized), which is also consistent with the high immunization coverage rates reported in Bhutan.
- For 13.8% of children, both parents were illiterate. Two children (1.1%) had both parents unemployed at the time of the study.
- A third of the children were living with at least five other persons in the same household.

- On average, families reported that it had taken around 15 minutes to reach the closest healthcare facility. However, it had taken over an hour for five families.
- Summer, fall, and spring each comprised around 30% of the recruited cases, while winter (21 December to 20 March), which is the coldest season in Bhutan, had the lowest number of pneumonia admissions (10.1%). This seasonality pattern differs from what is commonly seen in other settings, whereby hospitalizations due to pneumonia tends to peak during the coldest season. However, this finding is consistent with those reported by the national surveillance programme for SARI. This might be partially explained by the fact that winter coincides with the school break, leading to less contact between children, and families moving from the capital to the villages with lower population density.

Clinical characteristics upon admission

- Most children (79.4%) presented with severe pneumonia, as per the WHO criteria.
- Wasting was detected in 17 children (9.0%).
- Regarding vital signs, 77 children (41.2%) presented with fever, half of the children were breathing fast according to age, and three-quarters were hypoxemic. We defined hypoxemia as SpO2 < 90%, which is considered appropriate for altitudes under 2500 m, as is the case with Thimphu (2334 m). However, the proportion of children with hypoxemia in our study was much higher than that reported for similar cohorts in other settings.
- By inspection, over half of the children (54.3%) presented with low chest indrawing.
- On auscultation, typical lung consolidation-related sign (crackles) was most common (57.5%), followed by rhonchi (45.2%) and wheezing (25.0%).
- Regarding laboratory testing, 35.8% of the children were anaemic, 36.9% had leucocytosis, and 25.3% had neutrophilia.
- Among the 149 readable chest radiographs, 79 (53.0%) were normal, 39 (26.2%) were classified as primary endpoint pneumonia, and 31 (20.8%) as other infiltrates.

Microbiological characteristics

Blood cultures

- Blood culture was performed in 148/189 children (78.3%), of which 45 (30.4%) had received antibiotics prior to sample collection. Six different pathogens were isolated

among the eight non-contaminated positive blood cultures: *Streptococcus pneumoniae* (two cases), *Pseudomonas sp.* (two cases), *Escherichia coli*, *Acinetobacter sp*., *Salmonella typhi*, and *Serratia rubidaea* (one case each). *Streptococcus pneumoniae* was also isolated by RT-PCR in a dried blood sample from one child.

- The low proportion of confirmed bacterial cases in our cohort could be explained by several reasons. First, vaccination coverage was high, although the PCV was not in routine use during the recruitment period. Second, almost one-third of the children had received antibiotics prior to collection of the blood samples, which reduces the yield of blood culture by around 45%. Third, small blood volume (1 mL) was dedicated to blood culture in most cases, which is another factor known to compromise the sensitivity of blood culture. Nevertheless, these findings confirm the low yield of blood culture in hospitalized children with pneumonia and question both the need of blood culture for uncomplicated cases of pneumonia and using blood culture as the preferred screening tool for invasive bacterial disease in children with pneumonia. Molecular methods are more sensitive than blood culture to detect pneumococcal invasive disease, but this was not the case in this study (*Streptococcus pneumoniae* was detected by blood culture in two children and by RT-PCR in dried blood spot in one child).

NPW

- NPW was collected and subjected to molecular screening (17 viral targets and 4 bacterial targets) in 115/189 children (60.8%), of whom 52 (45.2%) had received antibiotics prior to sample collection. We recruited children over 12 consecutive months to cover the seasonal variation in the aetiology of respiratory infections that might exist.
- *Bordetella pertussis* was detected in three (2.6%) children, which is similar to the detection rate of hospitalized cases found in similar settings. One of these three children, aged five months, had a fatal outcome. This underlines the high fatality ratio of pertussis-infected pneumonia, especially in unvaccinated infants, and suggests the need for intervention, such as maternal vaccination to reduce morbi-mortality associated with pertussis in vulnerable populations.

- *Mycoplasma pneumoniae* was detected in one (0.9%) child, and *Chlamydophila pneumoniae* and *Legionella pneumophila* were not detected among respiratory samples.
- At least one virus was identified in 103/115 NPW samples (89.6%). Viral co-infection was detected in 35/103 children (34.0%): 22 presented double infection, 10 presented triple infection, and three children were infected with four viruses.
- The most commonly-isolated virus was RSV (52; 45.2%), followed by rhinovirus (42; 36.5%), human metapneumovirus (19; 16.5%), and influenza virus (16; 13.9%). Infection with coronavirus (Cor229E, CorHKU1, CorNL63, CorOC43) was low (2; 1.7%). Similarly, the new coronavirus (SARS-CoV-2) seems to cause a low infection rate in children. The reason why coronavirus infection rate in children is low is unknown.
- Although viral detection was common, attribution of causality is not straightforward, as viruses can commonly be found in symptomatic but also asymptomatic children. Besides, the interpretation of positive viral findings is challenging due to the identification of multiple co-existing viral infections.

Article 4. Pneumococcal nasopharyngeal carriage among Bhutanese children hospitalized with clinical pneumonia: serotypes and viral co-infection

The fourth article analysed data on PNC among the children recruited to the RIBhuC study. Of the 189 children recruited to the study, 121 (64.0%) had sufficient NPW sample for pneumococcal testing.

Pneumococcal nasopharyngeal carriers: prevalence and characteristics

- Overall, 76/121 children (62.8%) were carriers of *Streptococcus pneumoniae* in their nasopharynx, which is comparable to that of other LMICs before the introduction of PCV.
- Around half of the children were infants, with no differences between PNC and no PNC.
- There was a significantly higher proportion of females among PNC compared to no PNC (38/76, 50.0%; versus 13/45, 28.9%; p=0.023).

- There was no significant difference in the proportion of children with at least another child under 5 years of age in the same household between PNC and no PNC (42.1% versus 31.1%; p=0.229).
- Colonized children were less likely to have received antibiotics before admission (13.3% versus 31.1%; p=0.018) and before NPW specimen collection (31.6% versus 71.1%; p=<0.001), which was expected.
- There were no significant differences between colonizers and non-colonizers regarding laboratory findings, such as CRP or erythrocyte sedimentation rate (ESR), outcome, and prognosis.

Distribution of pneumococcal serotypes

- Thirty different serotypes (or groups of serotypes when it was not possible to differentiate them) were identified among the 76 children with PNC.
- Over half of the children (39/76; 51.3%) were colonized with at least two and up to five different serotypes.
- We considered the serotypes 1, 3, 4, 5, 7F, 14, 18C, and 19A as highly invasive according to findings from other studies and refer to the remaining serotypes as 'less-highly invasive'. Most children presented less-highly invasive serotypes; the most common were 7B/C or 40 (33/76; 43.4%), followed by 6A/B (12/76; 15.8%), and 23F (6/76; 7.9%). Around one-third of the children (24/76; 31.6%) presented with highly invasive serotypes; the most common were 14 (9/76; 11.8%), 3 (5/76; 6.6%), and 1 (5/76; 6.6%). This serotype distribution is rather different from that described in similar studies. We found a much higher proportion of children with the serotype 7B/C or 40, while the proportion of children we identified with the serotypes 19F, 6A, or 6B was considerably lower than in similar studies (proportion of serotypes in children sick with respiratory symptoms) or among healthy children in community-based carriage studies. Serotype 1 has been identified as an important cause of highly-invasive pneumococcal disease and is atypically found in carriage studies of healthy children. In our study, serotype 1 was the fifth most common serotype identified, which is aligned with findings of PNC in children sick with pneumonia.

- Over half of the children (44/76; 57.9%) presented at least one serotype included in PCV13, and half of the children presented at least one serotype included in any of the two PCV10 (38/76 [50.0%] for Synflorix® and 37/76 [48.7%] for Pneumosil®).

Association of PNC with viral co-infections

- Respiratory viruses were detected in a similar proportion among children with and without PNC (62/70; 88.6% versus 36/40; 90.0%; p=1.000).
- However, rhinovirus detection was more common among children without PNC (47.5% in non-colonized children versus 28.6%; p=0.046), whereas RSV was more common in the colonized group, although this did not reach statistical significance (50.0% in colonized children versus 32.5%; p=0.075). No further significant differences were found regarding the detection of other viruses.
- Previous studies found positive association of pneumococcal colonization with influenza, RSV, adenovirus, and rhinovirus, but findings have not been consistent.

Article 5. Association of clinical signs, host biomarkers, and aetiology with radiological findings in Bhutanese children hospitalized with pneumonia

The fifth article describes the radiological findings of the RIBhuC cohort, considering radiological endpoint pneumonia (consolidation, pleural effusion, or both) as a proxy for bacterial aetiology.

Radiological endpoints and demographic characteristics

- Among 149 children with readable chest radiographs, 39 (26.2%) presented with endpoint pneumonia, 31 (20.8%) with other infiltrates, and 79 (53.0%) with normal radiological findings. These findings are comparable to those of the recently conducted multicentre study (the PERCH study) that used the same criteria for radiological classification.
- The proportion of children living more than 30 minutes from healthcare facilities was higher among those with endpoint pneumonia (17.1% versus 2.8%, p=0.008).
- The rest of the demographic characteristics, in terms of age, gender, vaccine status, parental education, and parental employment, were similar among children with radiological endpoint and non-endpoint pneumonia.

Radiological endpoints and mortality

- Five children died, one with radiological endpoint pneumonia, and four with non-endpoint pneumonia. In other works, radiological endpoint pneumonia has been associated with higher mortality, reflecting the association between radiological endpoint pneumonia and bacterial aetiology, together with the tendency of bacterial pneumonia being more severe than viral ones. The lack of this association in our cohort is likely to be explained by access to prompt diagnosis and treatment with appropriate antibiotics. This lack of association between radiological endpoint pneumonia and mortality also questions the association of radiological findings with bacterial aetiology and overall prognosis.

Association of aetiology with radiological findings

- Bacteria were isolated by blood culture in six children; 2/31 (6.5%) with endpoint pneumonia and 4/63 (6.4%) with normal chest radiograph. There were no significant differences in the proportion of NPC and highly-invasive pneumococcal serotype distribution between children with endpoint and non-endpoint pneumonia.
- At least one virus was detected in respiratory secretions of most children and one-third of those had two or more viruses identified, with no significant differences by radiological outcome. All children with other infiltrates had at least one virus identified. Identification of respiratory virus in the nasopharynx of children with clinical pneumonia requires careful interpretation, as the distinction between nasopharyngeal carriage and causative agent is difficult and respiratory virus detection does not exclude a bacterial infection.

Association of clinical characteristics with radiological findings

- A higher proportion of children with endpoint pneumonia reported symptoms (64.1% versus 38.5%, p=0.007) or presented fever (42.1% versus 21.3%, p=0.045) for ≥5 days prior to admission. WHO severe pneumonia was more common among children with radiological pneumonia (92.3% versus 75.5%, p=0.033).
- Several works have assessed the association between clinical signs and radiological findings to identify children with pneumonia that need antibiotics. In accordance with some studies, we found that no single clinical sign was more suggestive of presenting endpoint pneumonia or non-endpoint pneumonia. However, other studies reported an association of increased respiratory rate, hypoxemia, crackles, fever on admission,

and duration of illness with endpoint pneumonia. These contradictory findings might be in part due to differences in the definition of clinical pneumonia and the interpretation methods and classification of chest radiographs.

- A high proportion of children presented with clinical signs usually considered more indicative of bacterial pneumonia (radiological endpoint pneumonia), including hypoxemia (79/108, 73.1%) or crackles (63/108, 58.3%), despite having chest radiograph that did not confirm the pneumonia endpoint. Therefore, a proportion of children with radiological non-endpoint pneumonia truly had pneumonia, supporting the notion that standardized definitions of radiological pneumonia have low predictive value for clinical management and decision on antibiotic needs.

Association of host-response biomarkers with radiological findings

- Despite clinical similarities between radiological outcomes, plasma levels of ESR, CRP, and PCT were higher among children with endpoint pneumonia and remained significantly higher after adjusting for observed confounders.
- At established thresholds commonly used in clinical work, neutrophilia (42.1% versus 24.6%, p=0.042), ESR ≥ 50 mm (33.3% versus 11.2%, p=0.005), CRP > 4 mg/dL (27.8% versus 11.2%, p=0.021), and PCT ≥ 250 pg/mL (56.0% versus 25.4%, p=0.007) were also significantly more frequent among children with endpoint pneumonia.
- PCT presented the best overall discriminatory ability with 72% (95% CI 50.6 to 87.9) sensitivity and 66.2% (95% CI 54.0 to 77.0) specificity.
- None of the other biomarkers (IL-6, IL-8, Angpt-2, sFlt1, sTREM-1, and sTNFR1) demonstrated good discriminatory ability between radiological endpoint and non-endpoint pneumonia.
- While the association between CRP and PCT with radiological endpoint pneumonia or microbiologically-confirmed bacterial pneumonia has been reported in other studies, findings are contradictory regarding the other biomarkers. This may be due to differences in study methods, biomarker kinetics during infection, and cohort demographics.
- A point-of-care biomarker fulfilling the WHO ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable to end-users) criteria to identify bacterial aetiology would help decision-making to start or discontinue antibiotics in children with clinical pneumonia, leading to better clinical

outcomes and reducing antibiotic overuse. In high-income countries, PCT at the point-of-care for guiding antibiotic therapy in children with pneumonia reduces antibiotic use, without negative influence on clinical outcomes. In our cohort, PCT was the most promising biomarker to identify radiological endpoint pneumonia as a proxy for bacterial aetiology, but PCT is not currently available in Bhutan.

Etiological or prognostic markers: moving forward

- We assessed associations of clinical signs and host-response biomarkers with radiological findings to identify aetiological markers that discern bacterial from viral pneumonia to guide decision on antibiotic therapy. However, an important question remains unanswered regarding care of childhood pneumonia. Which type of marker would best assist clinicians in decision-making: etiological markers or prognostic ones? The lack of single clinical signs or biomarkers, or a simple clinical algorithm that clearly discerns bacterial from viral pneumonia, may be explained by the common mixed aetiology. A biomarker able to identify children at risk of severe disease, that would benefit from prioritization of care from those (the majority) with a self-limited disease without antibiotics, is likely to improve clinical outcome and reduce the high mortality associated with pneumonia.

Article 6. Performance of host-response biomarkers versus standard clinical and laboratory parameters to risk-stratify children with clinical pneumonia in Bhutan

The sixth article assessed the performance of inflammatory, immune, and endothelial activation markers alone or in addition to clinical scoring scales to risk-stratify children hospitalized with pneumonia and predict their outcome.

We assessed four clinical scoring scales: the Respiratory Index of Severity in Children (RISC) score, based on hypoxemia, chest indrawing, wheezing, refusal to feed, and wasting; the RISC-Malawi score, a modified version of the RISC score that omits chest indrawing and refusal to feed but includes gender and unconsciousness; the Lambaréné Organ Dysfunction Score (LODS), based on prostration, coma, and deep breathing; and the WHO severity criteria that were used as inclusion criteria for the RIBhuC study. Prognosis outcome was defined as good if the child survived and did not require

supplemental oxygen or only received oxygen therapy for a maximum of five days; and as poor if the child died or received oxygen for more than five days. Of the 189 children recruited to the RIBhuC study, 118 (62.4%) had biomarker quantification and were included in the analyses. Overall, 23/118 (19.5%) children progressed to poor prognosis.

Association of demographic characteristics, clinical signs, and scoring scales with prognosis

- Parental education, parental employment, and time to access healthcare facilities were not associated with prognosis.
- Hypoxemia, high fever, lower chest retractions, upper chest retractions, nasal flaring, rhonchi, and prostration at presentation were all associated with poor prognosis, while auscultation of wheezing was associated with good prognosis.
- High score on each of the four clinical scoring scales (WHO severity, RISC, RISC-Malawi, and LODS) was associated with poor prognosis.

Association of host-response biomarkers with prognosis

- Results of routinely-ordered laboratory testing, including white blood cells (WBC), platelets, ESR, and CRP, were not associated with prognosis.
- Children with poor prognosis had higher PCT upon admission.
- The plasma levels of IL-8, sTREM-1, sTNFR1, Angpt-2, and sFlt1 were significantly higher at presentation in children progressing to poor prognosis. Differences remained statistically significant after adjusting for selected potential confounding factors, except for IL-8.

Performance of clinical characteristics, scoring scales, and biomarkers at predicting poor prognosis

- Of single clinical characteristics, oxygen saturation (area under the receiver operating characteristics [AUROC] 0.73, 95%CI 0.60 to 0.86) and lower chest retractions (AUROC 0.68, 95%CI 0.58 to 0.77) on admission displayed the best predictive accuracy for prognosis.
- Of the clinical scoring scales, RISC presented the best prognostic performance (AUROC 0.75, 95%CI 0.66 to 0.85) but was not significantly better than other single clinical characteristics at predicting prognosis.

- The best biomarkers for predicting poor prognosis were sFlt1 (AUROC 0.71, 95%CI 0.60 to 0.82), sTNFR1 (AUROC 0.69, 95%CI 0.55 to 0.82), and sTREM-1 (AUROC 0.68, 95%CI 0.55 to 0.82). These three immune and endothelial activation markers performed significantly better than WBC, ESR, and CRP, but no better than IL-6, IL-8, Angpt-2, or PCT (AUROC between 0.58 and 0.65).

Top performing biomarkers improve the prognostic performance of clinical characteristics

- The addition of either sFlt1, sTNFR1, or sTREM-1 significantly improved the prognostic performance of lower chest retractions or the RISC score, but these combinations did not perform better than sFlt1, sTNFR1, or sTREM-1 alone.

Prognostic performance of clinical characteristics, scoring scales, and biomarkers differ by age groups

- We investigated the performance of biomarkers by age groups (infants <12 months of age and older children ≥12 months of age), since inflammatory response varies by age.
- There was a trend towards higher performance of clinical characteristics in infants compared to older children and higher performance of biomarkers in older children compared to infants.
- The RISC score in infants (AUROC 0.80, 95%CI 0.69 to 0.92) performed significantly better than any of the biomarkers except for Angpt-2 and sFlt1 in predicting poor prognosis.
- In older children, the RISC score (AUROC 0.71, 95%CI 0.54 to 0.87) performance was similar to all biomarkers except sTNFR1 (AUROC 0.90, 95%CI 0.80 to 1.00; p=0.0557).

sTNFR1-based algorithms predict poor prognosis in children ≥12 months with pneumonia

- We performed classification and regression tree analysis to identify optimal cut-off points and variable ordering.
- Alone, sTNFR1 presented a sensitivity of 75.0% and specificity of 93.8%, and the positive and negative likelihood ratios were 12.1 and 0.27, respectively. The

performance of sTNFR1 alone was not significantly inferior to the performance of any combinations of clinical sign and biomarker that we assessed.

Conclusions and the way forward

Conclusions

- Reliable local data on the burden of diseases and identification of their health determinants are essential for targeting, implementing, and monitoring evidence-based policies and practices to reduce pneumonia mortality. While the health research capacity is developing in Bhutan, the main associated challenges are the lack of researchers with adequate methodological and critical appraisal skills, and the often overwhelming clinical workload for health workers.

- An ARI national surveillance system has been developed in Bhutan. This mainly focuses on influenza virus identification, although monitoring of other viral aetiology has recently been implemented. Some concerns on underreporting of cases have been reported. Despite progress from this national surveillance system, there are still considerable knowledge gaps regarding the aetiology of ARI in Bhutanese children.

- There is a dearth of data regarding the epidemiological, clinical, and radiological characteristics of childhood pneumonia in Bhutan. There are limited data on the burden of pneumococcal diseases and no data describing the circulating pneumococcal serotypes in Bhutanese children. Overall, national data are needed, as factors that influence the outcomes, such as aetiology and patient characteristics, vary geographically. In addition, Bhutan differs from its neighbouring countries and other LMICs in that the healthcare system relies on a primary healthcare approach through a strong net of healthcare facilities that deliver free essential health services to all Bhutanese citizens. This might impact disease outcomes and needs to be taken into consideration for the development and implementation of policies.

Conclusions from the RIBhuC study

- Over 12 consecutive months, 189 children were admitted at JDWNRH with WHO-defined clinical pneumonia, which corresponds to 11.9% of all children admitted to the paediatric department. Winter, which is the coldest season in Bhutan, had the lowest number of pneumonia admissions.

- The case fatality rate was 3.2%. All the children who died were referred from other centres far from Thimphu and reached the study hospital in critical condition.

- The burden of pneumonia requiring hospitalization was highest among infants. There was no known or suspected case of HIV infection. Immunization coverage was high but around 25% were only partially immunized according to age, and 9% of children were wasted. A high proportion of children presented with hypoxemia.

- Bacterial aetiology was infrequent, with only two cases of *Streptococcus pneumoniae* isolated by blood culture. Viruses were identified in a considerable proportion of children. RSV was the most commonly-identified virus, followed by rhinovirus. These microbiological findings coincide more with the aetiological profile of pneumonia in children from high-income countries and highlight the advanced stage of the epidemiological transition that Bhutan seems to have reached.

- Almost two-thirds of the children were pneumococcal nasopharyngeal carriers. Most common pneumococcal serotypes identified were 7B/7C/40, 6A/B, 14, and 23F, which differs from comparable studies and neighbouring countries. PCV13 and PCV10 could theoretically have averted up to 58% and 50% of the pneumococcal infections, respectively.

- Respiratory viruses were common among both pneumococcal nasopharyngeal carriers and non-carriers. Rhinovirus detection was higher in pneumococcal carriers, but we found no other significant differences regarding the other viruses between carriers and non-carriers.

- Around half of the children admitted with clinical pneumonia presented with a normal chest radiograph, while one-quarter presented with radiological endpoint pneumonia.

- We found no clear associations between the identification of respiratory viruses and radiological outcome.

- It remains challenging to identify children with pneumonia that require antibiotics even when using a combination of clinical, laboratory, and radiological parameters. We found no clinical sign that was suggestive of radiological pneumonia. Levels of ESR, CRP, and PCT were higher among children with radiological pneumonia, and PCT presented the best performance to discern between radiological outcome. Markers of endothelial and immune activation had little accuracy for the identification of radiological pneumonia.

- Parental education, parental employment, and time to access healthcare facilities were not associated with prognosis and fatal outcome.

- The RISC score was the clinical scoring scale that performed best at predicting prognosis, especially in infants <12 months of age. Nevertheless, none of the single clinical signs were good enough as a prognostic predictor to allow clinical decision-making for case management confidently.

- Our findings confirm that immune and endothelial activation markers have the potential to inform risk-stratification and clinical decision-making in children with pneumonia.

The way forward

The next steps consist of translating the findings of this thesis into easily implementable recommendations for policy changes so as to improve the care of children with pneumonia and to reduce the mortality associated with this disease. These useful data may support clinicians and policymakers to develop and improve evidence-based targeted strategies for the prevention and management of pneumonia in Bhutanese children. We encourage further efforts in the fight against childhood pneumonia, which includes accurate data collection through reliable national surveillance systems, health policies regulations and programme implementation, awareness and training among health workers, and further research at a national and international level.

The RIBhuC study provided findings that allow a comprehensive characterization of children with pneumonia admitted at JDWNRH. As most of the recruited children lived in the capital, all the study findings might not be applicable to the rest of the country. Bhutan is a small country but presents a wide diversity in terms of geography, with elevation ranging from 100 m in the Indian border to the high Himalayan peaks above 7500 m, corresponding to different climates, from tropical to very cold winters. The living conditions also vary between the urban population from those living in remote hamlets in the highest altitudes, leading to differences in economic status, alimentation and access to care. We encourage wider research across the country, from basic health units to reference hospitals, to obtain a comprehensive picture of pneumonia across Bhutan, including health determinants, and epidemiological and etiological characteristics.

During the study period, 11.9% of all children admitted to the paediatric department in the National Referral Hospital of Bhutan were recruited. These findings confirm the high burden that pneumonia constitutes to the Bhutanese healthcare system and serve as baseline to monitor progress from the already established preventive interventions and the implementation of new ones.

Immunization is the most effective strategy to prevent death from pneumonia, and wasting is a well-known risk factor associated with severity of the disease and death. We found that the proportion of partially immunized children according to age and the

number of children with malnutrition were not negligible, which highlights that there is room for improvement in the implementation of these essential preventive measures. In addition, these conditions might be more accentuated in rural and most remote areas, where nutritional status is usually poorer and access to care more challenging. Notably, a five-month old child with microbiological confirmation of *Bordetella pertussis* died. Policymakers may want to consider the implementation of interventions with proven efficacy to reduce the morbi-mortality associated with pertussis in vulnerable populations, such as vaccination during pregnancy.

Fostering robust pneumonia surveillance focusing on the burden and aetiology of the disease in children under five years of age appears essential. A good start might be to strengthen the existing ARI national surveillance system to improve data availability and reliability.

This study provided baseline information on the status of PNC and the circulating pneumococcal serotypes among Bhutanese children admitted with clinical pneumonia before the introduction of PCV13 in the country. We found that PCV13 could theoretically have averted up to 58% of the pneumococcal infections among the children in this study. It is highly recommended to monitor the impact of the vaccine, which was added to the routine immunization programme in January 2019. This could be achieved through a surveillance system or prospective study to determine circulating pneumococcal serotypes and to estimate the burden of the disease based on the number of hospitalizations attributed to pneumonia and pneumonia-related mortality. We recommend avoiding separate vertical surveillance systems and favour the incorporation of data collection on pneumococcal aetiology within the above-mentioned national aetiology surveillance system. Strengthening of the microbiological capacities at the different hospitals in the country may also be useful to provide a more thorough invasive bacterial disease surveillance.

In this thesis, we aimed to identify infectious aetiology of childhood pneumonia in Bhutan. We found no association between exposure to tobacco smoke, exposure to betel nut, exposure to heater with kerosene, or sociodemographic characteristics with

radiological outcome. We found no association either between these variables and poor prognosis. However, further research to determine the role of non-infectious risk factors of childhood pneumonia in Bhutan, including indoor and outdoor air pollution, would allow to further improve evidence-based targeted strategies for the prevention and management of pneumonia in this population.

This study reinforced that chest radiographs are an imperfect gold standard for the diagnosis of pneumonia and for discriminating between bacterial and viral aetiology. On the one hand, we question the utility of routinely performing a chest radiography to each child admitted with pneumonia. Unless some complications are suspected, the chest radiograph is unlikely to add any benefit for guiding the clinical management of the child, due to the lack of association between consolidation and bacterial aetiology, and as normal findings are also found in children with bacterial pneumonia. Although such practices are already supported by international guidelines, it is not strictly followed in the field and children still undergo unnecessary irradiation. On the other hand, researchers might need to move forward from using radiological findings as proxy of pneumonia aetiology, following the same rationale, or at least start exploring alternative diagnostic technologies, such as lung ultrasound, that do not irradiate and can be performed as point-of-care at the bedside.

The findings of this study might encourage policymakers to incorporate PCT in clinical practice in Bhutan to help decision-making to start or discontinue antibiotics in children with pneumonia, leading to better clinical outcomes and reducing antibiotic overuse. Although clinical efficacy and cost-effectiveness studies might be required to estimate its potential impact in the Bhutanese setting, rural and remote areas where laboratory facilities are difficult to access are likely to benefit from its readiness as a PCT point-of-care diagnostic tool.

The six children with fatal outcome were all referred from other health centres far from Thimphu and reached JDWNRH in critical conditions. These findings do not include children who may have died in remote areas before referral. It is known that early recognition of children with severe disease for timely referral and prompt intensive

management is associated with better outcome. This suggests room for care improvement at secondary and primary healthcare levels, where trained healthcare workers and resources remain scarcer. The geographical characteristics of the country, largely rural and at high altitudes, and the conditions of the roads, which worsen during the monsoon (leading to landslides and traffic interruption sometimes in the main axes of the country), are some of the challenges that a considerable proportion of the Bhutanese population face to access healthcare facilities. Despite these difficulties, Bhutan has made huge efforts to establish a strong network of healthcare facilities in even the most remote areas. While IMCI has been successfully implemented all over Bhutan, enhanced efforts might be needed to keep all healthcare workers trained and updated, with a particular emphasis in the recognition of clinical signs indicative of severity and risk for an adverse outcome. In the RIBhuC study, the RISC score presented a high sensitivity to predict children (especially infants) at risk of poor prognosis. Stakeholders might consider implementing this clinical scoring scale in community settings to identify children at risk for close monitoring and consideration for referral to superior healthcare centre. A sensitive tool with higher specificity than the one presented by the RISC score would avoid unnecessary referrals with subsequent healthcare facilities overload, unnecessary exposure to treatment, and misallocation of health resources. Immune and endothelial activation markers have great potential to become a simple, fast, and objective tool for risk-stratification of children with pneumonia. The novel tool should be developed for its use at the point-of-care, and fulfil the WHO ASSURED criteria, which are affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and deliverable to end-users. The use of such a triage tool to guide healthcare workers on decision-making for referral and prioritization of care is likely to help reduce the high mortality associated with childhood pneumonia.

We encourage further investigation to identify and validate a prognostic biomarker or biomarker signature allowing the risk-stratification of pneumonia in children at their first contact with the health system (both in the community settings and at the hospital level). Factors known to impact circulating biomarker concentrations such as duration of illness, prior administration of antibiotics, malnutrition, and other comorbidities are important considerations in biomarker discovery and validation studies. In addition, our

findings pointed to the importance of considering age-dependent differences in biomarker concentrations.

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