Epidemiological and clinical studies of viral pneumonia in young children in Bhaktapur, Nepal.

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Dissertation for the degree philosophiae doctor (PhD)
at the University of Bergen

2010

Dissertation date: November 12, 2010

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Acknowledgements

I wish to express my sincere gratitude to a lot of people who have contributed to this thesis in various ways. Most importantly, this work would not have been possible without the cooperation of all the children and their families in Bhaktapur who participated in the studies, for which I am truly grateful.

I first off all want to thank my supervisor Tor Strand for giving me the opportunity to join the research project in Nepal and for introducing me to the field of clinical research. His advice, trust and encouragement throughout this process have been invaluable to me. I feel very privileged to have been able to work with interesting and important research questions under his inspiring and qualified guidance.

I am also very grateful to my co-supervisor Halvor Sommerfelt for his enthusiasm and support, for patiently sharing his skills in epidemiology and for his invaluable feedback on important aspects of study design, methodological issues and manuscript writing.

I wish to thank my Nepalese colleagues in Kathmandu at the Child Health Department, Institute of Medicine, Tribhuvan University, Professor Prakash S. Shrestha, Associate Professor Sudha Basnet and Professor Ramesh K. Adhikari for their dedicated efforts in the implementation of the project and support of my work. I also thank Dr. Ram Krishna Chandyo, Dr. Manjeswori Ulak, and Dr. Meeru Gurung for their continuous efforts in the field clinic and for their support and friendship.

I also want to thank my colleague Dr. Palle Valentiner-Branth and his family for their hospitality and generosity during the two years we shared in Nepal during the project period. Thanks to Palle for sharing his experience with me, for the constructive discussions we had during the field trial, and for his input towards the manuscripts.

My thanks go to Shyam Dhaubhadel and his family for giving us the opportunity to conduct the research project at Siddhi Memorial Hospital in Bhaktapur. The support and efforts of the hospital staff throughout the project period is also most appreciated.

I thank Biswa Nath Sharma for his dedicated efforts and responsibility in running the PCR laboratory and Govinda Gurung for his diligent work in the laboratory and for administering the samples. Their extraordinary work with the PCR analyses was essential for the success

of this study. I also thank Subash Sherchan for excellent work with the PCR analyses. The Department of Microbiology at Tribhuvan University Teaching Hospital provided the laboratory facility at the university campus and thus made it possible for us to establish our virus PCR laboratory. Thanks to Professor Nhuchhe Ratna Tuladhar, Professor Bharat Mani Pokharel and Professor Jeevan Sherchand for their support in this process. I also thank all members of the Child Health Research Advisory Committee, including Professor Pushpa Raj Sharma, Professor Arun Syami, and Dr. Ratendra Nath Shrestha.

I am grateful to Dag Hvidsten, Håkon Haaheim, Ann Helen Helmersen, Maria Frost and Tore Jarl Gutteberg at the Department of Microbiology and Infection Control at the University Hospital of North Norway. Thanks to Tore and Dag for supporting our project and providing training in Tromsø for our Nepalese laboratory staff. Thanks to Dag also for the valuable discussions and his contribution to writing the manuscripts. Håkon and Ann Helen travelled to Nepal to provide technical assistance in the establishment and running of the PCR analyses. This was essential for the implementation of the project and their contribution is highly appreciated. Thanks to Ann Helen and Maria for the quality control analyses done in Tromsø.

I thank Professor Shobha Broor at the Department of Microbiology at All India Institute of Medical Sciences, New Delhi, and her PhD student Preeti Bharaj for the training in PCR methods they provided for the Nepalese laboratory team and myself. I also thank Dr. Nita Bhandari at Society of Applied Studies, New Delhi, for her valuable input on design and conduct of the pneumonia study in Bhaktapur.

I thank Andy Shrago, Karen Harrington and others at Prodesse for facilitating the transfer of the Hexaplex Plus assay to our laboratory in Nepal and for the training Håkon and I received in the premises of Prodesse in Waukesha, as well as technical support during the initiation of the project in Nepal.

I also thank others who have contributed to my academic progress or this thesis, especially Håkon Gjessing, Bjørn Bolann, Philippe Chevalier and Dorthe Jeppesen.

This PhD emerges from the Centre for International Health at the University of Bergen. I would like to thank the leadership and all my colleagues at CiH for creating a positive and inspiring work environment. Although nearly four years of my PhD-period was spent in

Nepal, CiH has served as an important base in between stays abroad and in the last phase of analyzing and writing.

And of course I wish to thank my parents Randi and Carl, my brother Henrik, and my husband Chijioke, for their love and support always, and all my friends who have encouraged me and cared for me.

I also wish to thank the many people who in various ways have contributed to my research work or made a positive impact on my life as a PhD student in Norway or outside Norway. Some were employed in the Child Health Research Project in Nepal as fieldworkers, supervisors, computer staff, administrative staff, doctors, or driver. Others have carried equipment to Nepal, advised me, helped me with practicalities, taught me Nepali, provided accommodation, invited me for dinner, served me dal bhat or chia, gone trekking with me, brewed coffee, or simply kept me company:

Dipendra Adhikari, Chantelle Allen, Sheldon Allen, Peter Andersen, Hans Arneberg, Shova Bista, Sama Bhandari, Chandrawati Chitrakar, Ashok Dangal, Krishneswori Datheputhe, Harald Eikeland, Helen Eikeland, Ingunn Engebretsen, Jan Fadnes, Ruth Foster, Punita Gauchan, Elisabeth Gullbrå, Kjartan Gullbrå, Magnus Hatlebakk, Anja Hem, Elin Hestvik, Solfrid Hornell, William Howlett, Marte Jürgensen, Bishnu Maya Kadel, Bimala Karmacharya, Bidhya Karmacharya, Sahilendra Karmacharya, Samir K.C., Lathaa Khadka, Nim Raj Khyaju, Padma Khayargoli, Ram Krishna Kuikel, Sukramani Kuikel, Unni Kvernhusvik, Allison Kwessel, Sudan Lama, Borgny Lavik, Inge Løvåsen, Mari Skar Manger, Devi Maharjan, Sushila Maharjan, Subhadra Malla, Alemnesh Mirkuzie, Mercy Njeru, Babu Ram Neupane, Kalpana Neupane, Nazik Nurelhuda, Annelies Ollieuz, Bjørg Evjen Olsen, Vegard Pedersen, Torunn Perstølen, Keshav Prasad Poudal, Shiva Poudel, Sunaina Poudel, Shova Pradhan, Pramila and Protima, Samjhana Premi, Ratna Rajthala, Ram Pyari Rana, Pashupati Bhakta Raya, Uma Regmi, Borghild Rønning, Shanti Sachin, Ingvild Fossgård Sandøy, Anne-Sylvie Saulnier, Bhim and Jharana Shahi, Bandhu Shrestha, Shyam Shrestha, Umesh Tami Shrestha, Tom Solberg, Nils Gunnar Songstad, Hans Steinsland, Bina Suwal, Indira Suwal, Dorjee Tamang, Shanta Tamang, Indira Twati, Sarah Webster, and Rachael Woloszyn.

Collaborations

This study emerged from Centre for International Health, Faculty of Medicine and Dentistry, University of Bergen. The existing collaboration with Child Health Department, Institute of Medicine, Tribhuvan University, Kathmandu, Nepal, provided the institutional framework for the research environment of this study. The research presented was part of the clinical trial: Community- and Health Facility-Based Intervention With Zinc as Adjuvant Therapy for Childhood Pneumonia (http://clinicaltrials.gov/ct2/show/NCT00148733). The research consortium for the trial included several additional institutions: Department of Epidemiology Research, Statens Serum Institut (SSI), Copenhagen, Denmark; Department of Pediatrics, All India Institute of Medical Sciences (AIIMS), New Delhi, India; Epidemiology, Prevention Research Unit, the Institute of Research for Development (IRD), Montpellier, France; and Society for Applied Studies (SAS), Calcutta, India; and Department of Microbiology and Infection Control, University Hospital of North Norway, Tromsø, Norway.

Funding for the study was provided by the Norwegian Council of Universities' Committee for Development Research and Education (NUFU project numbers 36/2002 and 2007/10177), the European Commission (EU-INCO-DC contract number INCO-FP6-003740), and the Research Council of Norway (RCN project number 151054 and 172226) as well as by a grant from the Danish Council of Developmental Research (91128).

List of publications

Paper I

Mathisen M, Strand TA, Sharma BN, Chandyo RK, Valentiner-Branth P, Basnet S, Adhikari RK, Hvidsten D, Shrestha PS, Sommerfelt H: RNA viruses in community-acquired childhood pneumonia in semi-urban Nepal; a cross-sectional study. BMC Medicine. 2009;7(35).

Paper II

Mathisen M, Strand TA, Sharma BN, Chandyo RK, Valentiner-Branth P, Basnet S, Adhikari RK, Hvidsten D, Shrestha PS, Sommerfelt H: Clinical presentation and severity of viral community-acquired pneumonia in young Nepalese children. Pediatr Infect Dis J. 2010;29(1):e1-6.

Paper III

Mathisen M, Strand TA, Valentiner-Branth P, Chandyo RK, Basnet S, Sharma BN, Adhikari RK, Hvidsten D, Shrestha PS, Sommerfelt H: Respiratory viruses in Nepalese children with and without pneumonia; a case-control study. Pediatr Infect Dis J. 2010;29:731-735.

Abbreviations

ALRI	acute lower respiratory tract infection
ARI	acute respiratory infection
BCG	Bacille Calmette-Guérin
CRP	C-reactive protein
DTP	Combined vaccine against diphtheria, tetanus and pertussis
EHA	enzyme hybridization assay
ELISA	enzyme-linked immunosorbent assay
EPI	Expanded Program on Immunization
GAPP	Global Action Plan for Pneumonia
GAVI	Global Alliance for Vaccines and Immunization
GPS	global positioning system
hBoV	human bocavirus
Hib	*Haemophilus Influenzae* type b
hMPV	human metapneumovirus
IF	immunofluorescence
IMCI	Integrated Management of Childhood Illness
LCI	lower chest wall indrawing
LMICs	low-and-middle-income countries
MOR	matched odds ratio
NA	nucleic acid
NPA	nasopharyngeal aspirate
OR	odds ratio
PCR	polymerase chain reaction
PIV	parainfluenza virus
RNA	ribonucleic acid
RR	respiratory rate
RSV	respiratory syncytial virus
SpO_2	oxygen saturation
under-5s	children under five years of age
UNICEF	United Nations Children's Fund
UNN	University Hospital of North Norway
URI	upper respiratory tract infection
UTM	Universal transport medium
VDC	village development committee
WHO	World Health Organization

Abstract

Pneumonia remains the leading cause of illness and death in children less than 5 years of age in low-and-middle-income countries. Both bacteria and viruses are major causes of pneumonia in children. The disease burden attributed to the different respiratory pathogens varies with season and between regions. Knowledge of the relative importance of each agent is essential for adequate case management as well as prevention strategies, such as development of vaccines. This thesis focuses on respiratory viruses as causes of pneumonia.

The basis for the present thesis is: 1) a cross-sectional study of 2,219 children with community-acquired pneumonia as defined under the Integrated Management of Childhood Illness (IMCI) program in the World Health Organization and 2) a case-control study of 680 pneumonia cases and 680 matched controls. Study subjects were included at a field clinic in Bhaktapur, Nepal. A nasopharyngeal aspirate was collected from each child at inclusion and examined for seven respiratory viruses using a commercial multiplex reverse transcription polymerase chain reaction (PCR) assay. The aim of the large cross-sectional study was to obtain information on the frequency of these seven common respiratory viruses and their seasonal distribution over a three-year period. Moreover, the study was designed to obtain information on clinical characteristics and outcomes of the pneumonia episodes and how the individual respiratory viruses were associated with these factors. The case-control study was undertaken to measure the degree to which the individual viruses were associated with IMCI defined pneumonia.

We identified at least one virus in a large proportion (40%) of the children with pneumonia. Respiratory syncytial virus (RSV), influenza A, and parainfluenza virus (PIV) type 3 were most frequently detected among the seven viruses in the three-year study. The epidemics of infection with individual respiratory viruses contributed substantially to the observed pneumonia epidemics. RSV occurred in yearly epidemics in relation to the rainy season or during the winter. We also found that RSV infection was associated with signs of severe illness; the children infected with RSV more frequently had severe pneumonia and, among infants, low oxygen saturation, compared to children who were RSV negative. Among cases with non-severe pneumonia, the children with RSV infection had longer time to recovery and increased risk of treatment failure compared to the other children. The case-control study revealed that all the seven viruses were associated with pneumonia but that the

strength of this association varied. RSV, PIV type 3 and influenza A were most strongly associated with pneumonia.

Our findings indicate that these viruses are important causes of pneumonia in young children in Bhaktapur. Although influenza A and PIV type 3, like RSV, were among the most common viruses and were strongly associated with pneumonia, RSV was by far the most frequently detected virus over the three-year period and children infected with RSV had the most severe clinical presentations and outcomes. This supports the notion that development of a safe and effective RSV vaccine should be a priority for prevention of pneumonia in young children in low-and-middle-income countries.

1. Introduction

The global burden of acute respiratory infection

Acute respiratory infection (ARI) is one of the leading causes of illness and death in children under five years of age (under-5s). According to World Health Organization (WHO) estimates, nearly 2 million under-5s die from ARI every year, corresponding to about 19% of all deaths in this age group (1). Pneumonia and bronchiolitis are considered to be leading contributors to the global burden of ARI in young children and responsible for the greater part of these deaths, of which the vast majority occurs in the developing world. The WHO algorithm for classification of ARI identifies children with acute lower respiratory tract infection (ALRI) as being in need of antibiotic treatment, acknowledging that a substantial part of the infections are actually viral. In this thesis, I use the term pneumonia as defined under WHO's Integrated Management of Childhood Illness (IMCI) program, which captures the clinical entities of both pneumonia and bronchiolitis and is sometimes referred to as "clinical pneumonia" (2). Aspects related to the challenges inherent in this classification of pneumonia are discussed in further detail below ("Diagnosing pneumonia"). Hereafter the terms pneumonia and ALRI will be used interchangeably.

The incidence of pneumonia in under-5s in industrialized countries is estimated at 0.05 episodes per child-year. In contrast, the incidence in low-and-middle-income countries (LMICs) is approximately 0.3 episodes per child-year, which translates into more than 150 million new episodes annually (3). The regions with the highest incidence are South-East Asia and sub-Saharan Africa. The incidence varies with the prevalence of several risk factors; including malnutrition, low birth weight, non-exclusive breastfeeding, indoor air pollution, and crowding (4). Incidence also varies with age and is higher in infants than in toddlers, i.e. young children ≥12 months old (3).

Etiological agents in childhood pneumonia

A variety of infectious agents cause pneumonia, but *Streptococcus pneumoniae* (pneumococcus), *Haemophilus influenzae, Staphylococcus aureus* and respiratory syncytial

virus (RSV) are considered to be the most important respiratory pathogens in areas without adequate pneumococcal and *H. influenza* type b (Hib) vaccine coverage, i.e. in most of the developing world. Other important respiratory viruses are influenza A and B, parainfluenza virus (PIV) type 1-3, human metapneumovirus (hMPV) and adenovirus. Until recent increases in measles vaccine coverage, measles still accounted for a substantial number of pneumonia deaths in children (5). In general, the true burden of the various organisms causing pneumonia is inadequately documented in LMICs due to lack of surveillance systems and diagnostic facilities (6).

Bacterial etiology

Etiology studies in the 1980s and 90s found pneumococcus to be the most common cause of severe pneumonia in LMICs, followed by *H. influenzae* and *S. aureus* (7-11). These studies were based on lung or pleural puncture combined with blood culture and included only a small number of children. Vaccine probe studies (12-17) have more recently been used to estimate disease burden attributable to pneumococcus and Hib (18, 19). It is estimated that nearly 14 million episodes of pneumococcal pneumonia and 8 million episodes of Hib pneumonia occur in under-5s annually, and pneumococcus alone cause around 700,000 deaths from pneumonia in this age group (18, 19). Estimates based on the proportion of radiographically confirmed pneumonia prevented in vaccine probe studies and supported by lung aspiration studies indicate that pneumococcus cause 17% to 37% of pneumonia cases among under-5s (20). The corresponding proportion for Hib is estimated at 0-31% (20).

Other important bacterial organisms with varying occurrence are *Staphylococcus aureus*, which may cause severe, necrotizing pneumonia with complicated effusion and rapid progression, non-type-b *H. influenzae*, and *Klebsiella pneumoniae* (3, 20, 21). Non-typhoid *Salmonella* species have been associated with non-severe pneumonia in malaria-endemic tropical regions of Africa, but its etiological role in pneumonia is still controversial (3). Several other gram-negative bacteria as well as atypical organisms such as *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* also cause pneumonia, but are not believed to be among the most common causes in the under-5 age group (21). Additionally, *Mycobacterium tuberculosis* has been identified in a proportion of acute pneumonia (7) and still continues to be an important cause of severe illness and death in children (6), especially

in areas with high HIV infection prevalence (22-25). Pneumonia due to opportunistic fungal infections with *Pneumocystis jirovecii* is also frequent in HIV endemic areas (26).

Viral etiology

Among the common respiratory viruses, which cause a wide range of illnesses from mild infections of the upper respiratory tract to pneumonia, RSV undoubtedly cause most severe illness and is responsible for a large proportion of hospitalizations in infants and young children attributable to these viruses in industrialized countries (27, 28). Hospitalization for RSV-associated illness in under-5s in the United States is three-fold more common than for influenza and PIVs (29-31). Globally, an estimated 34 million new episodes of RSV associated ALRI occurred in under-5s in 2005, of which 3.4 million required hospital admission and near 200,000 resulted in death (32). However, accurate information on the RSV disease burden in LMICs is lacking. Few population-based estimates of RSV incidence rates in LMICs are available (33-36), but existing data suggest that the incidence is high both in developing and in industrialized countries (29, 32). With limited and variable access to and quality of health care services in LMICs, morbidity and mortality are likely to be substantially higher (32, 37). The proportion of pneumonia cases that is caused by RSV in LMICs was estimated at a median of 20% (5^{th} to 95^{th} percentile 1 to 53) using data from children included in 87 studies (37).

PIVs, particularly type 1, 2 and 3, are second to RSV in causing severe viral lower respiratory infection in children (38). Parainfluenza viruses involve the lower airways less frequently and result in fewer hospitalizations than RSV (27, 31). The difference between hospitalization rates for RSV and PIV is particularly striking for the first six month of life (27). Hospitalization rates for RSV have been estimated to be ~3 per 1000 children/year for the age group below 5 years and ~17 per 1000 for those below 6 months (29), while the corresponding rates for PIV are ~1 and ~3 per 1000 (31). PIVs have been associated with pneumonia in LMICs (39), but the proportion of cases with PIV type 1, PIV type 2 and PIV type 3 in hospital- and community-based studies is not determined.

Seasonal influenza causes a significant number of acute respiratory infections, including pneumonia, among children (21). The disease burden has been largely under-recognized, especially in the community (30). In the Unites States, annual rates of outpatient visits attributable to influenza were reported to be around 95 fold higher than hospitalization rates

for children under 5 years, while the highest rates of hospitalization (4.5 per 1000 children) were reported for those below 6 months of age (30), similar to for RSV and parainfluenza (29, 31). The role of influenza in contributing to pneumonia has been uncertain, particularly in LMICs, but recent data from Bangladesh indicate that it could be substantial (40). In Hong Kong (41), population-based estimated hospitalization rates for influenza exceeded those reported in the United States (30). Respiratory viruses also play an important role in the pathogenesis of pneumonia by predisposing to bacterial infections, a feature especially associated with influenza virus (42).

In 2001, hMPV was detected in the Netherlands and is together with RSV a member of the subfamily *Pneumovirinae* within the *Paramyxoviridae* family (43). The virus is now recognized as an important causative agent of ARI in children, both in the community and in hospitalized cases (44). It seems to have a worldwide distribution, being detected in a large number of locations (45). The rate of hospitalization for hMPV infection has been found to be lower than for RSV infections but higher than that observed for influenza and parainfluenza viruses (46, 47). High incidence rates for hMPV-ALRI hospitalization are reported in South Africa and Hong Kong (48, 49). Available data show that hMPV account for approximately 5-8% of ARI hospitalizations (44, 50-52) and 2-6% of community cases of ARI in children below 5 years of age in industrialized countries (44, 50, 53). Hospital-based studies of children ≤5 years in LMICs have shown similar occurrence (54-57), but very few studies report on hMPV pneumonia in the community (58).

Seasonality of respiratory viral infections

Infections with these respiratory viruses exhibit distinct seasonal patterns in most temperate regions. Typically, RSV and influenza cause annual recurrent well-defined epidemics during the cold months (37, 59, 60). The activity of hMPV has been shown to be greatest in winter and spring in the northern hemisphere (44) and autumn through spring in the southern hemisphere (48, 53, 61), but data are still somewhat limited as year-round surveillance has not been extensively undertaken. In initial reports, the hMPV incidence varied substantially from year to year (62). There are now reports suggesting a biennial epidemic pattern of early and late hMPV occurrence in several European countries (59, 63, 64). PIV type 3 infections occur year round with outbreaks usually occurring in spring, while type 1 and 2 demonstrate

a biennial pattern with epidemics in the fall or early winter, sometimes in alternate years (65-67).

Although the seasonal variations of RSV and influenza infections have been extensively studied in various LMICs, especially for RSV, it is difficult to outline a clear pattern. A review by Weber and coworkers (39) revealed that RSV infections peaked during the cold months in temperate regions in the southern hemisphere, seemingly independent of rainfall. In sub-tropical and tropical locations with seasonal rainfall, RSV tended to occur in relation to the rainy season, however, in locations closer to the equator with perennial rainfall, RSV activity was almost continuous and peaks of infection varied (39). Influenza is also reported to be detectable throughout the year in tropical and sub-tropical regions with less predictable timing of outbreaks, although there are reports of a biannual pattern of outbreaks with considerable activity between epidemic periods (60). The peak hMPV season is reported to be during late winter to spring in Bangladesh (58) and India (54), while outbreaks have been observed in spring and autumn in South Korea (68) and in spring and summer in Hong Kong (49), but observation periods for these studies have only been 1-2 years. In a three-year study in South Africa, hMPV was seen in yearly epidemics, peaking during autumn and winter (48). There are few comprehensive reports on seasonality of PIVs from developing regions. Most studies have a short observation time and many studies did not distinguish between the different PIV types (69, 70). Seasonal observations in Singapore and Taiwan were largely similar to those in temperate regions described above (71, 72).

Clinical and epidemiological aspects of respiratory viral infections

RSV causes a wide spectrum of respiratory infections from rhinitis and otitis media to severe infections of the lower respiratory tract. The virus is the major cause of bronchiolitis in infancy and a significant cause of pneumonia during the first few years of life (73). Between 25 to 33% of primary RSV infections involve the lower airways (74), but this proportion is lower in reinfections and with increasing age (75). Infants are at highest risk of developing severe manifestations of the infection, especially before 6 months of age (75). Severe disease typically presents with fever, cough, expiratory wheeze, dyspnea and cyanosis (74). Spread of RSV from contaminated nasal secretions occurs via large respiratory droplets (76), which requires close person-to-person contact or contact with contaminated surface for

transmission. The virus persists on environmental surfaces for hours and is thus a frequent cause of nosocomial infections, especially in pediatric wards (6, 76). Primary infection is rarely asymptomatic and reinfections are frequent. In a prospective study in the United States, around two-thirds of children were infected during their first year of life, and by the age of two, nearly all children had experienced one infection and nearly half had been infected twice (75). Reinfections occur in all ages as immunity to RSV infection is incomplete and short-lived (77), but disease severity wanes with age (67). However, RSV may cause severe infections in immunocompromized adults and elderly people (78). Hospitalization for RSV bronchiolitis has been associated with subsequent asthma and wheezing in children (79, 80), but atopy and wheezing have also been shown to be risk factors for RSV hospitalization in young children (81). The majority of children who get severe RSV disease are otherwise healthy, but premature infants, infants with congenital heart disease, cystic fibrosis, bronchopulmonary dysplasia, or immunodeficiency are at particular high risk of severe illness (28, 82-84). Several other important risk factors for severe RSV illness related to the environment and the host have been identified, including male sex, age <6 months, birth in the first half of the RSV season, crowded living conditions, siblings, lack of breastfeeding, and day care exposure (85). Level of passively acquired maternal antibody to RSV could be an underlying factor in age of acquisition (86). A recent study of RSV burden in the United States found that only prematurity and young age were independent risk factors for hospitalization (29).

Influenza infection in children mainly manifests as febrile illness with respiratory symptoms, but can also cause severe respiratory illness, particularly in individuals with underlying cardiopulmonary conditions (6). High fever, rhinitis and cough are common features of influenza illness in children (40, 87-90), while adults frequently experience general malaise, headache, and myalgia as well. In young children influenza resembles other severe respiratory tract infections causing pneumonia, bronchiolitis, croup, otitis media, and, more rarely, febrile convulsions (74). Virus is transmitted via aerosols and droplets from respiratory secretions generated through coughing and sneezing, or by contaminated hands (6). Children experience the highest attack rates during seasonal epidemics (91), as they typically shed high amounts of viruses during infection and thus have an important role in the transmission in the community (92), while individuals aged 65 years and older experience most serious illness, complications and death from influenza (93). Among children, those younger than 2 years of age are most susceptible to severe consequences of

influenza infection (88, 90, 91) and estimates of hospitalization rates due to influenza are similar to those of adults at high risk (94, 95). Studies report no difference in clinical symptoms or signs between illness episodes caused by type A and B, but some have found children hospitalized with influenza A infection to be younger (89, 90, 96).

Like RSV, parainfluenza viruses cause infections restricted to the respiratory tract (74). While PIV type 1, 2 and 3 are the principal causes of croup, type 3 is also known to cause pneumonia and bronchiolitis in young children, typically in infants (67). The subglottal swelling in croup results in a barking cough, tachypena, tachycardia and suprasternal retraction (74). PIVs usually cause mild cold-like upper respiratory infection (URI) or pharyngitis, but approximately 15-25% of infections spread to the lower respiratory tract (66, 74). PIV type 3 is considered second to RSV in causing severe infections in infants, both with peak incidence of hospitalization before 6 months of age (29, 31). The virus is transmitted by respiratory droplets and person-to-person contact (74). Most children are infected with PIV type 3 by two years of age and with types 1 and 2 by five (67). Like for RSV, reinfections occur throughout life, as acquired immunity is short-lived (97). There are indications that croup is relatively less frequent in LMICs (38). Caucasian children have for instance been found to have higher incidence of croup compared to African-American (98).

The clinical manifestations of hMPV are similar to those of RSV (44, 99) and sometimes those of influenza (45). However, a number of studies report hMPV to cause less severe illness, more frequently manifest as pneumonia than bronchiolitis and infect slightly older children than those infected with RSV (100-107). Infections with hMPV have also been found to cause respiratory disease of similar severity as RSV infections (47). Seroprevalence surveys have shown that virtually all children are infected with hMPV by the age of 5 (43). The virus cause infection in all age groups, but has its greatest effect in children; those <2 years have the highest incidence and are at the highest risk of serious infections (44, 108). Pre-term infants also seem prone to severe disease (99). Adults usually suffer from relatively mild common cold-like respiratory symptoms (109), but like RSV and influenza virus infections, hMPV infections may also cause severe illness in the elderly and in patients with underlying disease (44, 109, 110). Several studies suggest that hMPV, like RSV, may be associated with episodes of acute wheezing and asthma exacerbations in children (44). Risk factors for severe hMPV disease and frequency of reinfections have not been extensively studied (111-114).

Control of pneumonia

Interventions targeting risk factors for pneumonia are required for primary prevention, whereas case management aims at reducing disease severity and case fatality. Both strategies are needed to reduce pneumonia mortality. The WHO ARI standard case management approach developed in the 1980s focuses on early detection and treatment with appropriate antibiotics (115) and has been the cornerstone of pneumonia control in low-income countries. The program was later incorporated into the Integrated Management of Childhood Illness (IMCI) guidelines (116). Community-based implementation of this case management strategy has greatly reduced overall and pneumonia mortality in young children (117), but implementation is lagging behind in many high-incidence countries and therefore has substantial potential for improvement (118). However, increasing antimicrobial resistance of pathogens causing pneumonia (25, 119) demonstrates the need for additional strategies. There are also areas for improvement in facility-based treatment. Hypoxia is associated with increased risk of mortality from pneumonia (120) and proper assessment and treatment of hypoxia has been shown to substantially reduce case fatality (121). The use of pulse oximetry is far more accurate than clinical signs in detecting hypoxia (122). Unfortunately, oximetry and oxygen therapy are unavailable in many developing country settings.

Vaccination against the important respiratory pathogens is effective in the prevention of childhood pneumonia and leads to a reduction in mortality; immunization against pertussis, measles, and pneumococcal infection being striking examples (5). While Hib and pneumococcal conjugate vaccines are licensed and recommended by WHO for inclusion in national programs (123, 124), LMICs can ill afford them. Special initiatives by the Global Alliance for Vaccines and Immunization (GAVI) may increase coverage (5). Vaccines against RSV and PIV type 3 are currently being developed (5), despite earlier setbacks, especially for RSV vaccines (38).

Malnutrition is an underlying factor in more than half of all under-5 deaths (4) and is strongly associated with an increased risk of dying from pneumonia (125). In fact, about a quarter of pneumonia deaths in LMICs are attributable to underweight or stunting alone (126). Promotion of exclusive breastfeeding, especially in the first month of life, and improving zinc nutriture are other potentially effective interventions in the prevention of pneumonia (126).

In 2007, WHO and UNICEF initiated a Global Action Plan for Pneumonia (GAPP) to increase awareness of pneumonia as a major killer of children and to develop a unified and equitable approach towards pneumonia control (127). In order to increase child survival, countries should focus on four areas that offer the best prospects for pneumonia control, namely vaccines, case management, nutrition and environment (128). Vaccines against measles, pertussis, pneumococcus and Hib, effective case management at both community and health facility levels, improvement of nutrition through promotion of exclusive breastfeeding and improving zinc nutriture, and reducing the prevalence of low birth weight are identified as key strategies for pneumonia control with the potential to substantially reduce pneumonia illness and death in under-5s (129). Environmental interventions, such as improvement of indoor air quality through cleaner fuels and better stoves, may prevent pneumonia and should be encouraged (129). In addition, prevention and management of HIV infection is also perceived as a major area that needs to be addressed to prevent pneumonia (129).

Diagnosing pneumonia

The diagnosis of true bacterial pneumonia in children remains a challenge, despite the frequency and severity of this condition. The reference standard for diagnosing pneumonia is an aspirate from the lower respiratory tract obtained by lung puncture or bronchoscopy (130). As a non-invasive proxy, radiography is considered a pragmatic reference standard for the diagnosis of pneumonia, but due to variability in interpretations by radiologists, this method also has its clear limitations (131). To improve the agreement of radiological categorization of pneumonia with alveolar consolidations in children, WHO established standardized criteria for interpretation of chest radiographs (132). This approach is limited by the fact that the classical radiologic feature of alveolar consolidation is not produced by all bacterial pneumonia episodes and may also be caused by non-bacterial pathogens (133). Moreover, chest x-ray may be negative in the early course of pneumonia and radiographic changes brought about by pneumonia may persist for weeks after recovery. Auscultatory findings, such as crepitations and bronchial breath sounds, used by doctors in the clinical assessment are largely subjective and have proven difficult to standardize (130).

Identifying cases of bacterial pneumonia is crucial to better target antibiotic treatment. This is especially a challenge in LMICs where limited resources imply that comprehensive individual investigation may not be feasible. The WHO ARI case management approach aims to facilitate and standardize clinical decision-making in resource-limited settings (134) and classifies pneumonia in order to inform case management. In contrast to the conventional diagnosis of pneumonia that uses a combination of clinical signs, chest x-ray or laboratory investigations, the WHO algorithm for classification of ARI is based on simple clinical signs only. These signs, which trained health workers can recognize accurately, have been validated and found to be sensitive and specific indicators of pneumonia (135). Thus, WHO defines pneumonia as an acute episode with fast breathing or lower chest indrawing in children with cough or difficult breathing. This approach identifies most children that potentially suffer from pneumonia and thereby require antibiotics, but in fact also encompasses those with bronchiolitis and a number of those suffering from reactive airways disease with superimposed respiratory infection (134). Global estimates of morbidity and mortality for clinical pneumonia are largely based on this definition (2).

Determining the etiology of pneumonia

Important rationale for pneumonia etiology research in LMICs is to establish evidence-based treatment guidelines (136) and direct the development of preventive strategies. Determining the etiology of childhood pneumonia has been attempted for decades, but has been hampered by the lack of sufficiently sensitive and specific tests. Most importantly, representative specimen from the lower respiratory tract is difficult to obtain. Children do not easily produce expectorate for examination, which even in adults is of questionable relevance for identifying the causative agents of pneumonia due to possible contamination by upper respiratory flora (137). Lung puncture is an invasive procedure and limited to those with a distinct area of consolidation on chest x-ray, thus, studies in LMICs based on such data are limited (138). Isolation of bacteria from blood of a child with signs of lung infection is highly specific for bacterial pneumonia but carries low sensitivity because the majority of cases are not bacteremic (139). The value of serology is often dependent on the availability of paired serum samples to assess any antibody titer increase, as well as the time of serum collection in relation to the onset of illness (140, 141). Some pathogens are difficult to culture and require advanced laboratory facilities that are not available in many hospitals.

Moreover, many widely employed methods for detection of pathogens causing pneumonia are flawed, leaving no adequate gold standard for testing performance of new diagnostic methods, such as nucleic acid detection. Rapid tests of bacterial etiology by antigen detection in urine are not able to differentiate between colonization and infection with bacteria (142). Since children in LMICs frequently are carriers of pneumococcus and Hib (143-145) (146), the tests have low specificity in children. It is not possible to clinically distinguish between bacterial and viral pneumonia in young children, and biomarkers, such as serum concentrations of acute phase proteins e.g. C-reactive protein (CRP) and procalcitonin, add little to the diagnostic accuracy (147-149). Vaccine probe studies are perhaps the best available means to determine the proportion of pneumonia attributable to a specific pathogen, but notably only estimate the role of the vaccine-type strains of a pathogen on a population level (150). There is also a possibility of serotype-replacement disease, as seen among Alaska native children (151).

The ability of the common epidemic respiratory viruses to cause lower respiratory tract infection is well established, also for the relatively recently discovered hMPV. As opposed to several bacteria, respiratory viruses do not colonize the upper respiratory tract, but rather replicates in mucosal epithelial cells in the upper airways (152). Virus isolation by tissue culture of nasopharyngeal specimens depends on the presence of viable virus and has traditionally been considered the gold standard for diagnosing respiratory viral infection (153). It has generally been assumed that a viral pathogen detected in upper respiratory tract secretions during ALRI is the cause of the illness (154). There are however, some problems inherent in this view. All the major viruses that cause pneumonia may cause a spectrum of clinical illness from inapparent infection of the upper airways to severe infection of the lower respiratory tract. In fact, acute respiratory infections initiate in the upper respiratory tract epithelium and in some cases descend to the lower respiratory tract. In general, infection is much more common in the upper than in the lower respiratory tract and on average, young children typically experience 5 episodes of URI yearly (155). A virus may be detected before the onset of symptoms and sometimes for a period after recovery, which means that a child may test positive for a respiratory virus for several weeks of the year. Thus, a virus present in a specimen from upper respiratory tract during pneumonia could be either causal or incidental, questioning its causal role in individual cases and making epidemiological estimates of causality across individuals prone to exaggeration.

The wide application of molecular methods in routine diagnostics of ARI has improved sensitivity compared to conventional methods, such as tissue culture and direct fluorescent antibody assays (153, 156-158). Moreover, molecular diagnostics have facilitated simultaneous detection of multiple pathogens in a single specimen and reduced analysis time. Polymerase chain reaction (PCR) assays have been developed for detection of rhinovirus and for newly discovered viruses such as hMPV, human bocavirus (hBoV) and subtypes of coronavirus. Combined with the use of comprehensive diagnostic testing protocols including a wide array of pathogens, this has resulted in an increase in the proportion of specimen from patients that test positive for any respiratory pathogen. However, there is a penalty to this increased sensitivity. An increase in the proportion of test positive specimen also from asymptomatic individuals (159), especially in young children (160) and the frequent detection of multiple pathogens in single specimens, particularly in studies utilizing multiple diagnostic methods including sensitive PCR assays to detect respiratory agents (59, 161, 162) have complicated the interpretation of positive PCR results.

These issues have raised concern regarding the clinical relevance of detecting certain viral pathogens in upper airways secretion and highlight the problem of ascribing the cause to individual agents. Consequently, it is essential to determine the proportion of virus positive nasopharyngeal specimens in a control group before making assumptions about causality. Most studies of viral etiology have failed to do so.

Focus of the thesis

Data on etiology and clinical presentation of childhood pneumonia are important for planning and assessment of pneumonia control strategies (163). In addition, data on etiology enable more accurate evaluation of the impact of new interventions, such as the introduction of vaccines (152). Such data are lacking for many LMICs, including Nepal. The focus of this thesis is to examine the epidemiological and clinical importance, in terms of frequency, seasonality and severity, of 7 respiratory RNA viruses in young Nepalese children with pneumonia in a community-based setting. The viruses were identified in nasopharyngeal aspirates (NPAs) using a validated (158, 164-166) commercial multiplex reverse transcription PCR assay. Using data from a cross-sectional study and a case-control study embedded therein, we measured the proportion of pneumonias with and calculated pathogenicity odds ratios (ORs) for these 7 viruses. These ORs measure the degree to which

the viruses are associated with pneumonia. Because we do not believe reverse causality is a relevant problem in this context, this is likely to be the best available measure of causality.

2. Objectives

Overall objective

To assess the role of RNA viruses in community-acquired pneumonia in young Nepalese children.

Specific objectives

In young Nepalese children;

1. Identify common viral pathogens in community-acquired pneumonia over a three-year period (Paper I);
2. Describe the seasonality of common viral pathogens in community-acquired pneumonia over a three-year period (Paper I);
3. Describe the clinical presentation, severity and course of viral community-acquired pneumonia (Paper II);
4. Measure the association between the presence of respiratory viruses in nasopharyngeal aspirates and pneumonia (Paper III).

3. Methods

Nepal demographics

Nepal is a landlocked country in South Asia bordered by China and India. It is commonly divided into three major areas that run east-west: the arid Mountain Region with the Himalayan Range in the north, the central Hill Region that includes the Kathmandu Valley, and the Terai Region, which refers to the southern fertile and densely populated lowland plains. The climatic zones corresponds to the altitude and range from tropical to arctic. The proximity to the Bay of Bengal makes Nepal influenced by the Indian monsoon in the summer.

The population exceeded 25 millions in 2006 (167). Nearly one quarter of the population lives below the poverty line (1 USD per day) (168). Labor migration to the Gulf, India and Malaysia is widespread. The overall adult literacy rate is 56.5% (169), but there are great disparities between genders and across regions (167). In 2001, only 14% of the population lived in urban areas (170), but this may have increased during the ten-year long Maoist led violent insurgency that ended in 2006. The infant mortality rate (per 1000 live births) has declined the last 15 years from 79 (1991-1995) to 48 (2001-2005) (167) and life expectancy at birth is around 66 years (169). Undernutrition is common, especially among children. About half of the children below 5 years were stunted and nearly 40% were under-weight in 2006 (167). The Expanded Program on Immunization (EPI) began in 1979 and official figures indicate that overall coverage for all basic vaccines (BCG, measles, and three doses each of DPT and polio vaccine) had reached 83% in 2006 (167).

Study area and population

The studies presented were undertaken in Bhaktapur district (Figure 1) in the eastern part of the Kathmandu Valley (27°N, 85°E)**.** The valley is situated at an altitude 1,300-1,350 meters above sea level and has a sub-tropical, temperate climate with four distinct seasons; pre-monsoon/spring (March-May), monsoon/summer (June-September), post-monsoon/autumn (October-November) and winter (December-February) (171). Temperatures may rise to

35°C in summer, while minimum temperatures can fall to 0°C in winter. The valley is the most densely populated area in the country.

Bhaktapur town is the district headquarters with a population of about 80,000. The municipality is divided into 17 administrative 'wadas' or neighborhoods. The Newars form the major ethnic group in the area and a large proportion is involved in subsistence farming. Migrant minority groups, such as the Lama and the Tamang, are more frequently engaged working in numerous carpet or brick factories, which makes them more dependent on purchasing food items and hence vulnerable to fluctuations of the prices in the market. Undernutrition, mainly manifest as stunting, and anemia, is common among children below 5 years of age (172). The vaccine coverage is >90% for all vaccines included in the national EPI (173).

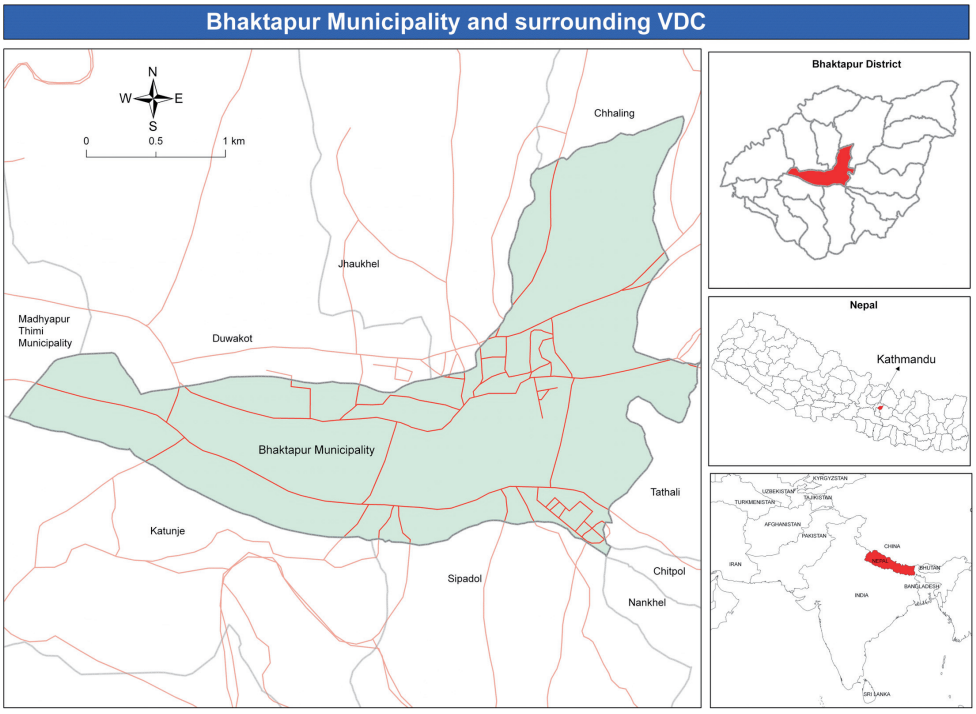

Figure 1. Map of Nepal with details of Bhaktapur district with municipality boundaries and surrounding village development committees (VDCs).

Prior to study start, we undertook a baseline census of children below three years of age living in households in the 17 wadas in Bhaktapur municipality. This census, which covered

8,398 households, showed that 41% of families with young children owned some agricultural land, while 22% owned domestic animals. Most of the households had access to piped drinking water (97%) and toilet with central drainage (88%). About half of the families owned their own accommodation (52%), while 46% lived in only one room. Although winters are cold and houses not isolated, heating of rooms is not common. Cooking is mainly done indoors and kerosene was used by 51%.

Fieldwork

One cross-sectional study and one case-control study form the basis of this thesis. A display of the field studies and methods in relation to the papers is given below (Table 1).

Table 1. Study design, period, topic and main analyses of the field studies presented in the respective papers.

Paper	Study type	Study period	Topic	Main analyses
I	Cross-sectional study of children with WHO-defined pneumonia (n=2,219)	29 June 2004 to 30 June 2007	Identification of common respiratory viruses and their seasonality	1) Descriptive statistics
II	Cross-sectional study of children with WHO-defined pneumonia (n=2,219)	29 June 2004 to 30 June 2007	Clinical presentation, severity and outcome of pneumonia episode	1) Logistic regression 2) Cox regression
III	Matched case-control study of children with and without WHO-defined pneumonia (n=1360)	25 March 2006 to 9 July 2007	Comparison of virus frequency in children with and without pneumonia	1) Conditional logistic regression

Recruitment area and strategy

The baseline census formed the basis for the surveillance system that was set up and maintained throughout the study period (Figure 2). We generated a list of all children below three years of age using the data from the census and regularly updated the list by identifying newborn babies and excluding children who had completed 36 months of age, moved away from the area or left the cohort for other reasons. The children in this open cohort were subject to monthly active surveillance and received a card that entitled them to free basic

health services at the project facility. At first encounter, the fieldworker collected detailed information about the household and the individual child. At the monthly visits, he or she obtained information, mainly from the mother, on symptoms of respiratory and diarrheal illness during the last seven days and referred children with symptoms of illness to the study clinic. In the area outside the municipality, no regular surveillance was undertaken and household information was obtained only when a child was included in the study.

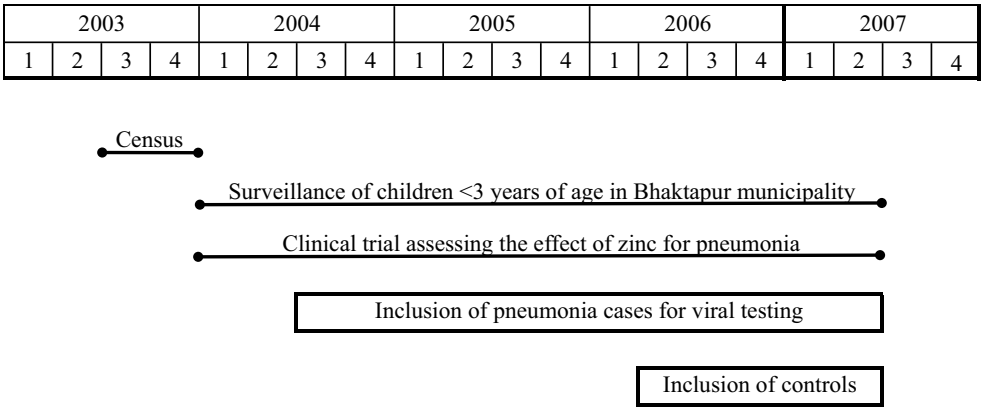

Figure 2. Time points for initiation and ending of the baseline census, the surveillance of children <3 years of age, and the clinical trial with inclusion periods for cases and controls in the virus studies. The virus studies that form the basis for the current thesis were embedded in the clinical trial.

For the cross-sectional study (paper I and II), the participants were recruited mainly from the municipality of Bhaktapur, i.e. from the open cohort of children that were under active surveillance. However, we also included eligible children with pneumonia from the surrounding district if brought to the study clinic. A total of 1,899 (85.6%) of the 2,219 cases were recruited from within the municipality, while the remaining came from the adjacent village development committees in the district. In the case-control study (paper III), both cases and controls were recruited solely from within the municipality of Bhaktapur.

The project staffed an outpatient department at Siddhi Memorial Hospital in the outskirts of Bhaktapur and families could bring their children for free treatment at our clinic for common childhood illnesses. In addition, the project ran a 10-bed pediatric ward with 24-hour service where mainly children with severe pneumonia were admitted.

Recruitment of cases, case definition and exclusion criteria

Children aged 2-35 months who came to our study clinic were screened for fast breathing or lower chest wall indrawing (LCI) and classified according to the standard WHO algorithm for ARI (174). Pneumonia was defined as cough or difficult breathing combined with fast breathing, i.e. ≥50 breaths/min for children 2-11 months old, and ≥40 breaths/min for children ≥12 months old. Severe pneumonia was defined as cough or difficult breathing combined with LCI. Children with auscultatory wheeze were given 2 doses of 2.5 mg nebulized salbutamol administered 15 minutes apart followed by reassessment after 30 minutes. A child was included only if he or she had fast breathing or LCI at reassessment. Cases with very severe pneumonia/disease, i.e. cough or difficult breathing with stridor when calm or any general danger signs (inability to drink/breastfeed, persistent vomiting, convulsions, lethargy or unconsciousness) were not included, but instead referred to a tertiary level hospital after initial treatment. Cases with other severe illness, documented tuberculosis, congenital heart disease, dysentery, severe anemia (defined as hemoglobin <7 mg/L), or severe malnutrition (defined as <70% National Health Care Surveys median weight for height) were not included in the study. Those with a history of cough for more than 14 days or who had received antibiotics within the last 48 hours were excluded. Children could not participate in the cross-sectional study again (paper I and II) until after 6 months because of restrictions imposed by the clinical trial protocol (175). Children included in the case-control study (paper III) could be enrolled as a case or as a control in the study again only after 2 months. The exclusion criteria for cases also applied to the controls, except that hemoglobin was not routinely measured in control children.

Paper I and II

We included 2,230 cases of pneumonia among 1,909 children from June 29, 2004 to June 30, 2007. Only for five days in September 2004 were we not able to include children due to lack of NPA collection equipment. The children were, after obtaining informed parental consent, enrolled in a clinical trial assessing the effect of zinc as adjuvant therapy in children with pneumonia (175). All included children were randomized to receive either zinc (10 mg for children aged 2–11 mo, 20 mg for children aged ≥12 mo) or placebo daily for 14 days adjuvant to antibiotics.

Paper III

We included children in the case-control study from March 25, 2006 in parallel with the undertaking of the zinc supplementation trial. The last case was included on June 30 and the last control on July 9, 2007. Among the 680 cases in the case-control study, 570 were also included in the zinc-pneumonia trial, while 110 cases were not because less than 6 months had lapsed from the previous enrolment in the trial. Hence, not all the laboratory investigations, such as CRP, were available at baseline for the cases that did not enroll in the zinc trial. A "grace period" of two months was set to ensure that cases were not included twice for the same episode.

Selection of controls (paper III)

Controls were matched by age (in months) of the case. One control was randomly selected for each case from the list of children under surveillance that was updated monthly. After inclusion of a case, a fieldworker visited the home of a potential control child on the same or the following day. If parents consented to the child's participation, he or she was referred to the study clinic to be examined for eligibility as a control. If the child did not come to the clinic after two home visits, or was not found or not eligible for other reasons, another randomly selected age-matched child was approached.

Case management

Children with non-severe pneumonia received oral antibiotic treatment with cotrimoxazole for five days according to the WHO's standard case management guidelines for pneumonia (12) and were examined daily by a fieldworker until recovery. The day of recovery was defined as the first of two consecutive days with a normal respiratory rate for age as assessed by the fieldworker. The fieldworker referred the child to the clinic if he or she still had fast breathing at 72 hours after inclusion. If the study physician confirmed pneumonia, treatment was changed to amoxycillin for 5 days. Treatment failure was defined as a change of antibiotic or hospitalization for pneumonia within the first three days after inclusion. Cases of severe pneumonia were hospitalized and received parenteral benzylpenicillin as first line treatment. Children with oxygen saturation (SpO_2) <90% received oxygen treatment.

Data collection

The fieldworkers involved in data collection were trained in standard case management according to the IMCI strategy (176) for one week, facilitated by Nepalese pediatricians and investigators from the study group in Nepal, who also trained and supervised doctors in study procedures.

The child's respiratory rate (RR) was assessed according to WHO guidelines (177), counting twice for one minute using a UNICEF timer. If only one of the counts were in the fast breathing range, counting was repeated and the two counts that were in the same category were recorded. The lower of the two counts was used in the analyses. We attempted to count the RR in children that were either awake and quiet or sleeping, as breastfeeding may increase the RR in some children and make assessment of LCI difficult. The majority of children were assessed while awake and quiet (96%), a small number while sleeping (3%), and very few while breastfeeding (<1%).

Arterial SpO_2 was measured either on a finger or a toe with a pulse oxymeter (Siemens MicrO2, Siemens Medical Systems Inc, Danvers, MA, USA) using a pediatric sensor (Nellcor, Pleasanton, CA, USA). It was recorded twice one minute apart after stabilization of the reading for one minute. The higher of the two measurements was used in the analyses. To determine the normal values for SpO_2 in children living in Kathmandu (at approximately 1,350 meters above sea level), we conducted a reference study among 425 healthy children aged 2 to 35 months attending the vaccination clinic in Kanti Children's Hospital in Kathmandu. SpO_2 was measured twice as described above. According to Duke and coworkers (121), the lowest value for normal oxygen saturation in children can be defined as the mean SpO_2 minus 2 standard deviations (SD). In our group of healthy children, the mean (SD) was 95.9% (1.50), which gives a lower limit of SpO_2 of 93% among normal children. Based on these data, we used SpO_2 <93% for defining hypoxia in the three papers, but we also present the proportions of children with SpO_2 <90%; this is the WHO threshold for oxygen administration (178).

We registered the location of the children's residence in Bhaktapur, i.e. children under surveillance (including the controls) as well as all included pneumonia cases, using handheld global positioning system (GPS) devises (eTrex®, Garmin Ltd., Olathe, KS, USA). The geographical location of the houses was visualized using a GPS-based computerized plot

(Google Earth Pro) and was utilized to map the distribution of viral infections in the community over time.

Collection, processing and storage of nasopharyngeal aspirates

NPA specimens were obtained using a sterile, disposable suction catheter (Pennine Healthcare Ltd., Derbyshire, UK) with a suction trap (trachea suction set, Unomedical a/s, Birkerød, Denmark) connected to a foot pump (Ambu® Uni-Suction Pump, Ambu A/S, Ballerup, Denmark). The catheter was inserted through the child's nostril to a distance equivalent of that from the nostril to the earlobe [21]. Suction was applied for minimum of ten seconds with maximum negative pressure of 200 mm Hg. Secretion remaining in the catheter after suction was recovered by rinsing 2-3 ml virus transport medium (DiagnoStick®, Department of Microbiology, University Hospital of North Norway, Tromsø, Norway) through the catheter into the suction trap. The trap was then disconnected and sealed. In March 2006, we changed transport medium to Universal Transport Medium (UTM) System (Copan Diagnostics Inc., Corona, CA) because the in-house product DiagnoStick® was no longer available. The new transport medium had the advantage of tolerating storage temperatures from 2-30°C before use, while the DiagnoStick® had to be kept frozen before use when stored for longer periods of time.

The specimens were refrigerated at 2-8°C following collection at the field clinic and transported on ice every working day to the main laboratory in Kathmandu, where they were vortexed and divided in three equal aliquots in sterile vials (CryoTubes™, Nunc AS, Roskilde, Denmark). The aliquots analyzed in Nepal were either frozen at –70°C or kept refrigerated at 2-8°C before analysis (paper I and II). Two aliquots were immediately frozen at –70°C and transported to Norway on dry ice and again stored at –70°C for quality control purpose. The specimens for the case-control study (paper III) were all refrigerated at 2-8°C before analysis (mean number of days of storage was approximately 10 days (range 0-37), median 6 days [IQR 3-12]). The storage conditions were identical for case and control specimens.

Comparative study of different storage temperatures

There is concern regarding degradation of viral RNA by storing specimens at temperatures of 2-8°C as compared to -70°C. We therefore undertook a separate study comparing results between samples refrigerated at 2-8°C for up to four months (i.e. 125 days) and samples frozen at -70°C immediately after processing. Assuming the frozen storage as gold standard, this comparative study showed that the sensitivity for samples refrigerated for up to four months was 93%. Moreover, the sensitivity did not differ substantially between samples refrigerated for periods of 2 months, 3 months and 4 months (data not shown). The specificity was 96% in the refrigerated samples compared to the frozen samples. It is not likely that specificity, as opposed to sensitivity, would be affected by prolonged storage at 2-8°C.

Setting up and running the virus laboratory in Kathmandu

The project hired a bus to shuttle fieldworkers from Kathmandu to Bhaktapur in the morning and back again in the evening five days a week. This bus also carried the NPA specimens from the field clinic to the project office in Kathmandu. Samples were received, processed and frozen by one of the project laboratory staff. Initially we used a -70°C freezer that was available in the research laboratory to store our samples, but to increase the freezer capacity we purchased a -86°C ultra-low temperature freezer that was shipped from Norway to Nepal. This freezer broke down within the first year and had to be replaced by a second freezer also shipped from Norway because there were no possibilities for repairing the broken freezer in Nepal. Despite these challenges, none of the specimens suffered accidental thawing.

The dry ice for transportation of NPA aliquots to Norway had to be ordered from New Delhi, India, through a local dealer in Nepal and was shipped to Kathmandu by air. We had a special bag made in Nepal for shipment purpose for transportation to Norway. There were always substantial amounts of dry ice remaining at arrival, indicating that temperatures during transport had been below -40°C.

The Department of Microbiology at Tribhuvan University Teaching Hospital provided the 3-room facility that we needed to set up a PCR laboratory. A lot of laboratory equipment and consumables were hand-carried from Norway to Nepal. The reagents for the PCR assays were imported directly from USA. This was divided into three major shipments over the

three-year period due to limited shelf life of the reagents. We also ordered pipette tips in bulk from France. Nepal charges a high tax on imported goods. This motivated an application for import tax exemption for our research material, which was a rather lengthy process that had to be repeated for every shipment. The body at the university that dealt with these applications was not operational for a longer period of time during the political unrest in the spring of 2006 when King Gyanendra was forced by the democracy movement to renounce his sovereign power. This delayed import of essential reagents for the laboratory for several months. Due to a general shortage of electricity in Nepal, the power supply is not continuous in Kathmandu at certain times of the year. The authorities scheduled local "load shedding", and the power could be discontinued for up to ~30 hours a week. To avoid interrupted power supply in our laboratory, we installed a back-up battery that would last for the required number of hours of the scheduled power cut, which rarely exceeded 4 hours. However, this battery was too small to serve as a back up for the freezers, which occasionally were without power. Therefore, we monitored the freezer temperature, which was never recorded to be above -40°C at any time.

Competence/capacity building

The Department of Microbiology and Infection Control at the University Hospital of North Norway (UNN) in Tromsø supported us in the planning and set up of the laboratory. Håkon Haaheim, a UNN staff, and myself (MM) went for a one-week training in the facilities of Prodesse in Waukeshaw, WI, USA in September 2004. Unfortunately, the two main Nepalese laboratory staffs, Biswa Nath Sharma and Govinda Gurung, were not granted a US visa for this trip. They received training in PCR analyses at the virus laboratory in the Department of Microbiology at All India Institute of Medical Sciences (AIIMS) under supervision of Professor Shobha Broor for ten days in April 2005 together with me. Håkon Haaheim travelled to Nepal in August 2005 to assist us in setting up the lab and to start the PCR analyses. The following year Ann Helen Helmersen from UNN visited Nepal for two weeks to provide assistance in making analysis procedures more efficient. This, and the introduction of multi-channel pipettes and PCR strips instead of individual PCR vials, resulted in a 3-fold increase in analysis capacity. In 2006 two of the Nepalese laboratory staff had a one-week stay at the Department of Microbiology and Infection Control at UNN for additional training.

Virus identification

One aliquot of each specimen was tested at our research laboratory in Nepal for RSV, influenza A and B, PIV type 1, 2 and 3, and hMPV using a commercially available multiplex reverse transcription PCR assay (Hexaplex Plus®, Prodesse Inc., Waukeshaw, WI) with minor modifications of the manufacturer's instructions (179) and previous descriptions (166). In brief, nucleic acids were extracted from 360 µl of NPA using a nucleic acid (NA) extraction kit (Roche High Pure Viral Nucleic Acid Kit, F. Hoffman-La Roche Ltd., Basel, Switzerland) according to the manufacturer's instructions. Each run of the assay included a positive RNA control and a negative control (virus transport medium), starting at NA isolation. Specimens and negative controls were individually spiked with 40 µl of internal control during NA isolation to identify any inhibition. Reverse transcription with random hexamers and multiplex PCR were performed according to the Hexaplex Plus protocol using GeneAmp® PCR System 2700 (ABI, Applied Biosystems, Foster City, CA, USA). The PCR supermix contained seven pairs of forward and backward primers flanking unique sequences of the seven viruses (the hemagglutinin neuraminidase gene of PIV type 1, 2 and 3, the matrix protein gene of influenza A, the NS1 and NS2 genes of influenza B, the NS1 and NS2 genes of RSV and the nucleocapsid gene of hMPV). After amplification, the PCR products were purified using Qiagen QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, CA, USA) and analyzed by enzyme hybridization assay (EHA) (166), measuring the optical density at 450 nm (OD_{450}) using a micro-plate reader (Stat Fax® 2100, Awareness Technology Inc., Palm City, FL, USA). The EHA was mainly run directly with individual probes discriminating between the different types of PIV and influenza. However, the EHA was during some periods run with pooled probes for PIV and influenza and then discriminated in a second EHA if the pooled probe yielded a positive result. Definitions of cut-off values and interpretation of PCR results were as described in paper III.

Three hundred and twenty-four NPA aliquots were stored beyond 3 months at 2-8°C before analysis in Nepal. Of these, we reanalyzed the 133 that yielded a negative result, now using the aliquot that had been frozen at –70°C and transported on dry ice to Norway. This was done at the Department of Microbiology and Infection Control, UNN, Tromsø, Norway, using the Hexaplex Plus assay and an automated extraction platform (NucliSens® easyMAG, bioMérieux, Durham, NC). Nucleic acids were extracted from 400 µl of sample,

negative and positive processing controls and amplification control using the extraction principle with magnetic particles of this platform.

Statistical analyses

Statistical analyses were performed using Stata/MP version 10.1 for Macintosh (Stata corporation, College Station, TX, USA). Anthropometric measures were expressed as Z-scores, which were generated using the WHO Child Growth Standards 2005 (180). Statistical significance was defined as a *P*-value <0.05.

In paper I and II, the 95% confidence intervals (CI) for proportions were calculated with binominal exact confidence interval using the "ci" command. Relative proportions were calculated using the "binreg" command. We used Cox proportional hazard models to estimate the association between viral status and duration of the non-severe pneumonia episodes. The odds ratio for treatment failure in non-severe cases was calculated using logistic regression. In the multiple regression models where each virus was used as the exposure variable we included age, breastfeeding status, whether the child belonged to the zinc or placebo arm of the trial, as well as presence of the seven different viruses. We also performed these analyses using high CRP (as an indirect marker of bacterial infection) as the exposure variable. In these latter analyses we included viral status (positive or negative) in the model instead of each of the seven viruses. Of the children included in these analysis, 274 were enrolled twice and 18 thrice. We used the "cluster" option in Stata to adjust the *P*-values and the confidence intervals of our estimates for repeated enrollments and thus allowed for possible dependence of observations in a child that was included more than once.

Meteorological data for the Kathmandu airport weather station (located approximately 10 km from Bhaktapur) were obtained from Department of Hydrology and Meteorology, Ministry of Environment, Science and Technology, Kathmandu, Nepal. Mean daily values for relative humidity and temperature were calculated as the average of two daily measurements (relative humidity at 8.45 AM and 5.45 PM, and maximum and minimum temperature). We estimated the Spearman rank order correlation coefficient to describe the association between the monthly number of infections with each virus and meteorological factors.

Proportions in the baseline table of paper III were compared using logistic regression. In an unmatched design, the pathogenicity odds ratio (OR) for each virus is the odds of detecting a pathogen-positive specimen from a child with pneumonia divided by the odds of detecting a pathogen-positive specimen from a control child. To take the matching into account, we estimated the pathogenicity using conditional logistic regression to calculate the matched OR (MOR). Because we sampled each control concurrently, i.e. shortly after the corresponding case had been identified, this OR is a direct estimate rather than a biased approximation of the incidence rate ratio for the given pathogen (181). These analyses included 1,360 specimens from 1,059 children of whom 808 were enrolled once, 210 twice, 33 three times, 7 four times, and one child five times. We used the "cluster" option in Stata to take repeated enrollments and thus possible dependence of observations in children that were included more than once into account when calculating the confidence intervals and *P*-values of these pathogenicity estimates. This adjustment only marginally affected the precision of the presented estimates. We sought to identify possible confounders, such as the presence of other viruses, sex, breastfeeding status, stunting, wasting, and whether the child had been delivered in a hospital, using unconditional logistic regression including age categories as factors. We also explored whether the MORs were different in children below and above 6 months of age for the two most common viruses. The *P*-values for such possible interactions were obtained from the unconditional models.

Ethical issues

The protocol for the study descried in paper I and II had ethical clearance from the Research Ethics Committee of the Institute of Medicine at Tribhuvan University in Kathmandu, Nepal as well as from the Regional Committee for Medical and Health Research Ethics (REK) of Western Norway (REK project no. 129.03). The protocol for the case-control study in paper III was approved by the Research Ethics Committee at Tribhuvan University, Nepal. Ethical approval was not sought in Norway, which was according to the Norwegian national guidelines at the time of application (2005). The storage of NPA specimens in Norway have been registered and approved by the Norwegian National Biobank Register, Norwegian Institute of Public Health (research biobank no 1832).

Informed consent for participation was obtained from the guardian of the child, usually one of the parents. A witnessed verbal informed consent was obtained from those who were

illiterate and a register of witnesses was kept at the field site. Children below three years of age received treatment for common childhood illnesses free of charge and this was not limited to study participants. Participants were informed that withdrawal of consent did not affect health care services offered by the project.

4. Results

The geographical location of houses of children <3 years old that underwent monthly surveillance and the pneumonia cases enrolled in the clinical trial are depicted in figure 3.

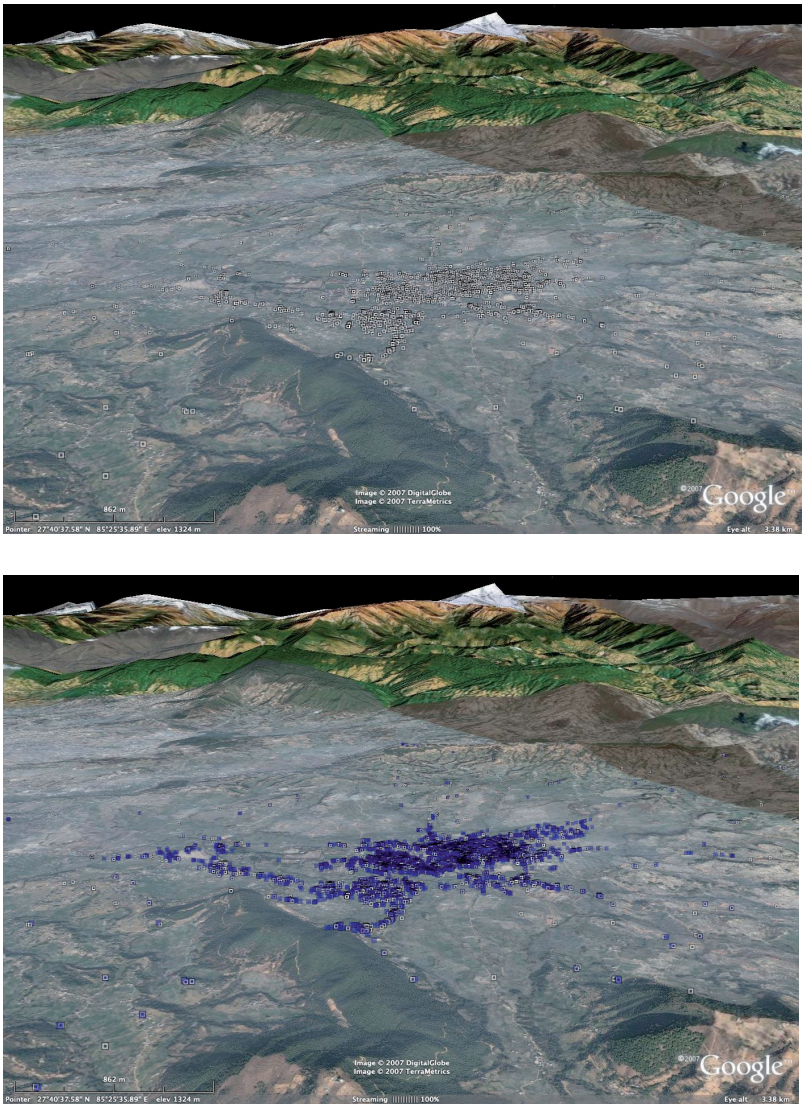

Figure 3. Google Earth map images of the eastern part of the Kathmandu Valley including Bhaktapur municipality showing the geographical distribution of households of children <3 years old under

monthly surveillance (white squares) and children with pneumonia (blue squares) enrolled in the clinical trial.

Subject characteristics

Cross-sectional study (paper I & II)

For this three-year study, we included 2,230 pneumonia cases. We excluded 11 cases from the analyses because of inhibition of the PCR. Of the remaining 2,219 cases, 56.9% were boys and 45.8% were infants (i.e. 2 to 11 months). The overall mean (SD) age of the study children was 13.4 (8.3) months but this was substantially lower among the children with severe pneumonia (8.6 [7.4] months). There were also more (62.6%) boys in the group with severe pneumonia. The majority (87.7%) of children were still breastfed at the time of inclusion in the study. Mean birth weight was 2,86 kg in the 1,585 children where this information was available, and 224 (14%) had low birth weight (<2,5 kg). Hospital delivery was reported in 1,718 (77.5%). Wasting was more frequently seen among cases with severe pneumonia than among non-severe cases (6.1% vs 3.6%), while the proportions of stunting were very similar (22% vs 24%). About a quarter of mothers reported not being able to read or write, while this was much less common among fathers (5%). Sixty-six percent of mothers did not work outside their home and almost half of the fathers (48.6%) were daily wage earners (i.e. work contracted on a day-to-day basis).

Case-control study (paper III)

The background characteristics of the cases in the case-control study did not differ from the larger population of cases described above with regard to age, sex, breastfeeding, birth weight, anthropometrical measures, or socio-economic variables. The controls were more often born in hospital than the cases (89% vs 81%), but their birth weights were nearly identical. A larger proportion of the cases were wasted and stunted compared to controls, but the differences were not substantial and the total numbers of children with wasting and stunting were small. There were a slightly higher proportion of controls that came from families owning agricultural land, belonged to the Newar caste, or lived in an extended family as compared to the cases (Table 1 in paper III). Other background characteristics were evenly distributed between the two groups.

Virus analyses

An overview of the main findings are shown in table 2.

Frequency of respiratory viral infections (paper I)

We detected at least one virus in 887 (40%) of the 2,219 pneumonia cases from June 2004 to July 2007. More than one virus was detected in only 29 (3.3%) of the positive specimens. RSV was the most common of the seven viruses being identified in 334 (15.1%) cases. Influenza A was second in frequency (164 or 7.4%) followed by PIV type 3 (129 or 5.8%) (Table 2).

Seasonality of respiratory viral infections (paper I)

We observed the largest epidemics of pneumonia during the end of the monsoon season and in winter, and most epidemic peaks coincided with epidemics, individual or compiled, of RSV, hMPV, influenza A and B, and PIV type 3. PIV type 1 occurred in an endemic pattern throughout the year with no major peaks. We only identified 17 cases with PIV type 2 infection during the entire study and these were sporadic. An annual RSV epidemic peaked either in September (2004 and 2006) or in December (2005). We observed a single hMPV epidemic, which occurred in December to January the first year, while the only substantial PIV type 3 outbreak peaked in June 2006. Influenza epidemics were predominantly observed during winter months and outbreaks of type A and B infections more or less overlapped. The spatial-temporal distribution of the individual viruses is shown online in the following website: http://folk.uib.no/mihtr/CHRP/Virus.html.

Respiratory viral infections and climatic parameters (paper I)

In the Spearman correlation analyses RSV and hMPV were positively associated with relative humidity (r=0.40; P=0.015 and 0.55; P 0.0005, respectively) but not with temperature or rainfall. In contrast, PIV type 3 was positively associated with temperature (r=0.65; P<0.0001) and rainfall (r=0.68; P<0.0001) but not with humidity. Influenza A did not correlate with any of the tree meteorological parameters, while influenza B exhibited

Table 2. Overview of the main features of the individual viral infections described in the three papers. PIV type 2 is not included due to few positive isolates.

Characteristic	RSV	hMPV	Influenza A	Influenza B	PIV type 1	PIV type 3
Paper I						
Positive isolates (%)	334 (15.1)	93 (4.2)	164 (7.4)	84 (3.8)	98 (4.4)	129 (5.8)
Study months detected	20/36	18/36	22/36	19/36	28/36	27/36
Epidemic season	last part of monsoon 2004/ 2006 and winter 2005	winter 2004/2005	winter 2004/2005	winter 2005	none	pre-monsoon 2006
Association with climatic parameters (Spearman)	positive with humidity	positive with humidity	none	negative with humidity and rainfall	negative with humidity	positive with temperature and rainfall
Paper II						
Infection in infants (2-11 months) (%)	154 (46.1)	37 (39.8)	70 (42.7)	33 (39.3)	41 (41.8)	63 (48.8)
Severe pneumonia (%)	36 (10.8)	4 (4.3)	5 (3.1)	10 (11.9)	2 (2.0)	3 (2.3)
Association with clinical signs and disease outcome						
axillary temperature ≥38.5 C	positive only in infants*	none	positive	none	none	none
wheezing	positive, more pronounced in infants**	none	negative	negative	none	none
crepitations	positive, more pronounced in infants**	none	none	none	none	none
lower chest indrawing	positive	none	none	positive	none	none
SpO_2 <93%	positive only in infants*	none	none	none	none	none
treatment failure	positive	none	none	none	none	none
prolonged time to recovery	positive	none	none	none	none	none
Paper III						
Association with pneumonia	positive	positive	positive	positive	positive	positive***

* The association was modified by age in that the association was positive in infants only (*P* for interaction between RSV and age category was 0.004 for high fever and 0.024 for SpO2 <93%).

** The association was modified by age in that the association was more pronounced in infants. *P* for interaction between RSV and age category was 0.036 for crepitations and 0.073 for wheezing. For LCI and disease outcomes *P* for interaction between RSV and age category was >0.2.

*** The association was modified by age in that the association was stronger in children 6 months of age and older (*P* for interaction between PIV type 3 and age category was 0.002).

RSV, respiratory syncytial virus; hMPV, human metapneumovirus; PIV, parainfluenza virus.

moderate negative correlation with all three (-0.31 to -0.39, $P<0.066$). As RSV infections peaked towards the end of the monsoon in the first and third year of the study but showed no correlation with rainfall, we further explored if there was an association with preceding rainfall. By introducing a 2-month lag after peak precipitation, we did indeed observe such an association (r=0.49; $P=0.003$).

Clinical features and outcomes of respiratory viral infections (paper II)

RSV was identified in 298 (14.3%) of the 2,088 cases with non-severe pneumonia, 36 (27.5%) of the 131 cases with severe pneumonia, and in 154 (15.2%) of the 1,016 infants 2-11 months of age. Mean age did not differ much between children infected with the different viruses (13.4 to 15.1 months) (Table 2), but was higher in those with non-severe compared to those with severe illness (13.7 vs 8.6 months). Half (53%) of the severe cases occurred in infants 2-5 months old and nearly one third of these were infected by RSV. Among those infected with PIV type 3 and RSV, nearly half were 2-11 months old (48.8% and 46.1%, respectively).

High fever (axillary temperature >38.5° C) was more common in those infected with influenza A compared to those who were not (OR 2.79; CI 1.89, 4.11). This tendency, albeit less pronounced, was also seen for Influenza B (1.45; CI 0.80, 2.62). A similar association between high fever and RSV infection was observed, but only in infants (Table 2 and 3). Being infected with RSV was also associated with LCI (i.e. severe pneumonia), hypoxia ($SpO_2<93\%$), wheezing, and crepitations. The associations between RSV infection and these clinical signs were more pronounced in children below 1 year of age (Table 2-4), but only for crepitations and hypoxia was there a significant age interaction with RSV (Table 2 and 4).

Among the 2,088 non-severe pneumonia cases, children infected with RSV had a higher risk of treatment failure and delayed time to recovery compared to children without RSV. RSV positive cases experienced treatment failure twice as often as the other children and had on average 18% longer duration of illness (Table 5). None of the total 2,219 cases of pneumonia died as a result of their disease.

CRP as exposure variable

We also analyzed the data using high CRP as a proxy for bacterial pneumonia. We used two different cut-offs (>40 mg/L and >80 mg/L) to define high CRP. CRP >40 mg/L was associated with high fever, crepitations, and LCI (Table 3 and 4). We found that the associations were even more pronounced using 80 mg/L as the cut-off. Neither CRP >40 mg/L nor CRP >80 mg/L were significantly associated with SpO_2 <93%. However, using SpO_2 <90% as the cut-off for hypoxia, having CRP >40mg/L and >80 mg/L were both substantially and statistically significantly associated with hypoxia, the OR being 4.0 (CI 2.1, 7.6) and 6.0 (CI 2.4, 15.0), respectively. Similar to what was observed for RSV, the association between CRP and crepitations was stronger for infants than for older children. Wheezing was highly associated with CRP >80 mg/L, but for this outcome we observed a qualitative interaction; wheezing was more common in infants with high CRP than in those with lower CRP (OR 1.8), while the opposite was found in older children (OR 0.50). For the remaining clinical signs and disease outcomes there were no other significant interactions; the lowest *P*-value for interaction with LCI being 0.11 for CRP >80 mg/L in infants (OR 4.3) versus in older children (OR 0.72) (Table 3 and 4).

Among the non-severe pneumonia cases, CRP >40 mg/L was associated with longer episode duration (HR 0.88; CI 0.80, 0.97; *P*=0.009). High CRP was also associated with increased risk of treatment failure. This association was significant both for CRP >40 (OR 1.4; CI 1.1, 1.9; *P*=0.012) and CRP >80 (OR 2.1; CI 2.3, 3.6; *P*=0.005). These associations were not confounded by the presence of virus.

Table 3. Association between RSV and CRP category and clinical signs.

Category		Axillary temperature ≥38.5° C n/N (%) 293/2,218 (13.2)				
		Positive n/N (%)	Negative n/N (%)	Odds ratio[a]	(95% CI)	*P*-value for interaction
RSV	**overall**	**53/334 (15.9)**	**240/1884 (12.7)**	**1.45**	**(1.03, 2.04)**	
	2-11 mo	27/154 (17.5)	75/861 (8.7)	**2.53**	**(1.56, 4.12)**	*P*=0.004
	12-35 mo	26/180 (14.4)	165/1023 (16.1)	0.97	(0.61, 1.54)	
CRP category						
>40 mg/L	**overall**	**73/334 (21.9)**	**220/1880 (11.7)**	**2.23**	**(1.65, 3.02)**	
	2-11 mo	26/137 (19.0)	76/874 (8.7)	2.64	(1.62, 4.31)	*P*=0.40
	12-35 mo	47/197 (23.9)	114/1006 (14.3)	2.02	(1.38, 2.97)	
>80 mg/L	**overall**	**29/79 (36.7)**	**264/2135 (12.4)**	**4.47**	**(2.75, 7.27)**	
	2-11 mo	8/29 (27.6)	94/982 (9.6)	3.62	(1.59, 8.20)	*P*=0.54
	12-35 mo	21/50 (42.0)	170/1153 (14.7)	4.98	(2.69, 9.23)	

Category		Wheeze n/N (%) 994/2,219 (44.8)				
		Positive n/N (%)	Negative n/N (%)	Odds ratio[a]	(95% CI)	*P*-value for interaction
RSV	**overall**	**182/334 (54.5)**	**812/1885 (43.1)**	**1.40**	**(1.09, 1.80)**	
	2-11 mo	96/154 (62.3)	393/862 (45.6)	1.80	(1.26, 2.58)	*P*=0.073
	12-35 mo	86/180 (47.8)	419/1023 (41.0)	1.17	(0.85, 1.62)	
CRP category						
>40 mg/L	**overall**	**143/334 (42.8)**	**850/1881 (45.2)**	**0.89**	**(0.70, 1.13)**	
	2-11 mo	68/137 (49.6)	420/875 (48.0)	1.04	(0.72, 1.50)	*P*=0.289
	12-35 mo	75/197 (38.1)	430/1006 (42.7)	0.80	(0.58, 1.10)	
>80 mg/L	**overall**	**32/79 (40.5)**	**961/2136 (45.0)**	**0.81**	**(0.51, 1.28)**	
	2-11 mo	18/29 (62.1)	470/983 (47.8)	1.77	(0.82, 3.81)	*P*=0.012
	12-35 mo	14/50 (28.0)	491/1153 (42.6)	0.50	(0.26, 0.94)	

Category		Crepitations n/N (%) 651/2219 (29.3)				
		Positive n/N (%)	Negative n/N (%)	Odds ratio[a]	(95% CI)	*P*-value for interaction
RSV	**overall**	**133/334 (39.8)**	**518/1885 (27.5)**	**1.71**	**(1.33, 2.02)**	
	2-11 mo	62/154 (40.3)	194/862 (22.5)	2.28	(1.59, 3.27)	*P*=0.036
	12-35 mo	71/180 (39.4)	324/1023 (31.7)	1.36	(0.97, 1.90)	
CRP category						
>40 mg/L	**overall**	**118/334 (35.3)**	**533/1881 (28.3)**	**1.39**	**(1.08, 1.78)**	
	2-11 mo	53/137 (38.7)	203/875 (23.2)	2.12	(1.46, 3.09)	*P*=0.005
	12-35 mo	65/197 (33.0)	330/1006 (32.8)	1.03	(0.73, 1.44)	
>80 mg/L	**overall**	**35/79 (44.3)**	**616/2136 (28.8)**	**1.97**	**(1.23, 3.16)**	
	2-11 mo	18/29 (62.1)	238/983 (24.2)	5.12	(2.37, 11.07)	*P*=0.002
	12-35 mo	17/50 (34.0)	378/1153 (32.8)	1.10	(0.60, 2.03)	

[a] Adjusted for age, breastfeeding status and treatment group. The estimates for RSV were adjusted for presence of the other six viruses, while viral status (positive or negative) was included in the model for CRP categories. The result of the CRP analysis was missing in four cases.

Table 4. Association between RSV and CRP category and clinical severity signs.

Category		Positive n/N (%)	Negative n/N (%)	Odds ratio[a]	(95% CI)	*P*-value for interaction
		Lower chest indrawing n/N (%) 131/2,219 (5.9)				
RSV	**overall**	**36/334 (10.8)**	**95/1885 (5.0)**	**2.17**	**(1.43, 3.28)**	
	2-11 mo	28/154 (18.2)	67/863 (7.8)	2.42	(1.50, 3.91)	*P*=0.41
	12-35 mo	8/180 (4.4)	28/1022 (2.7)	1.63	(0.72, 3.72)	
CRP category						
>40 mg/L	**overall**	**34/334 (10.2)**	**97/1881 (5.2)**	**2.52**	**(1.65, 3.85)**	
	2-11 mo	25/137 (18.3)	70/875 (8.0)	2.85	(1.73, 4.69)	*P*=0.40
	12-35 mo	9/197 (4.6)	27/1006 (2.7)	1.92	(0.88, 4.20)	
>80 mg/L	**overall**	**9/79 (11.4)**	**122/2136 (5.7)**	**2.68**	**(1.33, 5.42)**	
	2-11 mo	8/29 (27.6)	87/983 (8.9)	4.31	(1.91, 9.72)	*P*=0.11
	12-35 mo	1/50 (2.0)	35/1153 (3.0)	0.72	(0.10, 5.37)	
		Oxygen saturation <93% n/N (%) 670/2,219 (30.2)				
Category		Positive n/N (%)	Negative n/N (%)	Odds ratio[a]	(95% CI)	*P*-value for interaction
RSV	**overall**	**123/334 (36.8)**	**547/1885 (29.0)**	**1.40**	**(1.09, 1.80)**	
	2-11 mo	66/154 (42.9)	242/862 (28.1)	1.89	(1.32, 2.69)	*P*=0.024
	12-35 mo	57/180 (31.7)	305/1023 (29.8)	1.07	(0.76, 1.52)	
CRP category						
>40 mg/L	**overall**	**111/334 (33.2)**	**557/1881 (29.6)**	**1.21**	**(0.94, 1.55)**	
	2-11 mo	48/137 (35.0)	258/875 (29.5)	1.31	(0.90, 1.92)	*P*=0.56
	12-35 mo	63/197 (32.0)	299/1006 (29.7)	1.13	(0.81, 1.58)	
>80 mg/L	**overall**	**29/79 (36.7)**	**639/2136 (29.9)**	**1.38**	**(0.87, 2.21)**	
	2-11 mo	13/29 (44.8)	293/983 (29.8)	1.94	(0.93, 4.07)	*P*=0.26
	12-35 mo	16/50 (32.0)	346/1153 (30.0)	1.12	(0.61, 2.01)	
		Oxygen saturation <90% n/N (%) 42/2,219 (1.9)				
Category		Positive n/N (%)	Negative n/N (%)	Odds ratio[a]	(95% CI)	*P*-value for interaction
RSV	**overall**	**11/334 (3.3)**	**31/1885 (1.6)**	**2.18**	**(1.05, 4.52)**	
	2-11 mo	8/154 (5.2)	20/862 (2.3)	2.33	(1.00, 5.45)	*P*=0.79
	12-35 mo	3/180 (1.7)	11/1023 (1.1)	1.88	(0.49, 7.20)	
CRP category						
>40 mg/L	**overall**	**15/334 (4.5)**	**27/1881 (1.4)**	**3.98**	**(2.08, 7.62)**	
	2-11 mo	10/137 (7.3)	18/875 (2.1)	4.38	(1.97, 9.76)	*P*=0.69
	12-35 mo	5/197 (2.5)	9/1006 (0.9)	3.33	(1.12, 9.89)	
>80 mg/L	**overall**	**6/79 (7.6)**	**36/2136 (1.7)**	**6.04**	**(2.44, 14.96)**	
	2-11 mo	4/29 (13.8)	24/983 (2.4)	7.02	(2.22, 22.13)	*P*=0.69
	12-35 mo	2/50 (4.0)	12/1153 (1.0)	4.69	(1.00, 21.97)	

[a] Adjusted for age, breastfeeding status and treatment group. The estimates for RSV were adjusted for presence of the other six viruses, while viral status (positive or negative) was included in the model for CRP categories. The result of the CRP analysis was missing in four cases.

Table 5. Association between RSV and CRP category and outcomes of non-severe pneumonia episodes.

Category	No. of infections	Treatment failure[a] n/N (%) 466/2,070 (22.5)				
		Positive n/N (%)	Negative n/N (%)	Odds ratio[c]	(95% CI)	*P*-value for interaction
RSV	**overall**	**100/296 (33.8)**	**366/1774 (20.6)**	**2.01**	**(1.52, 2.65)**	
	2-11 mo	50/125 (40.0)	172/788 (21.8)	2.4	(1.62, 3.61)	*P*=0.245
	12-35 mo	50/171 (29.2)	194/986 (19.7)	1.76	(1.21, 2.56)	
CRP category						
>40 mg/L	**overall**	**80/298 (26.9)**	**386/1768 (21.8)**	**1.44**	**(1.08, 1.91)**	
	2-11 mo	31/112 (27.7)	191/797 (24.0)	1.33	(0.85, 2.07)	*P*=0.662
	12-35 mo	49/186 (26.3)	195/971 (20.1)	1.51	(1.04, 2.19)	
>80 mg/L	**overall**	**24/69 (34.8)**	**442/1997 (22.1)**	**2.11**	**(1.25, 3.55)**	
	2-11 mo	8/21 (38.1)	214/888 (24.1)	2.10	(0.86, 5.13)	*P*=0.970
	12-35 mo	16/48 (33.3)	228/1109 (20.6)	2.06	(1.07, 3.97)	

Category	No. of infections	Time to recovery from pneumonia[b] N=2,088				
		Positive Median (IQR)	Negative Median (IQR)	Hazard ratio[c]	(95% CI)	*P*-value for interaction
RSV	**overall**	**3 (2-5)**	**2 (1-4)**	**0.82**	**(0.75, 0.90)**	
	2-11 mo	3 (2-5)	2 (1-4)	0.85	(0.75, 0.96)	*P*=0.412
	12-35 mo	3 (2-4)	2 (1-4)	0.79	(0.69, 0.89)	
CRP category						
>40 mg/L	**overall**	**3 (1-5)**	2 (1-4)	**0.88**	**(0.80, 0.97)**	
	2-11 mo	3 (1-5)	2 (1-4)	0.85	(0.73, 0.99)	*P*=0.578
	12-35 mo	2.5 (1-4)	2 (1-4)	0.89	(0.79, 1.02)	
>80 mg/L	**overall**	**3 (2-5)**	**2 (1-4)**	**0.84**	**(0.70, 1.01)**	
	2-11 mo	3 (2-5)	2 (1-4)	0.91	(0.71, 1.16)	*P*=0.597
	12-35 mo	3 (2-4.5)	2 (1-4)	0.83	(0.66, 1.04)	

[a] Treatment failure defined as change in antibiotic or hospitalization for pneumonia within the first three days.

[b] The data was analyzed using Cox proportional hazards regression models. Recovery day was defined as the first of two consecutive days with a return of respiratory rate to normal range for age.

[c] Adjusted for age, breastfeeding status and treatment group. The estimates for RSV were adjusted for presence of the other six viruses, while viral status (positive or negative) was included in the model for CRP categories. The result of the CRP analysis was missing in four cases.

Matching (paper III)

We approached 1,955 potential control children for the 726 pneumonia cases enrolled in the case-control study. For 60% of the cases, we approached 1-2 potential controls, while for the remaining 40%, three or more children were asked to participate before we could select an eligible control (Figure 4). The most common reasons for not being selected as a control were that the family were travelling or had moved, in addition to not bringing the child to the clinic for evaluation of eligibility despite having agreed to do so (Figure 5). We included 75% of the controls within the first week of having included the corresponding case, 90% within two weeks and 98% within three weeks (Figure 6). For two pairs, the case and control were included 33 and 37 days apart. In 20 cases, no control was obtained within an acceptable time.

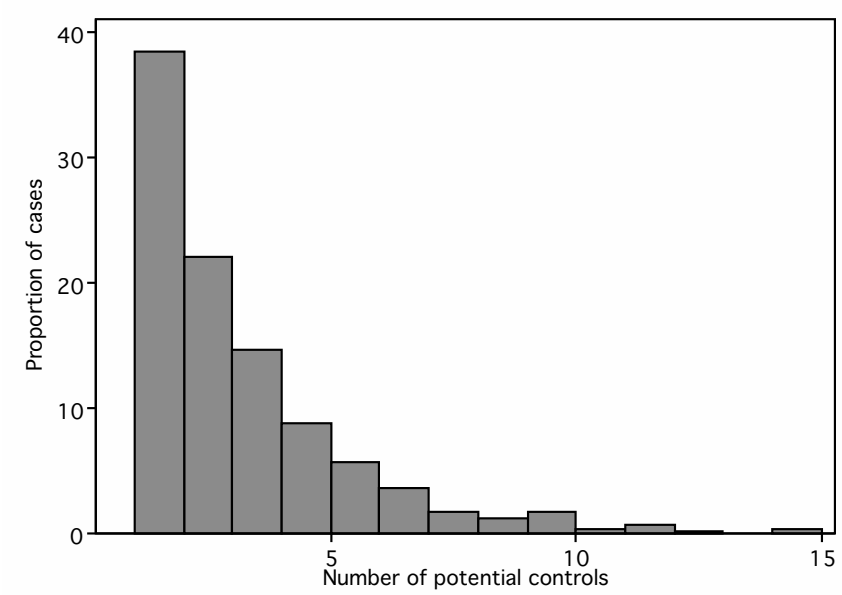

Figure 4. Number of potential controls approached for cases.

The presented results are based on the analyses of 680 matched cases-control pairs (Figure 5). Despite that the age matching was done according to protocol (month and year of birth), the age of the case and the control differed by 2 months in ten pairs. This was a result of age being calculated as age in completed months, not age in days, combined with some delay in

enrolment of the controls as compared to the corresponding cases. We used the age of the case (in completed months) when stratifying the analysis according to age group.

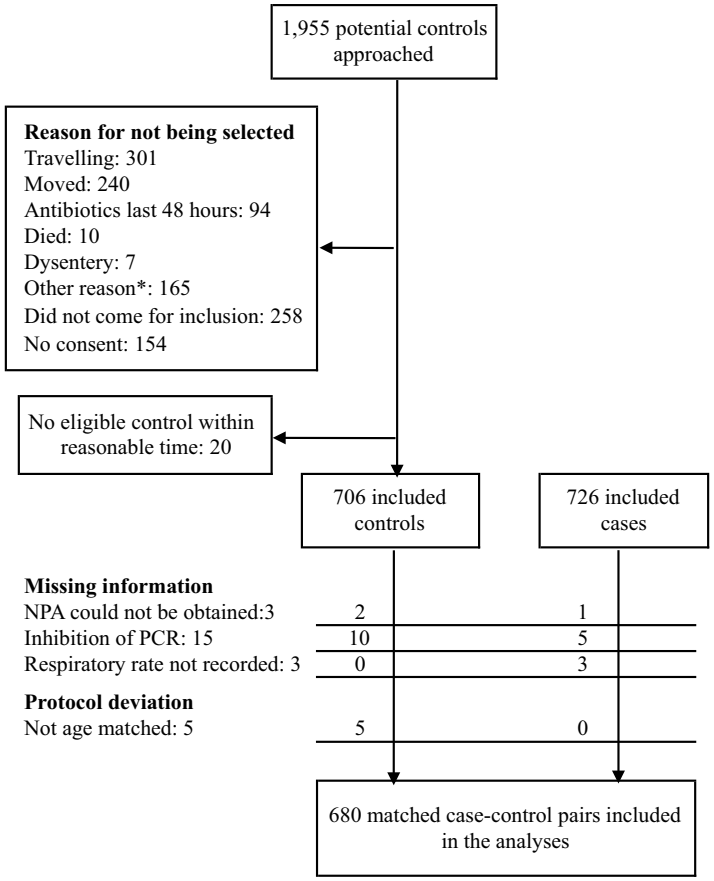

Figure 5. Flow chart of control selection and matching. *Other reasons were mainly previous or current enrolment in one of the studies.

Association between respiratory viral infections and pneumonia (paper III)

We detected at least one virus in 248 (36.5%) of the cases and 48 (7.1%) of the controls. In the conditional logistic regression analyses, we found that all the seven viruses were associated with pneumonia; the matched odds ratios (MORs) for the individual viruses ranging from 2.0 to 13.0. The viruses most strongly associated with pneumonia were PIV type 3, RSV and influenza A, with MORs of 13.0, 10.7 and 6.3, respectively. PIV type 3 and

RSV were the most prevalent viruses in this case-control study, while influenza A was the second most detected virus after RSV in the three-year cross-sectional study. The proportion of the individual viruses in the controls ranged from 0.4% to 1.8%, whereas these proportions were even lower in the controls without any respiratory symptoms and ranged from 0% to 1.1%.

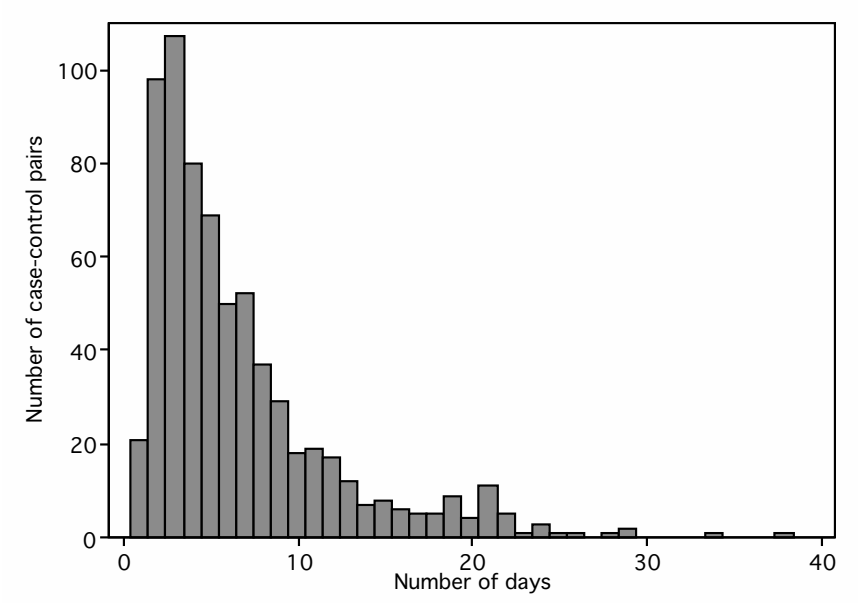

Figure 6. Time lag from inclusion of case to the inclusion of the matched control.

5. Discussion

Frequency of respiratory viral infections

The etiology of viral childhood pneumonias have been studied extensively during the last 3-4 decades and there is a large body of research contributing to this field both in industrialized countries as well as in LMICs. Comparing studies is difficult because pneumonia case definitions, study settings and method of case ascertainment, as well as age group of the study populations differ. Many hospital-based studies have had limited observation time, and some were performed during one or only a few seasons (105, 182-184). Hospital-based studies will tend to include cases with more severe illness compared with longitudinal community-based studies. In addition, various clinical specimens and diagnostic assays with different sensitivity and specificity have been used in order to identify a varying number of viral agents (185-189). Finally, a number of newly identified viruses have recently emerged as pathogens with the potential to cause pneumonia (161, 162, 190-192). This has contributed to great variations in the reported overall frequency of viruses detected in children with pneumonia.

We identified at least one respiratory RNA virus in 40% of the pneumonia cases. Studies of viral etiology of ALRI in tropical and developing countries published up to 1996 identified virus in 9% to 64% of hospital-based studies and 11% to 36% of community-based studies (39). Notably, measles played a major role in some of the studies undertaken up to the mid 1980s. Importantly, studies before 1980 mainly depended on classical diagnostic techniques such as culture and serology for viral identification. Rapid antigen tests, such as immunofluorescence (IF) and enzyme-linked immunosorbent assay (ELISA), were more widely used in later studies, IF often in combination with culture (39). The rapid antigen tests were more sensitive than culture for RSV (193, 194) but less sensitive for some of the other common respiratory viruses, in particular adenovirus (195, 196).

The epidemiological setting of our study could be compared to two rather large studies in Pakistan and Thailand that included both ambulant and admitted under-5s with ALRI, albeit over a period of only 17 and 12 months, respectively. The study in Pakistan detected virus in 37% of cases using viral culture and IF, while the study in Thailand identified virus in 45%

using the same methods in addition to serology (188, 189). In addition to the viruses we detected, these studies also identified adenovirus, and the study in Thailand identified another four additional respiratory viruses. Two recently published community-based studies reported comparable results; a cohort study of children followed till 24 months of age in Bangladesh detected virus in 45% of ALRI cases (197), while a study in India that followed children up till 42 months of age identified virus in 26% of ALRI cases (35). Both studies identified adenovirus in addition to the viruses included in our assay using antigen detection and the latter also performed rapid culture assay for RSV.

There are few comprehensive studies of common respiratory viruses from LMICs that have employed sensitive molecular methods for virus identification (55). A study in Brazil detected a virus in 60% of hospitalized under-5s with radiologically diagnosed pneumonia using PCR for rhinovirus and serology and antigen tests for 7 other common respiratory viruses (187). A study of hospitalized under-5s conducted over 14 months in Vietnam detected viruses using multiplex PCR for 13 organisms in 70% of ALRI cases, notably with a high proportion of rhinovirus (55). A similar 5-year study in Korea, which is a industrialized country in Asia, reported identification of virus in 60% of ALRI cases (191).

Frequency of RSV

RSV has been the predominating virus in the great majority of etiology studies in LMICs (39). We identified RSV in 15.1% (CI 13.6%, 16.6%) of all pneumonia cases (14% [CI 12.8% to 15.8%] of non-severe cases and 27% [CI 20% to 36%] of severe cases) over three years and three RSV epidemics. In comparison, a community-based study that followed 635 Kenyan children from birth until all had experienced three RSV epidemics identified RSV using IF in 13% of those with ALRI, 19% of severe ALRI cases and 5 % of hospitalized ALRI cases (36).

However, the proportion of RSV positive specimens in children with ALRI varies greatly between studies (39). Even studies of under-5s in four LMICs based on a standardized WHO protocol using antigen detection (ELISA) found the proportions of ALRI and severe ALRI with RSV to be 18% to 35% and 7% to 45%, respectively (34). In a comprehensive review, Stensballe and co-workers estimated the proportion of RSV positive tests in children with ALRI at a median of 20% (5^{th} to 95^{th} percentile 1 to 53) in LMICs and 25% (5^{th} to 95^{th} percentile 1 to 75) in industrialized countries (37). They reported the proportion of RSV

positive samples in studies performed before 1979 and after 1980 at a median of 17% and 23%, respectively, and in field studies and hospital-based studies at a median of 15% and 21%, respectively. From 1980 onwards, rapid antigen tests that were more sensitive for RSV became available. In the PCR era, the data on the proportion of RSV positive tests in children with ALRI in LMICs derive mostly from hospital-based studies. Two studies in India and Vietnam employing multiplex PCR identified RSV in 20% of 301 and 22% of 557 under-5s with ALRI, respectively (54, 55), the former study spanning 2 years and the latter 14 months. These results are similar to findings in under-5s in industrialized countries (51, 191).

We detected RSV somewhat less frequently than in the study in India (54) and in Vietnam (55). The reasons for this could be differences in study design and setting, pneumonia definition, validity of the diagnostic assays and age groups. While our study included mainly (94%) non-severe pneumonia visiting a field clinic, the Indian study included almost equal proportions of inpatients and outpatients; the Vietnamese study only inpatients. Both studies included children up to five years of age. Our study encompassed children 2-35 months, and the fact that we did not include cases <2 months of age, who also face a considerable RSV burden (36), could also have contributed to this difference. This could possibly also explain why the proportion of RSV was not higher in infants than in toddlers in our study. Studies of infants typically report higher proportions of RSV compared to older children (34).

For five days during the RSV epidemic that peaked in September 2004 we were unable to collect NPA from 25 pneumonia cases due to lack of collection equipment. These cases were consequently not included in the virus study, but because of their modest number they could only have had a slight impact on the final proportion of RSV in the study. Even in the extreme situation of all of these cases having RSV infection, including them would have increased our proportion with only 1 percentage point.

Frequency of influenza, parainfluenza and human metapneumovirus

In the current study, influenza A (7.4%) was second in frequency after RSV followed by PIV type 3 (5.8%), PIV type 1 (4.4%), hMPV (4.2%) and influenza B (3.8%). The longitudinal study of children followed from birth until 42 months in rural India detected influenza A in 6.6% of ALRI cases and PIV type 3 in 9% using IF and rapid culture (35) (and personal communication Shobha Broor, AIIMS, New Delhi). Comparing our results

with those of the two hospital-based studies using multiplex PCR in Vietnam (55) and India (54) mentioned above, we identified influenza A less frequently than in Vietnam (16.7%) and more frequently than in India (3%), while both studies identified none or very few cases of influenza B. Proportions of samples positive for PIV type 3 and hMPV were very similar to our findings. The proportion of hMPV positive specimens in the current study is also comparable with the results from studies of hospitalized children <6 years of age in Hong Kong (hMPV positive 5.5%) (49) and <3 years of age in Mexico (hMPV positive 6.1%) (56); both studies lasted more than one full year. In contrast, a three-year study in South Africa detected hMPV in as much as 11% of HIV-uninfected children <4 years old hospitalized for ALRI (48). No community-based studies of hMPV pneumonia in LMICs covering at least one year could be found for comparison with our data. A community study of febrile children <13 years of age detected hMPV by serologic testing in 8 of the 20 pneumonia cases identified over a year (58).

Undiagnosed infections

We did not identify any virus in 60% of the pneumonia cases included in the three-year study (paper I). The proportion of cases that tested positive for virus varied greatly from month to month, ranging from 2.3% (November 2006) to 84.5% (September 2004). Our ability to detect a viral pathogen was highest when there was most pneumonia, which could indicate that the pneumonia epidemics we observed to some extent were driven by viral infections. We could have failed to identify some cases who were actually infected with the viruses included in our PCR assay. However, studies identifying a wider array of viral agents than the 7 seven targeted by our Hexaplex Plus assay, identified virus in up to 70% of pneumonia cases (55). Thus, viruses not included in our assay, as well as bacteria, will have caused many of the undiagnosed infections. In fact, bacteria could also be present in the cases were we did detect a virus. Viral-bacterial mixed infections are known to be common in childhood pneumonia (185). Numerous clinical and experimental studies indicate that infections with respiratory viruses, in particular influenza, predispose individuals to bacterial superinfection (198). Vaccine studies have shown that influenza vaccination reduces the incidence of bacterial respiratory infections (198) and immunization with

a 9-valent pneumococcal conjugate vaccine prevented pneumonia hospitalizations associated with respiratory virus in children (199).

Seasonality of respiratory viral infections

RSV

Our study spanned over three years, which, despite being of longer duration than most other studies, is a short time period to draw any conclusions regarding seasonality patterns of the different viral infections. However, we observed three annual outbreaks of RSV infections. In 2004 and 2006, the RSV epidemics peaked in September, i.e. in relation to the rainy season, while in 2005 the epidemic peaked in December, and this peak was smaller compared to the two other years. Interestingly, RSV activity was completely absent for 3-6 months between epidemics. Similarly, in Dhaka epidemic peaks of RSV infection were seen both in relation to the monsoon and the cold season, but in contrast to our findings, RSV activity was evident almost throughout the year (200). RSV epidemics in relation to the wet season have previously been reported from other locations on the Indian subcontinent (201, 202). There are also descriptions of outbreaks during cold but also rainy winter months in Pakistan (188). Similar associations with rainfall are reported for RSV in a number of tropical locations (203).

Association with meteorological parameters

RSV activity in a community is influenced by transmission conditions, which may be influenced by changes in climate (204). Investigating possible correlation between monthly number of RSV cases and meteorological variables, we found that RSV infections were positively associated with relative humidity, a finding that also has been reported from tropical climates (205, 206). This is in contrast to most temperate locations where RSV infections are inversely correlated with temperature and not with humidity. One study in temperate Argentina reported RSV frequency to correlate positively with relative humidity, like ours, and inversely with temperature (207). Even though two of the three observed RSV epidemics in the current study occurred in relation to the rainy season, we found no correlation between monthly frequency of RSV and monthly rainfall. By introducing a two-month lag after peak precipitation we did identify such an association (data not shown),

which is, however, likely to be influenced by other unknown factors. Due to the epidemic nature of the viral infections under study, observations based on monthly number of episodes are correlated. Therefore, the precision of the observed correlations is overestimated. Further studies are required to examine the degree to which the observed associations between viral infections and meteorological factors are actually caused by clustering of individual infections over limited time periods.

Climatic parameters are likely to be interrelated, which may obscure conclusions drawn from the univariable analyses employed by us. Moreover, RSV activity has been shown to relate to temperature in a bimodal fashion (204), i.e. the number of RSV cases increased when the mean temperature was in the range of 2-6°C and then again when the temperature was above 24-30°C. We observed that rainfall and temperature were strongly correlated, while relative humidity was not correlated with any of the two other variables (data not shown). Under controlled experimental conditions, both temperature and humidity have an impact on survival of RSV in aerosols (204), but if this is important in the transmission between humans is not known. Moreover, seasonal changes in social behavior may modify associations with climatic factors. Indoor crowding, which occurs during cold months in temperate climates and during the rainy season in tropical climates, is a known risk factor for RSV infection (85). The relationship between climate and virus activity may be subject to a number of other unknown confounding factors.

Interference

Interference of other viruses, i.e. that major respiratory viruses (and possibly other common epidemic viruses) do not reach their epidemic peak at the same time (208, 209), could be another possible determinant for timing and magnitude of an outbreak with RSV. A recent example of such possible interference between respiratory viruses was observed in Scandinavia during the autumn of 2009 when rhinovirus seemed to interrupt the spread of swine flu and delayed the outbreak until later in the winter (210). In fact, the pneumonia outbreak during winter 2005/2006 in the current study was compiled by RSV and influenza (both type A and B). During influenza infection, infected cells produce interferon and other cytokines, which causes the cells to enter an antiviral state (210). Thus, spread of influenza in the community may have limited the spread of other viruses, in this case RSV.

Biennial epidemic pattern

Although the most typical pattern of RSV infections in temperate locations is regular annual epidemics during the cold season, there are also reports of alternating cycles of large and small annual epidemics with different timing of the peak activity in several European countries (59, 211-213). The reasons for such dual rhythm of biennially alternating large winter peaks and smaller spring peaks are unknown, but climatic factors, alternating dominating subtypes and interference of other viruses have been suggested (59, 212, 213). We have too short observation time to determine if the observed RSV epidemics fit into a similar biennial pattern.

Parainfluenza virus

PIV type 1 activity was endemic with no major peaks throughout the study period, which deviates from the pattern of biennial autumn epidemics frequently described elsewhere (66, 71). We observed only a single, not yearly, outbreak of PIV type 3, but the timing/season of the epidemic was in line with what has been reported in temperate (66) and subtropical regions (72). The PIV type 3 epidemic occurred in spring 2006 after the termination of the influenza epidemic, a phenomenon also observed over 6 consecutive years in the US (97). We also saw clusters of PIV type 3 infection at the same time of the year during the previous and the following year that did not reach epidemic proportions. PIV type 3 predominated during the wet and warm summer months in the current study. In tropical locations, such as Singapore and Papua New Guinea, PIV type 3 infections have also been described as endemic with annual epidemics occurring during the first half of the year and occasionally in autumn (71, 214).

Influenza virus

We found that peaks of influenza infections were greatest during winter months with overlapping activity of type A and B. We detected influenza in 25 of the 36 months of the study, and our findings are in line with those from tropical and sub-tropical locations where influenza is diagnosed greater parts of the year (215, 216). There are several reports from tropical areas of influenza epidemics occurring during the rainy season (203). We, however, only observed moderate activity during the months of rain. A summer outbreak of influenza

has been described in the plains of southern Nepal (217), where the climate is different from that in the hill region.

Human metapneumovirus

The single hMPV winter epidemic seen in the current study peaked in January 2005 after a major RSV epidemic. A winter outbreak of hMPV is consistent with observations in Europe (44) and South Africa (48). In India (54) and Bangladesh (58) hMPV peaks occurred in March and April, which is the dry but warm pre-monsoon season. The incidence of hMPV has previously been reported to vary substantially from year to year (62). There is a lack of studies with surveillance of several years' duration in LMICs. Studies with longer observation time, primarily in Europe, have reported a biennial pattern of alternating early and late occurrence of epidemics (59, 63, 64, 218); similar to what has been reported for RSV. Moreover, in these studies hMPV epidemics occurred anti-cyclical to RSV, i.e. small hMPV epidemics occurred in years with large RSV epidemics, and *vice versa*, an observation that supports the concept of interference by competing pathogens.

Association between etiology and clinical signs and outcomes of infection

Children with RSV infection were twice as likely to present with severe pneumonia (i.e. LCI) or SpO_2 <90% compared to the other children. Apart from influenza B that also was associated with LCI, none of the other viruses were associated with any of these severity signs. It is known that RSV involves the lower airways more frequently than other common respiratory viruses (67). Among children with RSV infection, we also noted that several clinical signs were more common or only observed in infants.

Similar to RSV, high CRP (used as an indirect marker of suspected bacterial pneumonia) was associated with LCI, crepitations), high fever and SpO_2 <90%. The associations were most pronounced using a cut-off for CRP of 80 mg/L and for infants, but the age interaction for these clinical signs was only significant for crepitations and high fever. These findings are plausible, as severe presentation of pneumonia is more common in infancy. Although CRP has generally low sensitivity and specificity in differentiating between bacterial and

viral pneumonia, a CRP concentration >80 mg/L has been shown to predict bacterial pneumonia well (specificity 0.90) in children <2 years of age (133).

Among the 2,088 non-severe cases, longer duration of illness and increased risk of treatment failure was observed for cases with RSV and for cases with high CRP. A high CRP would usually indicate a more severe infection that could take longer than three days to resolve despite effective antibiotic treatment. Moreover, antimicrobial resistance to cotrimoxazole (the first line antibiotic used) is common in Nepal (138). The antibiotic treatment would have no impact on a viral infection unless there is a concomitant bacterial infection. The observation that RSV was associated with treatment failure probably reflects that RSV infections took longer to resolve than the other infections, which may have tended to resolve spontaneously within 3 days of treatment.

Although the assumed bacterial pneumonia cases more frequently presented with a higher degree of hypoxia (SpO_2<90%) compared with the RSV cases, our findings suggest that the presentation and outcome of RSV infections was on the whole similar to that of assumed bacterial infection, underscoring the paramount position of RSV among the respiratory pathogens. Although we cannot rule out the possibility of a concomitant bacterial infection, adjusting for high CRP in the analyses did not change the estimates for RSV or the other viruses (data not shown). Most previous studies demonstrating the severity of RSV infections with regard to clinical presentation and outcome are from hospitalized children (83, 186, 219). The current findings indicate that a sizable proportion of children with RSV infections exhibit signs of severe illness also in a community setting with predominantly ambulant patients. In the United States, the outpatient load of RSV infection is 10-25 times higher than the inpatient load for children <5 years (29). If this is the case also in Nepal and other LMICs, the burden of ALRI caused by RSV is high.

Association between respiratory viral infections and pneumonia

PIV type 3, RSV, Influenza A, PIV type 1, hMPV and Influenza B were all significantly associated with pneumonia, with matched odds ratios ranging from 13.0 to 2.2. We detected PIV type 3, RSV, and Influenza A in 14%, 10% and 3% of cases, while the corresponding proportions in the controls were 1.8%, 1.2% and 0.6%, respectively. According to a recent

review, RSV positive specimens have been identified using PCR in 0% to 5% of asymptomatic children (159). For other common viruses such as PIV, influenza and hMPV, proportions have ranged from 0% to 2% (51, 159). Similar results have been reported from previous studies in LMICs using culture, IF or serology (10, 214). These viruses are not frequently present in control children, which is an indicator of their causal role in pneumonia when detected from the upper airways during illness. PIV type 2 had a matched odds ratio of 2.0, and was the most infrequent of the viruses with only 9 infections during the entire case-control study. Accordingly, the corresponding precision was lower than for the other viruses; its 95% confidence interval spanning 0.5 to 8.0. Our findings are in line with those reported for Australian children during the first year of life (220). We were not able to identify reports on such associations from other LMICs.

The viruses most strongly associated with pneumonia in the case-control study (paper III) were also the most prevalent viruses detected in pneumonia cases in this community during the three-year cross-sectional study (paper I). Because the studies were clinic-based, these two observations will necessarily be closely related. It is likely that the most pathogenic viruses give disease that prompts visits to a health facility. A cohort study with frequent sampling of children in the homes may have given somewhat different estimates for the viruses. However, our estimates are consistent with those obtained in such a longitudinal study (220).

We identified at least one virus in 7.1% of controls. As for cases, the overall proportion of viruses detected in controls will depend upon the number and kind of agents tested for. Viral pathogens or atypical bacteria have been found in up to 68% of control subjects less than 5 years in Netherlands, but the study employed a broader diagnostic panel than ours and the major agents detected (for all age groups) were rhinovirus, coronavirus and *Chlamydophila* species (160). The availability of PCR for rhinovirus has increased the proportion of positive findings in asymptomatic subjects (159). In the Netherlands, rhinovirus has been detected in 22% of children aged up to 7 years by biweekly sampling (221) and 20% of asymptomatic children aged up to two years (222). Studies on some newly identified viruses such as hBoV and hMPV are mainly based on PCR detection. In Israeli children, hMPV was identified in 2.2% of season-matched controls (51), while hBoV was detected in 8.6% of asymptomatic infants in a Danish birth cohort followed for 1 year with monthly sampling (223), but none of 68 healthy children <5 years of age in France (224, 225). Studies including PCR assays

for rhinovirus and hBoV tend to report higher proportions of overall virus positive specimens in sick children (55, 161, 162, 185, 191, 221), but these viruses are also among the viruses detected as frequently in asymptomatic controls as in cases (221, 223). Quantization of viral load has been suggested in the assessment of their role in lower respiratory tract infection (161, 226, 227). There are no reports so far from LMICs on frequency of detection in controls using PCR for these viruses.

We found a higher association between PIV type 3 and pneumonia for children six months and older compared with 2-5 months old children. A similar, albeit statistically non-significant, age-dependent difference was also found for RSV. The explanation for this difference between infants and older children is uncertain, but could partly be due to maternal antibodies protecting the infants during the first 6 months of life (228-230).

Limitations of the study

As we did not know the exact number of children in the target age group in this population, we were not able to estimate true incidence rates of pneumonia and consequently not disease burden in the community. Our clinic was not the sole health facility in Bhaktapur, and we conducted an active monthly surveillance of an open cohort of children below three years of age and have reasons to believe that the majority of pneumonia cases were brought to our clinic. The proportion of severe cases (5.9%, CI 5% to 7%) was only slightly lower than the estimated average of 8.7% (IQR 7% to 13%) (3). Some children with severe illness were probably transported directly to a tertiary level hospital in the capital and thereby bypassed our clinic. Moreover, we excluded cases of pneumonia that had received antibiotics within the last 48 hours prior to inclusion. This would lead to a bias toward inclusion of milder cases. In fact, among those that met our inclusion criteria we found that cases with severe pneumonia were more likely to have received antibiotic treatment in the previous 48 hours compared with non-severe cases (19% vs 8%, respectively), which would result in underestimation of viruses that cause severe pneumonia.

Although fieldworkers referred children with respiratory symptoms, the inclusion of cases for the studies relied on parents actually bringing their child to the project clinic. The baseline census and the subsequent active surveillance of children below three years of age in all the neighborhoods of Bhaktapur municipality made the project well known and the

free treatment offered by our study clinic for common childhood illnesses encouraged parents to use this facility. One could argue that this could motivate the poorer part of the population to utilize our services and bias the study population towards a lower socio-economic level. However, the socio-economic features of the study population did not differ from those of the surveilled population (data not shown). We therefore have no reason to believe that there was any substantial selection bias in the inclusion of cases for the studies. The controls in paper III were randomly selected from a list of children under surveillance. Children from migrant families could in theory be underrepresented on this list and any difference in socioeconomic status between migrant families and the indigenous population could have introduced a bias in our pathogenicity odds ratio estimates. We observed a difference in anthropometric measures between the cases and the controls, as well as in the proportion of children who were delivered in hospital. Adjusting for these factors in the unconditional logistic regression analyses did not, however, substantially alter the estimates (data not shown), making such selection an unlikely source of bias.

The political instability during the democracy movement in the spring of 2006 led to several days of nationwide general strike in the beginning of April. Due to the demonstrations in Kathmandu the government imposed daytime curfew, which failed to curb the protests. In the following days crowds of several hundred thousands participated in the demonstrations against King Gyanendra and his government and violent clashes with armed police took place in the streets. This culminated on April 21, when the king announced that he would step down from power and called for general elections. Bhaktapur was less affected by the emergency measures and our field activities were not interrupted despite that public transportation in the valley came to a halt during these weeks. Our field clinic was within walking distance from most parts of the municipality and disruption of public transport should not have had a major impact on people's ability to access the clinic. Yet the general political situation and emergency measures in April 2006 may have impeded parents from seeking medical treatment for their sick children. Notably, the inclusion of cases during April that year was the lowest during the whole study period and approximately half of the inclusions for April the other two years. If this can be attributed to the political situation or to a period of genuinely low pneumonia incidence is uncertain.

The aim of the WHO pneumonia definition has been to ensure high sensitivity and simultaneously attempting to maintain adequate specificity in order to avoid failing to treat

bacterial pneumonia and minimize the number of children with non-bacterial pneumonia receiving unnecessary antibiotics. Prior to revision in 2008 (176), the WHO ARI algorithm detected about 80% of the children that required antibiotic treatment (231, 232). However, 20-30% of children who met the criteria were unnecessarily treated with antibiotics, and many of these children presented with wheeze (232). Most children with non-recurrent wheeze are likely to have a viral infection and hence will not benefit from the use of antibiotics (232). Thus, to improve the specificity of the criteria, revised guidelines recommended a trial of rapid acting bronchodilator in children with wheeze and fast breathing and/or lower chest indrawing before being classified as pneumonia (176). In the current study, we treated all wheezing children with salbutamol, which is according to the revised 2008 guidelines, and excluded the child if he or she no longer fulfilled the criteria for pneumonia at reassessment. However, despite of the relatively low specificity of the WHO definition of pneumonia and that we allowed controls to have respiratory symptoms, we did demonstrate a very strong association between the presence of virus in NPA and WHO defined pneumonia. Any lack of specificity in diagnostic criteria for measuring study outcomes tends to bias the odds ratio towards 1 (233), thus, our estimates of association are rather under- than overestimated.

We used a commercially available multiplex PCR kit for our analyses undertaken in Nepal. This assay detects the most common viruses causing pneumonia in children and has proven highly sensitive and specific for this purpose (166) compared to conventional methods (157, 158). Drawbacks of the assay are that it is both costly and relatively labor intensive. We estimated the reagent cost per sample to approximately 50 USD and this makes it not feasible for routine diagnostics in a low-income country. Development of new or establishment of existing in-house PCR assays could increase sustainability in a LMIC setting, if adequate training and infrastructure is ensured. Efforts in laboratory diagnostics of respiratory viruses should perhaps focus on epidemiologic surveillance rather than clinical routine analysis, as a positive PCR test has limited implications for the individual patient, i.e. it does not rule out the presence of bacterial agents.

Sensitivity of PCR analyses

Even though we primarily included new cases of pneumonia and most cases presented early in the course of illness (95% within 7 days of illness), some specimen collected from cases

will not contain detectable viral RNA. This could be due to timing of specimen collection in relation to onset of symptoms, inadequate collection procedures, or loss or degradation of RNA during transport, processing or storage. Using a multiplex PCR implies some loss in sensitivity compared to PCR assays for single agents, but an advantage is the possibility of co-detections in a single specimen. However, the detection of more than one virus was relatively low, at 3.3%, in our study.

Three-hundred-and-twenty four of the 2,219 NPAs were stored for up to 16 months at 2-8°C. The samples refrigerated beyond three months that yielded a negative result (n=133) were reanalyzed in the laboratory at the University Hospital of North Norway in Tromsø using the aliquot that was frozen at -70°C immediately after processing in Nepal, while positive samples (n=191) were not. Thus, we were probably unable to identify some co-detections among the positive samples that were not reanalyzed. Assuming a proportion of viral co-infections similar to what we observed in the rest of the study, the estimated co-detection would have been 4.1% instead of the currently reported 3.3%. Moreover, the comparative study described under the "Methods" section showed that refrigeration at 2-8°C for up to three months resulted in a 7% loss in sensitivity compared to storage at -70°C. However, this concerned approximately 10% (243/2,219) of our samples only, but indicates that the proportion of children that tested positive for any virus could be slightly underestimated in the current study.

Other pathogens

The detection of other respiratory pathogens, notably *S. pneumonia*, *H. influenzae* and *S. aureus*, would also have been of great interest, but was not feasible within our project setting. In particular in the case-control study, detection of rhinovirus, adenovirus, hBoV, coronavirus, and enterovirus, viruses that are also frequently identified in healthy children (159), would have enabled us to estimate individual pathogenicity odds ratios for each virus and thereby better define their role in childhood pneumonia.

6. Conclusions

The studies presented in this thesis show the importance of RSV, PIV type 3, and influenza virus in childhood pneumonia in this Nepalese community. The cross-sectional study contributed information on seasonal patterns and clinical features of the different viral infections. The observed pneumonia epidemics were to a considerable extent driven by viral epidemics. RSV was found to be the most common among the seven viruses identified over the period of three years. Annual epidemics with RSV occurred in relation to the rainy season or during the cold months. The newly identified virus, hMPV, was shown to circulate in the community and an outbreak with hMPV occurred one of the winter seasons. RSV infection was associated with the most severe clinical presentation and outcome among all the viral infections we identified. The high pathogenicity estimates for the commonly occurring PIV type 3, RSV and influenza viruses make them important targets for preventive measures, such as vaccination.

7. Research challenges

The important role of RSV in community-acquired pneumonia both with regards to frequency and severity in young children underscore the need for continued effort to develop of a safe and effective RSV vaccine, as this could substantially reduce the burden of pneumonia in children of LMICs.

Population-based studies should be undertaken in order to define the proportion of pneumonia cases attributable to each virus and to estimate the disease burden of the most important viral agents.

Efforts should be made to gain better regional data on the viral and bacterial causes of childhood pneumonia. New viral respiratory pathogens have emerged and their exact causal role in pneumonia needs further investigation.

Local epidemiologic surveillance of respiratory viruses causing pneumonia in children should be undertaken to enable prediction of outbreaks and for planning of preventive and therapeutic control measures.

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