ACUTE VIRUS INFECTIONS IN PAEDIATRIC PATIENTS

ADMITTED TO INTENSIVE CARE

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Fleur M. Moesker

Colofon

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Acute virus infecties bij kinderen opgenomen op intensive care

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Voor mijn leergierige en altijd lezende Oma Iet †

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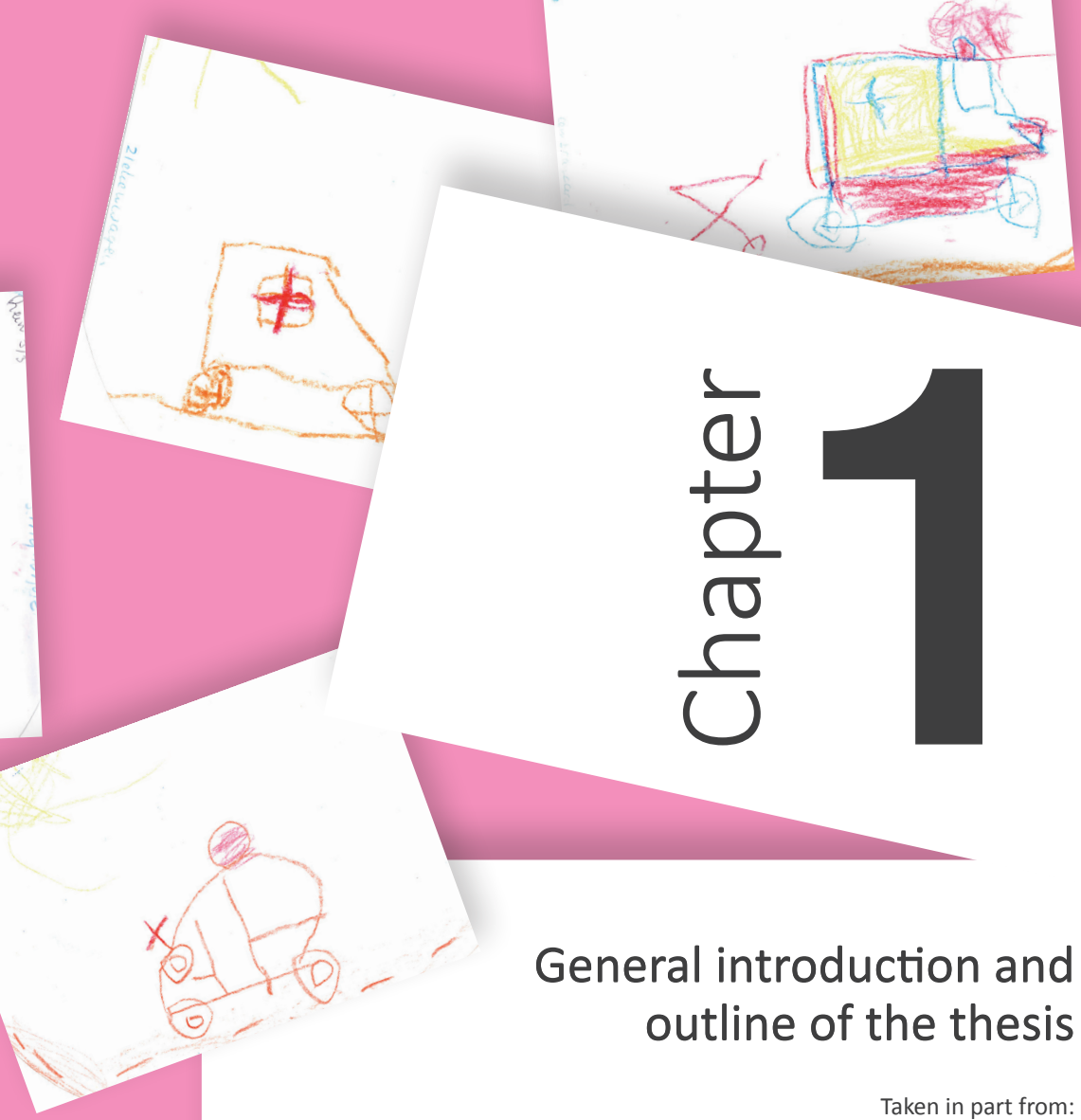

Chapter 1

General introduction and outline of the thesis

Taken in part from:

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Fleur M. Moesker, Pieter L.A. Fraaij, Albert D.M.E. Osterhaus
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Fleur M. Moesker, Pieter L.A. Fraaij, Albert D.M.E. Osterhaus
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Paediatric infectious respiratory diseases

Infectious diseases still form a significant burden of disease in children. In 2013, WHO reported that approximately 50% of deaths of children under the age of five were due to infectious causes (1). Especially acute respiratory tract infections (ARTIs) are a frequent cause of morbidity and mortality (2,3). ARTIs can be caused by a multitude of pathogens including bacteria, viruses and fungi. Recently Jain et al. showed in a large study in the USA that in the pneumocci vaccination era viruses are the most frequent cause of radiologically confirmed pneumonia (4). Indeed ARTIs may be caused by well-known seasonal viruses such as respiratory syncytial virus (RSV) and influenza virus, but may also result from infection with a large number of other viruses as well as from infection with emerging and re-emerging viruses. The latter also may be associated with severe disease and therefore remains a significant threat to children (5–7). An important example is avian influenza virus (H5N1) which was initially identified in a 3-year-old boy in Hong Kong who was admitted to hospital and subsequently died from a combination of influenza pneumonia, acute respiratory distress syndrome, multi-organ failure, and disseminated intravascular coagulation (8). H5N1 has since continued to be a serious cause of concern for adults and children resulting in 850 cases of which 449 patients died so far (53%) (9).

Another major threat, which has dramatically increased over the past decades, is the re-emergence of dengue virus (DENV) infection. A recent study estimated that 390 million dengue virus infections per year in adults and children are likely to occur (10). An estimated 500 000 patients with severe dengue infection require hospitalization, many of which are children (11). These examples illustrate that infectious diseases are definitely not a phenomenon of the past and will continue to pose a threat to human and in particular also child health. The focus of this thesis is on the impact and management of paediatric respiratory virus infections and of newly discovered, emerging, and re-emerging viruses in children.

Acute respiratory tract infections (ARTIs) in children

WHO reports that children under the age of five can suffer from up to six viral ARTIs each year (12–14). For children less than one year of age, ARTIs are the most common reason for emergency department visits because of breathing difficulties (15–18). Based on their clinical presentation ARTIs are differentiated into upper respiratory tract infections (URTI) and lower respiratory tract infections (LRTI). This division is arbitrarily chosen as being above and under the laryngo-tracheal transition in the human respiratory tract (**Figure 1**). URTI include: common cold, acute otitis media, viral pharyngitis, and viral laryngotracheitis. LRTI include: acute bronchitis, bronchiolitis, viral pneumonia, and acute respiratory distress syndrome, all of which can progress into a severe acute respiratory tract infection (SARI) or are referred to as such.

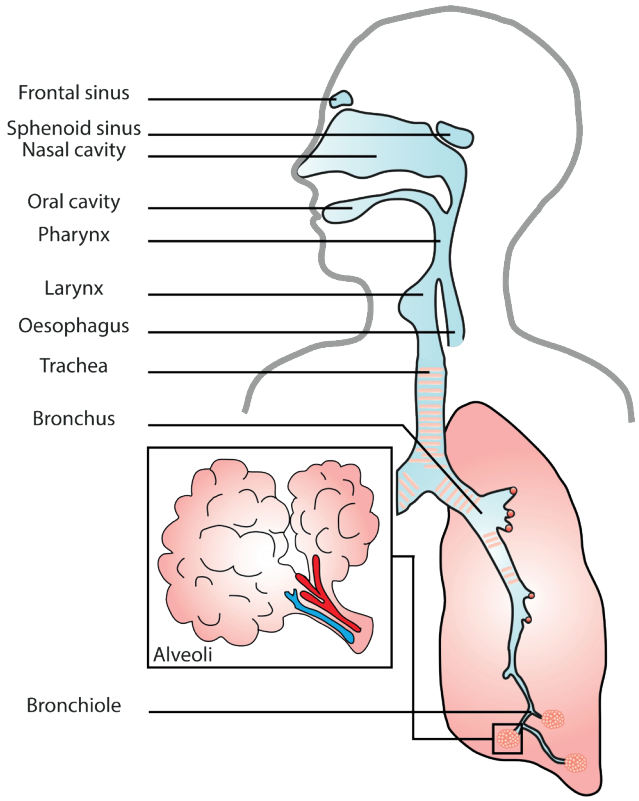

Figure 1. Anatomy respiratory tract (courtesy of J.Y. Siegers).

Causative viral agents of ARTIs

Respiratory viruses are transmitted through aerosols, respiratory droplets, via indirect contact with infected secretions, or through contact with contaminated environmental surfaces (21). Most common causative agents for respiratory tract infections in children are: RSV, human metapneumovirus (HMPV), influenza viruses, human rhinovirus (HRV), human bocavirus (HBoV) and parainfluenza viruses type 1-4 (22–24). Other viruses associated with ARTI include adenovirus (ADV), and human coronaviruses OC43 (HCoV-OC43), NL63 (HCoV-NL63) and 229E (HCoV-229E) (25) (**Figure 2**). Some of these viruses were only discovered in the past two decades, such as HMPV in 2001 and HBoV in 2005 (14,26). Viruses that are difficult to isolate *in vitro* such as HBoV, have been discovered by recently developed virus discovery technologies, such as random amplification and next-generation sequencing (NGS). With the advent of these new molecular methods an ever increasing number of new animal and human viruses, including respiratory viruses has been discovered (for review see (27–34)).

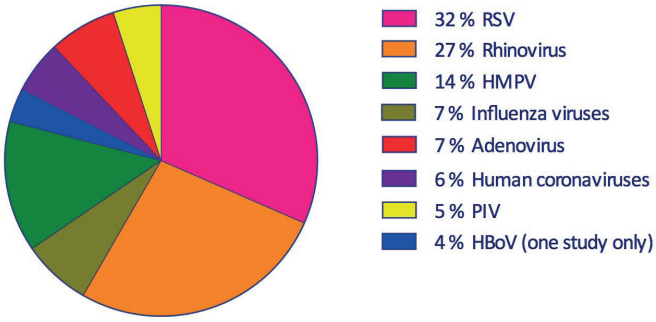

Figure 2. Commonly detected respiratory viruses in children adapted from Jain et al. NEJM 2015 (4), Self et al. JID 2015 (19), Esposito et al. Influenza and other respiratory viruses 2013 (20).

The epidemiology of respiratory viruses varies considerably. In temperate climate zones, RSV infections and influenza are known to cause seasonal outbreaks that may start from October onward until March at the northern hemisphere, and in our summer months at the southern hemisphere (7,35). These infections affect individuals of all ages, but the major disease burden is found in high-risk individuals and those at the extremes of the age spectrum (18,35,36). The seasonality of the other respiratory viruses has been studied to a lesser extent, although these also have defined incidence patterns with usually more infections during fall, winter, or spring (37).

Underlying disorders and risk factors for ARTI in children

The respiratory disease burden of viral infections in children with underlying high-risk conditions is high (2,4). In addition to risk factors such as neuromuscular disorders, immune deficiencies, and cardiopulmonary disease several factors have been identified to contribute to the burden of disease caused by respiratory viruses in children. Underlying medical conditions such as pre-term birth (children born before 37 weeks of gestation), asthma/reactive airway disease, lung abnormalities such as congenital hernia diaphragmatica, congenital heart disease, neurological disorders, skeletal malformations (scoliosis), and chromosomal disorders (including Down syndrome), have been documented to lead to an increased risk for hospitalization due to ARTI (38,39).

Diagnostics of seasonal virus infections

Accurate aetiological diagnosis of respiratory virus infections may be important in the hospital setting as for some of these viruses specific interventions are available. It can also help determine whether a viral or bacterial agent is the cause of disease, which may influence decisions about clinical management, like starting specific, symptomatic or antibiotic treatment, and patient or hospital management, like patient isolation and

tailored hygienic measures. Aetiological diagnosis alone on the basis of clinical symptoms and signs which often include rhinorrhea, nasal congestion, fever, and coughing at different levels of severity, is virtually impossible due to their limited specificity for the individual virus infections. Therefore laboratory based diagnostic assays are needed to confirm suspicions on clinical and epidemiological grounds and to come to a final aetiologal diagnosis (40,41).

Over the last decades new virus diagnostic methods have been developed using real-time reverse transcriptase polymerase chain reactions (RT-PCR), which have gradually replaced more classical and laborious techniques to have become the current gold standard for virus detection in patients including children with ARTI (40). RT-PCR has been shown to have higher sensitivity and specificity in diagnosing viruses than virus isolation, direct immunofluorescence (D-IF), and other antigen detection tests. RT-PCR based virological diagnostics are however not routinely performed in all hospitals, as with the currently available technology, these still require a molecular diagnostic laboratory with specialized personnel and equipment (42). Instead, commercially available rapid antigen detection tests (RADTs) are used in predominantly primary and secondary care settings as these are still easier and cheaper to perform and less time-consuming (43–45). Although molecular and antigen detection methods are rapidly improving and therefore aetiological data become more accessible, clinical presentation and epidemiological data remain a major factor in clinical decision-making.

Intervention strategies: prevention and treatment of seasonal virus infections

Treatment of ARTIs is still primarily and largely based on supportive care, i.e. hydration and oxygenation, as specific antiviral therapy is not yet available for most respiratory viruses. However, prevention and treatment options are available for RSV and influenza viruses. Preventive passive immunization with the RSV specific humanized monoclonal antibody palivizumab (Synagis®) is predominantly used in pre-term born children at risk of developing severe infection and has now been practised successfully for several years (35,46–49). Still, it is important to realize that complete protection is not possible and monthly injection of palivizumab is necessary (35,46,50). Unfortunately, decades of attempts to develop a RSV vaccine have not resulted in a safe and effective vaccine. Problems encountered in the early days of RSV vaccine development in the early 1960s came from studies, in which whole inactivated virus vaccinated children, upon later exposure to the virus, developed more severe (and occasionally even fatal) disease, than placebo vaccinated children (51). Since the underlying mechanisms of this unexpected phenomenon were poorly understood, these findings caused a major setback to the RSV vaccine field. Over the past decade however, at least a dozen promising vaccine candidates and therapeutic agents have been developed, which are currently in different stages of

clinical development (46,52). Their eventual introduction would indeed have the potential to change paediatric clinical practice in the respiratory field considerably (46,52).

For specific treatment of influenza virus infections there are currently two classes of anti-virals commercially available: neuraminidase inhibitors (Oseltamivir, Zanamivir, and in some countries also Peramivir and Laninamivir) and M2-channel blockers (Amantadine and Rimantadine) (53,54). Neuraminidase inhibitors prevent the release of virions from infected (host) cells and can be used both prophylactically and for treatment of acute influenza. It is important that treatment is started as soon as possible, and if possible within 48 hours after onset of disease (55). M2-channel blockers target the M2 protein of influenza A virus. Unfortunately, due to rapid development of viral resistance and often prominent side effects, the clinical use of M2-channel blockers is currently no longer advised (56).

Preventive vaccination offers the most cost-effective and successful protection from seasonal influenza. Based on predicted annual differences in the antigenic make-up of seasonal influenza viruses, vaccine strains represented in the annual vaccination strategies need to be subject to updating every season (57). Current influenza vaccines are developed to induce immune responses against two types of seasonal Influenza A virus (H1N1 and H3N2), and one or two lineages of Influenza B virus (Victoria or Yamagata) (58).

For other respiratory viruses no vaccines or specific treatment are available. However, promising developments have recently taken place. For instance a cross-neutralizing antibody showed protection against several human and animal paramyxoviruses (RSV, HMPV, pneumonia virus of mice and bovine RSV) which may form the basis for prophylaxis of human RSV and HMPV infections (59). This monoclonal antibody also identified a new candidate antigenic site that could be included in a vaccine (pre F-fusion protein). In addition, ongoing studies testing HMPV candidate vaccines recently revealed a promising strategy towards a live-attenuated vaccine (59,60). Overall, apart from symptomatic treatment based on supportive care, there are still limited treatment options for respiratory virus infections in children (47).

Emerging and re-emerging virus infections

Although in the seventies of the last century it was assumed by several policymakers and scientists, that most bacterial and viral infections were under control in the industrialized world with the use of vaccines, antibiotics and antivirals, the unprecedented pace of emergence of previously unknown or re-emerging virus infections in the past decades proved otherwise (61). Emerging and re-emerging infectious diseases are caused by newly discovered pathogens and by pathogens that have recently become more

widespread. Most of these infections are of viral and eventually all of zoonotic origin, like influenza viruses, severe acute respiratory syndrome coronavirus (SARS-CoV), Middle-East respiratory syndrome coronavirus (MERS-CoV), Ebola virus, Dengue virus and Zika virus (62–71). Taking advantage of the complex relationships between human and animal species, also known as the human-animal interface, these viruses manage to cross-species barriers (61,62,70,71). People become infected via direct contact with infected animals or via other intermediate hosts such as birds, rodents, bats or arthropods (61,62,70,71).

Previously unknown or believed to be contained viruses have caused new outbreaks. For example, the severe disease caused by SARS-CoV, rapidly became a pandemic threat. SARS was first identified in China and subsequently new cases were found in other countries and continents (72). An unprecedented well coordinated and collaborative response by scientists from all over the world led to the rapid identification of SARS-CoV as the causative agent and by a quick and well-coordinated public health response, an emerging pandemic was stopped in its wake for the first time in human history (73–76). More recently, another coronavirus has spilled over from the animal world, again causing severe and often fatal ARTI in humans. It was named after the region where the first case was described in 2012: MERS-CoV (31). Currently, 1714 laboratory-confirmed cases have been described in 26 countries and approximately 36% of the reported cases with MERS died (77). The route of transmission is not fully proven, but dromedary camels are most likely to be a major reservoir host for MERS-CoV and an animal source of infections in humans (66,78). Predominantly nosocomial human-to-human transmission of MERS-CoV has been reported upon close contact (79). Among recent study reports on MERS vaccine development is that on a Modified Vaccinia virus Ankara (MVA) based MERS-CoV vaccine, which was able to induce protective immunity resulting in reduction of excreted infectious MERS-CoV in dromedary camels (80). This MVA-based vaccine will now also be tested for protection of humans at risk, such as people with camel contacts and healthcare workers. These newly discovered coronaviruses, SARS-CoV and MERS-CoV, mainly affected adult or elderly patients (72,81). Smits et al. described, with the use of an animal model for SARS, that older macaques developed a more severe pro-inflammatory response resulting in more extensive lung damage compared to younger macaques (82). However, the other recently discovered coronaviruses: HCoV-NL63, HCoV-HKU1, but also the longer known HCoV-229E and HCoV-OC43 are known to usually cause mild disease in paediatric patients (6,25,83,84). The severity of the disease caused in children depends on several factors. For influenza viruses this has been studied in more detail. Maternal antibodies may be an important first line of defence that however lasts for the first months to a year only (85). In the absence of such antibodies the still immature immune system in the first months has to deal with the infection (86). Thereafter a more

mature immune system may have to deal with these infections for the first time, which in the absence of memory responses will be more severe than in adults (87).

Recent geographical extensions of areas where arthropod-borne viruses such as DENV, West-Nile virus, Chikungunya virus and Zika virus occur, has created major public health concerns (11,88–90). For none of these virus infections, vaccines are available and treatment options are limited and largely symptomatic (88–93). Currently, vector control and use of repellents with e.g. DEET (N,N-di-ethyl-meta-tolueenamide) are among the limited prevention methods (88–90,93). Children, pregnant women and immune-compromised patients form high-risk groups that may develop severe disease upon infection with these viruses (88–90,93). It is interesting to note that some of these viruses such as Chikungunya virus and West Nile virus, do not seem to cause as many major clinical symptoms in children compared to adults (94–96). In contrast for Zika virus infection pregnant women and more specifically their unborn babies currently form a high-risk group, as this infection has been associated with an unexplained increase of microcephaly in newborn babies from mothers infected with Zika virus in the first trimester of their pregnancy (90,97). The WHO has defined priority studies on the understanding of Zika virus disease (90,98,99). For several decades however, the most problematic paediatric re-emerging viral infection has been DENV.

Dengue virus, a re-emerging infectious disease

DENV circulates in tropical and subtropical areas around the world and is transmitted by the bite of infected mosquitoes of the *Aedes* species. There are four different DENV serotypes (1-4), which may co-circulate in endemic areas (100). Outbreaks occur mostly during the rainy season due to the increased number of mosquitoes (101). DENV affects humans of all age groups, although in some parts of the world it is mainly a paediatric public health problem (11,102). Severe DENV infections mostly affect children in South-East Asia, which may present with dengue shock syndrome, while in the Americas, adults are more likely to develop severe disease (103,104). However, recent epidemics in the Americas have shown an increased number of paediatric patients with severe and fatal DENV infections (105,106). DENV infections present with different clinical manifestations. A large number of infected people will not show clear clinical symptoms, while others develop dengue haemorrhagic fever or dengue shock syndrome, which may be fatal (10). Clinical manifestations of severe dengue are characterized by systemic capillary leakage, thrombocytopaenia and hypovolaemic shock (100,107). Factors that may predispose for severe dengue are: age, previous exposure to a heterologous DENV subtype, and underlying medical conditions such as chronic aenemia, diabetes mellitus, hypertension, congestive heart failure, other cardiovascular diseases, chronic obstructive pulmonary disease, asthma, renal disease, and multiple co-morbidities (108).

Until now treatment options for DENV are limited as no specific antiviral treatment is available and treatment is based on supportive care and intense monitoring of patients. Recently, the first dengue vaccine, Dengvaxia (CYD-TDV) by Sanofi Pasteur, was registered in several countries for use in individuals between 9-45 years of age living in endemic areas (11,106,109). Soon recommendations of the Strategic Advisory Group of Experts (SAGE) will follow concerning immunization with this new dengue vaccine. Moreover, clinical trials with other candidate vaccines are still on-going and more research is needed for vaccination of children under the age of nine (110,111).

AIM AND OUTLINE OF THIS THESIS

This thesis primarily aims to study the impact of newly discovered respiratory viruses in children as well as the role of emerging and re-emerging viruses in the development of severe disease. In addition the performance of currently used viral diagnostic and prevention methods for acute virus infections in children are addressed.

After presenting an introduction to the field of acute paediatric virus infections in children in **Chapter 1**, the capacity of viruses as sole causative agents to cause severe acute respiratory tract infections (SARIs) in children is evaluated in **Chapters 2** and **Chapter 3**. In **Chapter 4** the potential role of underlying sickle cell anaemia in the development of severe dengue is addressed by identifying and studying two juvenile cases with this combination of predisposition and infection. In **Chapter 5** the diagnostic performance of commercial rapid antigen detection tests for RSV and influenza in a tertiary care paediatric hospital is evaluated, with RT-PCR as gold standard and other currently used diagnostic assays as comparators. Subsequently in **Chapter 6** a candidate H5 influenza vaccine based upon the MVA vector system, is tested for safety and breadth of immunogenicity in healthy young adult volunteers. Finally **Chapter 7** presents a summarizing discussion of the thesis.

Chapter 2

Human bocavirus infection as a cause of severe acute respiratory tract infection in children

Fleur M. Moesker, Jeroen J.A. van Kampen, Annemiek A. van der Eijk, Annemarie M.C. van Rossum, Matthijs de Hoog, Martin Schutten, Saskia L. Smits, Rogier Bodewes, Albert D.M.E. Osterhaus, Pieter L.A. Fraaij

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ABSTRACT

In 2005 human bocavirus (HBoV) was discovered in respiratory tract samples of children. The role of HBoV as single causative agent for respiratory tract infections remains unclear. Detection of HBoV in children with respiratory disease is frequently in combination with other viruses or bacteria. We set up an algorithm to study whether HBoV alone can cause severe acute respiratory tract infection (SARI) in children. The algorithm was developed to exclude cases with no other likely cause than HBoV for the need for paediatric intensive care unit (PICU) admission with SARI. We searched for other viruses by next-generation sequencing (NGS) in these cases and studied their HBoV viral loads. To benchmark our algorithm, the same was applied to respiratory syncytial virus (RSV) positive patients.

From our total group of 990 patients who tested positive for a respiratory virus by means of RT-PCR, HBoV and RSV were detected in 178 and 366 children admitted to our hospital. Forty-nine HBoV positive patients and 72 RSV positive patients were admitted to the PICU.

We found seven single HBoV infected cases with SARI admitted to PICU (7/49, 14%). They had no other detectable virus by NGS. They had much higher HBoV loads than other patients positive for HBoV. We identified fourteen RSV infected SARI patients with a single RSV infection (14/72, 19%). We conclude that our study provides strong support that HBoV can cause SARI in children in the absence of viral and bacterial co-infections.

INTRODUCTION

In the past decade, many unknown viruses have been identified using novel molecular pathogen discovery techniques (112). After detection, it is not always clear if these newly discovered viruses are able to cause disease. For some viruses it remains difficult to rule out simultaneous infections in the patient with other known or yet unknown pathogens. Human bocavirus (HBoV) is a clear example of such a newly discovered virus.

HBoV was discovered in 2005 in respiratory tract samples from children suffering acute respiratory tract infections (113). Currently, four genotypes have been described (HBoV 1-4) (114). Based on seroprevalence studies, HBoV specific antibodies in adults ranges from 64 to 95%, indicating the high number of HBoV encounters (115). HBoV infection in children has been associated with respiratory tract infections (HBoV1-2) and gastrointestinal disease (HBoV 2-4) (114). HBoV infection results often in a mild self-limiting respiratory tract infection and may even be asymptomatic (114,116). Many children shed HBoV in the respiratory tract for prolonged periods, making interpretation of a positive test result in a patient difficult (117). Nonetheless, a few case reports describe HBoV as cause of severe acute respiratory tract infection (SARI) in children requiring intensive care. Although the role of other pathogens was addressed, co-infections were not excluded in a structured method (114,118–125). Altogether, this has resulted in the on-going debate whether HBoV as single causative agent can cause disease.

To address this issue, we set up an algorithm to exclude cases with no other likely cause than HBoV for the need for intensive care. We searched for overlooked viruses by next-generation sequencing (NGS) in these cases and studied their HBoV viral loads. To benchmark this method, the same algorithm was applied to patients with respiratory syncytial virus (RSV) infection. As RSV is generally considered to be a well-established paediatric pathogen that can cause SARI in children (126–128).

METHODS AND MATERIALS

Patient and sample selection

We conducted a retrospective cohort study during 5 consecutive years from April 2007 through March 2012. We selected paediatric patients (<18 years) admitted to the Paediatric Intensive Care Unit (PICU) of Erasmus MC-Sophia, which is a tertiary paediatric referral centre. This is the sole PICU for a region with a general population of ~4 million and an annual birth cohort of ~47,000 children (129).

We selected respiratory tract samples of these patients that tested positive with real-

time reverse transcriptase polymerase chain reactions (RT-PCRs) for respiratory viral diagnostics. We used the electronic laboratory information management system of the Viroscience Department to obtain all test results. Samples included nasal washings, sputum, throat swabs and broncho-alveolar lavages, which were obtained during routine clinical practice. We only used the first available sample of each patient. A viral pathogen was defined if a cycle threshold value (Ct-value) < 40 was detected for adenoviruses, coronaviruses (OC43, 229E and NL63), HBoV 1-4, human metapneumovirus, influenza A and B viruses, parainfluenza viruses 1-4, RSV and/or rhinoviruses (42). The Ct-value represents the number of cycles required for the fluorescent signal to cross the threshold (exceeds background level) and is inversely related to viral load.

Algorithm

All HBoV RT-PCR positive cases, with or without co-infections, were originally included. Next four selection steps were applied: first, we selected all PICU admitted patients, as these patients are critically ill and key for testing our hypothesis. Second, patients with a viral co-detection upon RT-PCR were excluded. Third, selected clinical data were extracted from electronic medical files of the patients. Only patients with SARI as reason for admission were included (see **Table 1** and **supporting Table 2**). Fourth, absence of bacterial co-infections was considered likely if C-reactive protein (CRP) levels were ≤ 40 mg/l upon admission and bacterial cultures tested negative, such as sputum, blood or cerebrospinal fluid cultures. We defined a sputum culture negative if ≤ 10 bacteria per ocular field were present in gram staining, if no respiratory pathogenic bacteria were cultured, and/or when commensal bacterial growth was ≤ 2/4. Sputa containing < 10 epithelial cells per ocular field (10x10 magnification) were considered quality sputa. Sputa containing ≥ 10 epithelial cells per ocular field were only considered quality sputa if the leucocyte-to-epithelial cell ratio was ≥ 10 and > 6 bacteria with the same morphology were present. Absence of a sputum culture was not a reason for exclusion, as sputum is not easily obtained from children. A reason for exclusion was if other bacterial cultures tested positive, such as a cerebrospinal fluid cultures or blood cultures.

Lastly, selected samples were tested for *Mycoplasma pneumoniae* using RT-PCR and only negative samples were used for NGS. Confirmatory serology detecting antibodies against HBoV or RSV was not performed, as serum samples were not collected routinely from these patients.

Three investigators independently reviewed the previously described selection steps and disagreement was resolved by consensus (FM, JVK and PF).

Next-generation sequencing

After applying the algorithm, samples were analysed for the presence of known and unknown viruses. We used a viral metagenomics procedure as previously described, which includes random RNA and DNA amplification in combination with NGS (454 Life Sciences®, Branford, Connecticut, USA)(130,131). In brief, the amplicons of random RNA and DNA amplification were pooled and purified, after which rapid library preparation, emulsion PCR and NGS were performed. Iterative exhaustive assembly of sequences was applied according to our virus discovery pipeline written in the python programming language (Python 2.7). An absolute minimum of two reads was required for a distinct hit to be reported. Hits positive for endogenous retroviruses or anelloviruses were excluded from analysis. Presence of genome material for bovine viruses was discarded because of bovine reagents used. Sequences were deposited in GenBank and given accession numbers (see supplemental data).

Phylogenetic analysis of HBoV

We performed phylogenetic analyses to study whether a specific HBoV subtype could be linked to a more virulent HBoV infection resulting in PICU admission. We used the VP1/VP2 genes for sequence analysis, after which a phylogenetic tree was reconstructed. Samples tested were HBoV RT-PCR positive selected cases for NGS and selected controls. Controls were samples of patients with Ct-value < 30, which is the minimum requirement to sequence HBoV in this method. These control samples were obtained from children whether or not admitted to PICU, without SARI and with co-infections. Two samples were obtained from children born in hospital and admitted for more than 90 days and were called nosocomially infected controls. All samples were amplified and sequenced as described previously (132). Phylogenetic analyses were conducted with MEGA version 5.

HBoV Ct-values in respiratory tract samples

To study the role of HBoV Ct-values in patients with SARI, we compared median Ct-values in three groups of patients. Groups were defined as; all hospitalized HBoV patients, all PICU admitted patients with HBoV and viral co-detection and selected PICU admitted patients with a single HBoV infection.

Validation Method using RSV infections

To validate our algorithm we applied the exact same selection steps to RSV positive samples of patients admitted to PICU. We chose RSV for validation because it's role in children with SARI is well-established compared to other pathogens and we therefore expected that RSV related SARI admissions would remain to be identifiable after using our algorithm (126–128).

Statistical analysis

Data were analysed using SPSS version 20.0 (IBM, SPSS, Chicago, IL, USA) and GraphPad Prism version 6. For continuous data, medians, interquartile range (IQR), lower IQR and upper IQR (LIQR and UIQR) were calculated. For the assessment of HBoV viral loads, median Ct-values were compared using the Mann-Whitney U test. A p-value <0.05 was taken as threshold of statistical significance.

Ethical considerations

The Medical Ethical Committee of the Erasmus MC approved this study (MEC 2013-221). Informed consent was waived because this is a retrospective cohort study. Data were stored anonymously and cannot be retraced to individual patients.

RESULTS

Patient and sample inclusion

In total 990 paediatric patients with a median age of 0.82 years (IQR 3.13), were identified for whom respiratory viral diagnostics were performed. Of those, 178 patients (18%) were HBoV RT-PCR positive. PICU admission was necessary for 49 HBoV cases (28%), of which 20/49 were positive for HBoV alone (41%) and 29/49 were positive for HBoV and other viruses (59%) (see **supporting Table 3**). Subsequently, clinical charts were reviewed and only patients admitted to PICU because of SARI were selected (11/20, 55%). Next, samples of patients with CRP levels ≤ 40 mg/L upon admission were included (8/11, 73%). Lastly, cases with negative blood, liquor and sputum cultures (if available) and negative RT-PCR test results for *M. pneumoniae* were selected. Sputa were obtained from intubated children. A total of 7/8 cases (88%) were considered with a single HBoV infection resulting in SARI and their samples were tested with NGS (see **Figure 1**).

Next-generation sequencing of HBoV cases

The seven identified respiratory tract samples were subjected to NGS and near full-length genomes were obtained in most samples (see **supporting Table 4**). No other viral pathogens besides HBoV were detected with NGS in 7 cases selected with our algorithm (2.8-44.3 % of total number of analysed reads).

Clinical data from children with HBoV

The median age of the seven children with HBoV was 24 months (min 4.4 – max 31.4, LIQR 15.7 – UIQR 28) and four patients were female (4/7, 57%). One patient was four-months-old upon admission and was born prematurely with a gestational age of 27 weeks and was previously diagnosed with broncho-pulmonary dysplasia. Due to the small sample

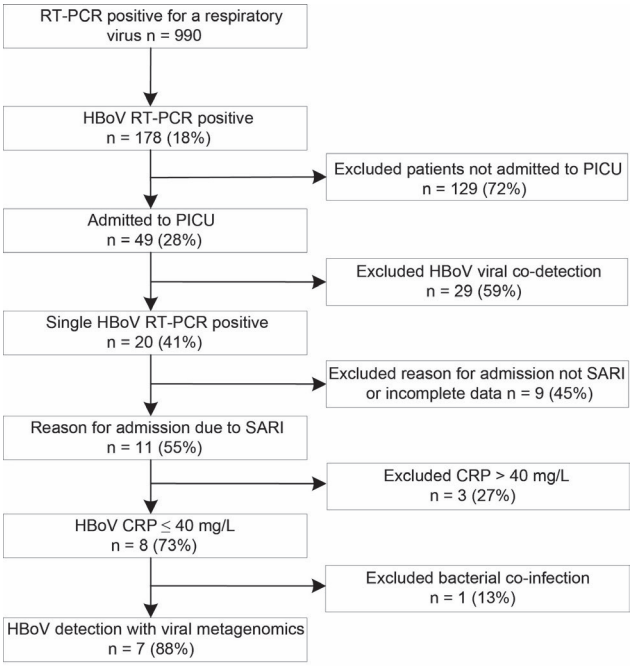

Figure 1. Flowchart for patient selection of HBoV RT-PCR positive patients admitted to the Erasmus MC-Sophia from 2007 -2012

size we referred from further comparative statistical testing. The length of symptoms before PICU admission was 1-7 days. Upon PICU admission CRP levels with a median of 8 mg/L (min 1 – max 36, LIQR 5 – UIQR 28) were tested within a median PICU stay of 2.5 hours (min 0 – max 6 hours). For 6/7 children follow-up CRP levels were obtained. In two cases there was an increase in CRP, none of these CRP-levels were ≥ 80 mg/L (see **Table 2**). For patient 2, CRP levels increased after initiation of ECMO (from 18 to 58 mg/L respectively). Of the other patient negative bacterial sputum samples were obtained in the absence of antibiotics at day 1 of PICU stay. Three of the seven patients (43%) with a single HBoV infection had no pre-existing medical history. All seven patients received antibiotics during admission and required supplemental oxygen. Mechanical ventilation was performed in five patients (71%) and extracorporeal membrane oxygenation (ECMO) treatment was needed in one patient (14%). All patients survived. The median duration of PICU admission was 4 days (IQR 2-7 days) (see **Table 1**).

Phylogenetic analysis of HBoV

To study whether a specific subtype of HBoV was associated with severe disease, phylogenetic analysis were performed. The VP1/VP2 region of HBoV was successfully amplified in all 7 selected cases (100%) and 40 controls (87%) (see **supporting Figure 4**).

Table 1. Baseline characteristics of selected HBoV and RSV RT-PCR positive PICU admitted patients at the Erasmus MC-Sophia from 2007-2012

Characteristics	HBoV positive PICU cases (n = 7)	RSV positive PICU cases (n = 17)
Age		
Months†	24 (4-31, 14.3-31.3)	2 (0-39, 0-7.5)
Years†	2 (0.3-2.62, 1.19-2.60)	0.18 (0.02-3.26, 0.06-0.68)
Female	4 (57)	5 (29)
Male	3 (43)	12 (71)
Reason for admission		
Respiratory failure	3 (43)	16 (94)
ECMO indication due to respiratory failure	1 (14)	-
Bronchiolitis / PSA	3 (43)	-
ALTE with ARTI	-	1 (6)
Clinical diagnosis at admittance		
URTI	-	1 (6)
LRTI	1 (14)	1 (6)
BHR / PSA	4 (57)	-
ARDS	1 (14)	-
Severe atelectasis with ARTI	1 (14)	-
Bronchiolitis	-	15 (88)
Medical history		
None	3 (43)	6 (35)
Pulmonary disease	3 (43)	2 (12)
GSA < 37 weeks	-	2 (12)
GSA < 37 weeks and pulmonary disease	1 (14)	-
Cardiac disease	-	1 (6)
Congenital anatomical malformations	-	4 (24)
Macrosome or dysmature	-	2 (12)
Laboratory testing		
CRP (mg/L) †	8 (1-36, 5-28)	8 (2-22, 4-11.5)
WBC count (x 10^9 /L) †	13.6 (8.1-27, 9.3-20)	13.5 (8.7-33, 10.5-15)
Sputum obtained and start antibiotics		
≤ 12 hours before sputum obtained	1 (14)	3 (17)
> 12 hours after sputum obtained	4 (57)	4 (24)
Sputum not obtained	2 (28)	10 (59)
Respiratory support		
Supplemental	1 (14)	7 (41)
Invasive	5 (72)	10 (59)
ECMO	1 (14)	-
PICU admission duration		
Days (median, min-max)	4 (2-7)	4 (2-29)
Survival	7 (100)	17 (100)
Viral metagenomics		
HBoV as sole viral pathogen detected	7 (100)	-
RSV as sole viral pathogen detected	-	14(82) ‡

* Values are no. % cases. ALTE; acute life threating event, ARDS; acute respiratory distress syndrome, BHR; bronchial hyperreactivity, CRP; C-reactive protein, ECMO; extracorporeal membrane oxygenation, GSA; gestational age, HBoV; human bocavirus, LRTI; lower respiratory tract infection, PSA; paediatric status asthmaticus, PICU; paediatric intensive care unit, RSV; respiratory syncytial virus, URTI; upper respiratory tract infection, WBC; white blood cells.
† median, min-max, LIQR-UIQR. ‡ One sample could not be processed, one tested negative for viruses, one tested positive for rhinovirus.

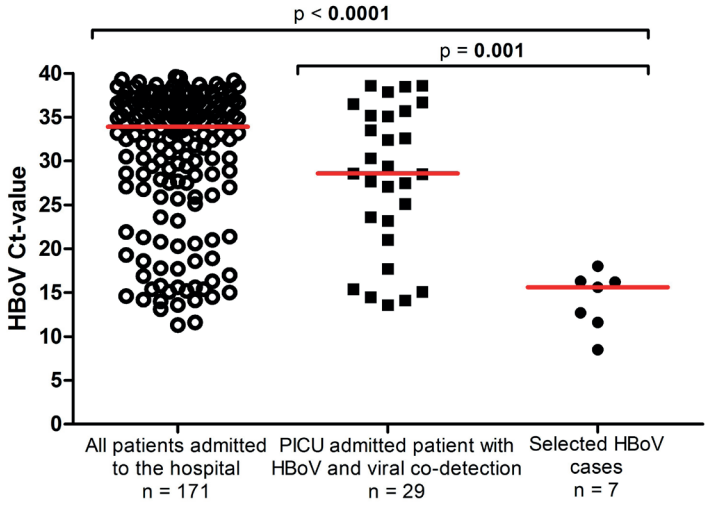

Figure 2. Comparison between median Ct-values of HBoV RT-PCR positive respiratory tract samples of paediatric patient admitted to the Erasmus MC-Sophia from 2007-2012; all hospital admitted paediatric patients versus Paediatric Intensive Care Unit (PICU) admitted patients with HBoV and viral co-detection versus PICU admitted patients with a single HBoV infection. Horizontal bars represent group medians

Blast and phylogenetic analyses showed that all HBoV strains of cases and controls were closely related to a total of two reference genotypes corresponding to HBoV genotype 1 (Stockholm: ST1 and ST2)(132).

HBoV Ct-values in respiratory tract samples

We compared the median HBoV Ct-values to study whether a low Ct-value resulted in severe disease and if viral co-detection resulted in higher Ct-values. We found significantly lower Ct-values in the seven-selected HBoV positive cases (median Ct-value 15.6, LIQR 11.6 –UIQR 16.3) compared to all HBoV positive patients admitted to hospital (median Ct-value 33.9, LIQR 11.3-UIQR 36.8) ($p < 0.0001$) (see **Figure 2**).

Furthermore, HBoV Ct-values were significantly lower compared to 29 PICU patients with HBoV and viral co-detection (median Ct-value 28.6, LIQR 13.6 – UIQR 35.45) ($p=0.001$) (see **Figure 2**).

Validation Method using RSV infections

Of the 990 paediatric patients, 366 tested RSV RT-PCR positive (37%). PICU admission was required in 72/366 of RSV cases (20%). After applying our selection steps, 17/24 RSV RT-PCR positive samples were tested with NGS (71%). Of these, 15/17 samples yielded

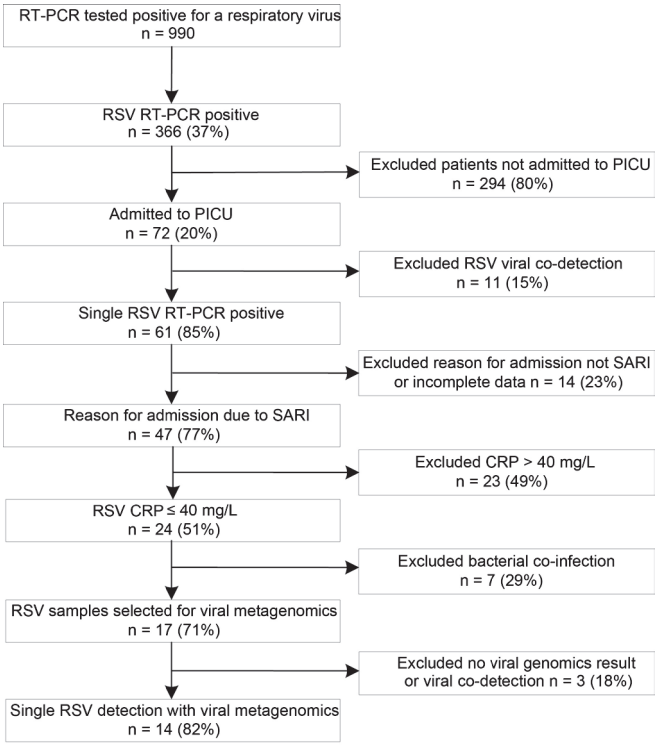

Figure 3. Flowchart for patient selection of RSV RT-PCR positive patients admitted to the Erasmus MC-Sophia from 2007 -2012

results (88%). One sample could not be processed; another had a limited number of reads. In all samples RSV was detected (0.01-31.7 % of total number of analysed reads). In addition, in one sample a rhinovirus was co-detected (1.17 % reads; see Figure 3 and supporting Table 5). Overall, in 14/72 SARI PICU patients RSV was found to be the sole pathogen (19%).

DISCUSSION

We here present a study on the relation between a recently discovered viral pathogen - HBoV - and SARI in children. We analysed HBoV RT-PCR positive samples from paediatric patients in whom viral and bacterial co-infections were considered unlikely after a rigid selection process. Pivotal to our approach is the addition of NGS to the clinical and conventional laboratory data. Based on the algorithm and NGS, we showed that a single HBoV infection caused SARI in seven patients requiring PICU admission. Which is in concordance with case reports on severely ill children with HBoV infection (118–

125). Although, in these reports viral and bacterial co-infections were described, they were not structurally assessed and NGS was not performed to detect overlooked viruses (118–125).

By adding NGS to our selection method, we bypassed the limitation of testing with RT-PCR alone, which relies on the use of selected primers for suspected pathogens. NGS allows detection of variant viruses that would otherwise escape detection (133,134). It is specifically designed to detect viruses and is optimized to decrease the number of other nucleid acids from i.e. host and bacteria. We showed previously that the sensitivity of detection with this method is approaching that of RT-PCRs in respiratory specimens (33). The routine clinical use of NGS alone is still in development, but it may well be that it will replace RT-PCR in the future (134).

In addition to the NGS findings, we also studied the difference between median HBoV Ct-values of selected cases and other patients with HBoV detection. We found a significantly lower median Ct-value in the selected cases than that of patients also admitted to PICU, but with viral co-detection. This may indicate that a single recent HBoV infection is associated with a higher viral load than in combination with other viruses as suggested previously by other studies (124,135,136). Based on the age of the patients and the low Ct-values we assume that the severe HBoV infection was a consequence of a primary infection (124). Furthermore, we compared the median Ct-values of selected cases with patients not admitted to PICU with HBoV and found that the Ct-values of the selected cases were also significantly lower suggesting a direct relation between disease severity and viral load as reported by others (121,124,136). Interestingly, others have also found a relation between Ct-values gender and underlying disease. Unfortunately our samples size was too small to confirm or refute these findings (124).

In order to benchmark our algorithm used to exclude patients with viral and bacterial co-infections, we also applied it to patients with RSV infection. We only found 14 cases with RSV as single causative agent for SARI. Considering the incidence of RSV-associated hospitalizations, this is lower than expected (128). We were able to benchmark our algorithm, although it seems to be very stringent and results in an underestimation of the true HBoV epidemiology. It is therefore of note that we did not set out to estimate the true burden of disease of HBoV, but to establish whether HBoV by itself can cause severe acute respiratory tract infections. Our study is subject to several limitations. It may be argued that the retrospective nature of this study and its inherent limitations may have resulted in a sample bias. Blood samples for HBoV serology or PCR were not obtained, because these are not routinely tested at our hospital. Furthermore, not all bacteriology samples were obtained, as more invasive procedures should have been

carried out to fully rule out the role of bacteria in lower respiratory tract infections. These are currently not routinely practiced in critically ill children for ethical and technical reasons. As an alternative we set strict CRP levels to our inclusion criteria. Based on previously published data we set a lower cut-off for bacterial co-infections (40 mg/L) than the usually applied 80 mg/L, to minimize the contribution of bacterial infections to the observed disease. In addition, during non-structured follow-up none of the patients showed an increase in CRP levels > 80 mg/L. Still, in two cases a moderate increase was observed and it should be noted that despite these low CRP levels, bacterial involvement cannot be fully ruled out based on CRP levels alone (137–139). Other biomarkers such as pro-calcitonin and interleukins could have been used to differentiate between bacterial and viral infections, but these were not carried out in our hospital (139,140).

Based on our findings we conclude that a single HBoV infection can cause SARI in children in the absence of viral and bacterial co-infection.

Acknowledgements

We thank Claudia Schapendonk for excellent technical support and Hans Kruining for help with obtaining the data for the database.

SUPPORTING INFORMATION

Table 2. Overview non-structured follow-up CRP levels obtained during PICU-stay

PICU stay (days)	1	1.5	2	2.5	3	3.5	4
Patient 1 CRP levels (mg/L)	5	-	-	-	-	-	-
Patient 2 CRP levels	36	18	55	58	41	-	-
Patient 3 CRP levels	8	-	4	-	-	-	-
Patient 4 CRP levels	11	9	-	5	-	-	-
Patient 5 CRP levels	28	-	79	-	66	-	10
Patient 6 CRP levels	7	-	1	-	-	-	-
Patient 7 CRP levels	1	-	5	-	6	-	4

- represents missing data

Supporting Table 2. Reasons for admission because of a Severe Acute Respiratory tract Infection (SARI) or not because of SARI

Reasons for admission defined as SARI	Reason for admission not defined as SARI
Respiratory failure with possible need for mechanical ventilation	Neurologis disorders Ischaemia
Extracorporeal membrane oxygenation (ECMO) indication due to respiratory failure	Erythema multiforme and shock Nosocomial infection
Bronchiolitis / Paediatric status astmaticus	Gastro-intestinal disease
Acute life threatening event (ALTE) with ARTI	Cardiac disease
Acute respiratory distress syndrome (ARDS)	Congenital malformations
	Admission data missing

Supporting Table 3. HBoV RT-PCR positive and viral co-detection with RT-PCR of PICU admitted patients from 2007 till 2012

Pathogen detected with RT-PCR	HBoV and one other virus n = 24 (%)
Rhinovirus	9 (38)
Adenovirus	4 (17)
RSV	4 (17)
Human coronavirus NL 63	3 (13)
Human coronavirus OC43	1 (4)
Parainfluenza virus type 3	1 (4)
Influenza A	2 (8)

Pathogen detected with RT-PCR	HBoV and ≥ 2 viruses n = 5
Parainfluenza virus type 1 + rhinovirus	2
Parainfluenza virus type 2 + human coronavirus NL63	1
Adenovirus + human coronavirus NL63	1
Adenovirus + RSV	1

Supporting Table 4. Viral metagenomics results of selected HBoV cases

Sample number HBoV	Total number of analysed reads	No. HBoV reads	% HBoV reads / total analysed reads
1	6823	1382	20.26
2	30736	2634	8.57
3	17305	7670	44.32
4	27437	1570	5.72
5	6903	2057	29.80
6	10471	2681	25.60
7	15848	444	2.8

Supporting Table 5. Viral metagenomics results of selected RSV cases

Sample number RSV	Total number of analysed reads	No. RSV reads	No. Rhinovirus reads	% RSV reads / total analysed reads	% Rhinovirus reads / total analysed reads
1	6736	1641	-	24.36	
2	11417	1898	-	16.62	
3	14207	74	167	0.5	1.17
4	13463	4	-	0.03	
5	7942	652	-	8.2	
6	6937	2197	-	31.7	
7	10350	2568	-	24.8	
8	12390	602	-	4.85	
9	13846	3321	-	24	
10	16271	22	-	0.14	
11	584	0	-	0	
12	12161	2284	-	18.8	
13	0*	n/a	-	n/a	
14	17636	2	-	0.01	
15	17190	1429	-	8.3	
16	16061	293	-	1.8	1.2
17	9661	2007	-	20.8	0.3

* Sample could not be processed.

Supporting information gene bank numbers phylogenetic tree

BankIt1747547	k2007001255	KM257816
BankIt1747547	k2007001443	KM257817
BankIt1747547	K2007001535	KM257818
BankIt1747547	k2007001654	KM257819
BankIt1747547	K2007001674	KM257820
BankIt1747547	K2007001679	KM257821
BankIt1747547	K2007001732	KM257822
BankIt1747547	K2007001919	KM257823
BankIt1747547	K2007002149	KM257824
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BankIt1747547	K2007002841	KM257826
BankIt1747547	K2007002845	KM257827
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BankIt1747547	K2009002589	KM257843
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BankIt1747547	K2010000191	KM257845
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BankIt1747547	K2010001030	KM257847
BankIt1747547	K2010001188	KM257848
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BankIt1747547	K2010001713	KM257851
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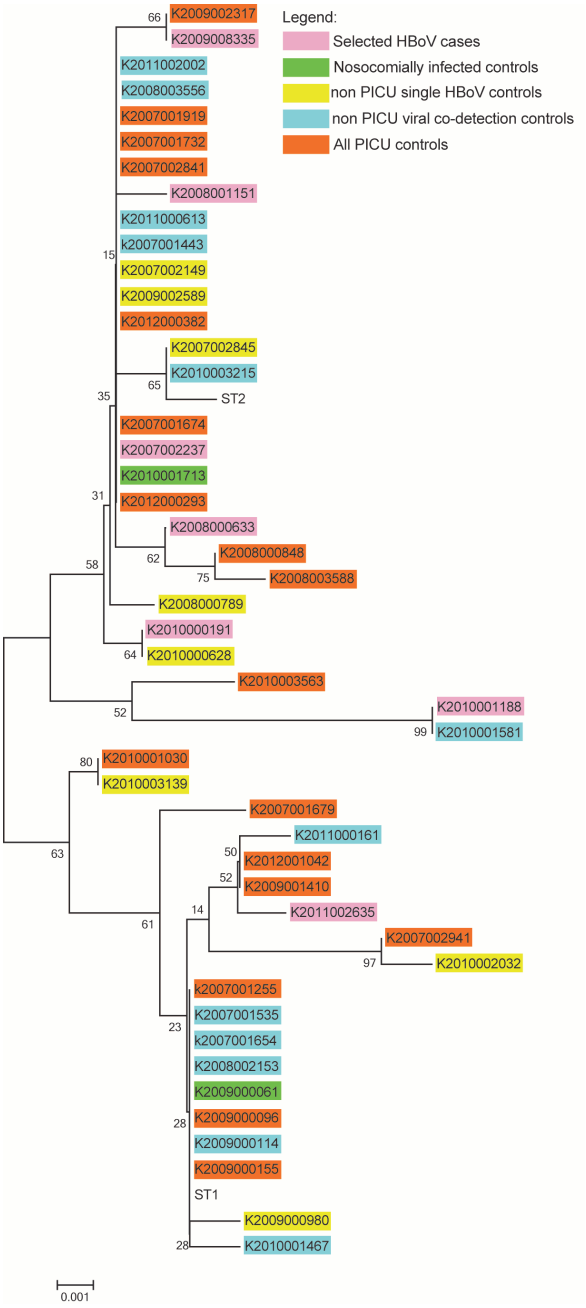
BankIt1747547	K2011000613	KM257857
BankIt1747547	K2011002002	KM257858
BankIt1747547	K2011002635	KM257859
BankIt1747547	K2012000382	KM257860
BankIt1747547	K2012001042	KM257861
BankIt1747547	K2012000293	KM257862

Supporting Figure 4

Figure 4. Phylogenetic analysis of the partial VP1/VP2 nucleotide sequences of HBoV RT-PCR positive samples of patients admitted to the Erasmus MC-Sophia from 2007 until 2012. Phylogenetic tree was constructed with MEGA 5 software using the neighbor-joining method. Bootstrap values are shown at the branch nodes. Prototype strain ST1 and ST2 were also included (page 23).



Chapter 3

Viruses as sole causative agents of severe acute respiratory tract infections in children

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ABSTRACT

Background: Respiratory syncytial virus (RSV) and influenza A viruses are known to cause severe acute respiratory tract infections (SARIs) in children. For other viruses like human rhinoviruses (HRVs) this is less well established. Viral or bacterial co-infections are often considered essential for severe manifestations of these virus infections.

Objective: The study aims at identifying viruses that may cause SARI in children in the absence of viral and bacterial co-infections, at identifying disease characteristics associated with these single virus infections, and at identifying a possible correlation between viral loads and disease severities.

Study design: Between April 2007 and March 2012, we identified children (<18 year) with or without a medical history, admitted to our paediatric intensive care unit (PICU) with SARI or to the medium care (MC) with an acute respiratory tract infection (ARTI) (controls). Data were extracted from the clinical and laboratory databases of our tertiary care paediatric hospital. Patient specimens were tested for fifteen respiratory viruses with real-time reverse transcriptase PCR assays and we selected patients with a single virus infection only. Typical bacterial co-infections were considered unlikely to have contributed to the PICU or MC admission based on C-reactive protein-levels or bacteriological test results if performed.

Results: We identified 44 patients admitted to PICU with SARI and 40 patients admitted to MC with ARTI. Twelve viruses were associated with SARI, ten of which were also associated with ARTI in the absence of typical bacterial and viral co-infections, with RSV and HRV being the most frequent causes. Viral loads were not different between PICU-SARI patients and MC-ARTI patients.

Conclusion: Both SARI and ARTI may be caused by single viral pathogens in previously healthy children as well as in children with a medical history. No relationship between viral load and disease severity was identified.

INTRODUCTION

Worldwide acute respiratory tract infections (ARTIs) are among the most important causes of morbidity and mortality in children (3,141). While most ARTIs result in relatively mild and self-limiting disease, sometimes disease progresses to severe acute respiratory tract infections (SARIs). SARIs are characterized by respiratory failure and are an indication for admission to a paediatric intensive care unit (PICU) because of the need of respiratory support. Some viruses such as respiratory syncytial virus (RSV) and influenza viruses have been shown to be the single cause of SARI, but for other viruses like human rhinoviruses (HRVs) and human coronavirus-NL63 (HCoV-NL63) this is under debate (84,142,143). It may be that these viruses cause minimal or mild disease only, but in combination with bacterial co-infections, viral co-infections or alternatively, underlying high-risk conditions infection may result into SARI (144–146). This hypothesis follows observations of detection of viruses in the nasopharynx of children in absence of clinical signs and symptoms (19,116,147,148). Another explanation for the variable association between infection and severe disease could be the extent of infection, as reflected by viral loads. High viral loads may be associated with increased host cell damage and a more profound immune response, but this relationship has not been confirmed consistently (126,149,150). To address the possible causative role of virus infections in SARIs, we performed a retrospective study in our hospital, which has implemented routine testing for fifteen respiratory viruses over the study period.

MATERIALS AND METHODS

Patient and sample selection

We conducted a retrospective study analysing data of paediatric patients (age <18 years) with or without a medical history that were admitted to Erasmus MC-Sophia from April 2007 through March 2012. Erasmus MC-Sophia is a tertiary paediatric referral centre. Clinical and virological data were extracted from the clinical and laboratory databases. We collected all qualitative and quantitative diagnostic virological data obtained with real-time reverse transcriptase PCR (RT-PCR) on respiratory tract samples (nasal washing, sputum, throat swab, nose swab, or bronchoalveolar lavage specimen (BAL)) of patients admitted to PICU or medium care (MC). Nasal washings were obtained by infusing 1-2 ml NaCl intranasally. Samples were obtained on clinical indication. Only samples obtained upon admission or within 72 hours after admission were included. Patients could be included more than once if re-admitted with SARI or ARTI, and a sample was obtained > 35 days after the first discharge and tested positive for a different virus. For further analysis these patients (SARI: n=1; ARTI: n=1) were considered new patients. The

following clinical data was obtained: age, gender, reason for admission, clinical diagnosis, underlying medical condition, specimen used for RT-PCR, C-reactive protein (CRP)-levels, bacteriological test results, types of respiratory support, length of PICU stay and hospital stay, and final outcome. Based on this clinical data, we subsequently grouped patients with SARI or ARTI as primary reason for PICU or MC admission respectively.

Patients were also categorized according to presence or absence of known risk factors for SARI: pulmonary disease, pre-term birth (born before 37 weeks of gestation), anatomical malformations, syndromal disorders, cardiovascular disease, oncology, immunology, neuro-muscular impairment and other disorders including scoliosis.

Study groups

We defined SARI as a severe acute respiratory tract infection with the potential need for invasive respiratory support and therefore requiring PICU admission. We defined ARTI as acute respiratory tract infection without the need for intensive care admission and we used this group as a control to study severe disease caused by a single virus infection. In the present study we specifically focused on samples obtained from patients admitted to PICU or MC with SARI or ARTI, caused by a single virus infection in the absence of typical bacterial and viral co-infections (PICU-SARI patients and MC-ARTI patients respectively). We further defined respiratory support into supplemental oxygen need (nasal cannula), non-invasive respiratory support (non-rebreathing mask and optiflow), invasive respiratory support with endotracheal intubation (nasopharyngeal tube, trachea tube, trachea-cannula) and extracorporeal membrane oxygenation (ECMO).

The laboratory diagnostic work-up was RT-PCR testing for fifteen respiratory viruses: adenoviruses (ADV), HCoV-NL63, HCoV-OC43 and HCoV-229E, human bocavirus (HBoV), human metapneumovirus (HMPV), influenza A and B viruses, parainfluenza viruses 1-4 (PIV), RSV type A and B and HRV (42). Samples with a cycle threshold (Ct)-value < 40 were considered positive. Of note Ct-values are inversely correlated to viral load, and only provide semi-quantitative results. For the purpose of this study we combined RSV type A and B results and we excluded patient samples obtained during an emergency department visit, out-patient-clinic visit or from patients admitted to the neonatal intensive care unit. Positive viral test results were traced back to patients admitted to PICU or MC. We compared all patients with a single virus detected and admitted to PICU with patients admitted to PICU with SARI (PICU-SARI patients), and all patients admitted to MC with patients with ARTI admitted to MC (MC-ARTI patients).

Exclusion of typical bacterial co-infections

We excluded samples from patients with evidence of typical bacterial co-infections upon admission and within 24 hours after admission when: CRP-levels were > 40 mg/L or tested first time > 24 hours after admission and/or in case of positive bacteriological test results. We defined a sputum positive for bacterial infection when > 10 bacteria per ocular field (/OF) were present in gram staining performed on the sputum (direct examination), respiratory pathogenic bacteria were cultured, and/or commensal bacteria were cultured with growth of > 2 on a scale of 4. The quality of sputum was optimal for testing when it contained < 10 epithelial cells/OF (10 x 10 magnification). Sputum samples containing ≥ 10 epithelial cells/OF observed by direct examination, were only considered sputum of good quality when the leucocyte-to-epithelial cell ratio was ≥ 10/OF. Absence of sputum for bacteriological testing was not a reason for exclusion, as sputum could not be obtained from all children. Furthermore, we analysed other bacterial cultures if performed and a positive culture of cerebrospinal fluid, blood or BAL were defined as bacterial co-infection and thus a reason for exclusion from further analysis. Routine testing for atypical bacterial infections caused by *Chlamydophila pneumoniae, Mycoplasma pneumoniae* or *Bordetella pertussis* was not performed.

Three investigators (FM, JVK and PF) independently reviewed the combined laboratory and clinical data from all PICU patients, based on the previously defined selection criteria, to decide on inclusion. Disagreement was resolved by consensus.

Statistical analyses

Data were analysed using SPSS version 20.0 (IBM, SPSS, Chicago, IL, USA) and GraphPad Prism version 6. For continuous data, medians, interquartile ranges (IQR), lower IQR (LIQR) and upper IQR (UIQR) were calculated. To analyse categorical data we used Fisher's-exact tests or Chi-square tests depending on the sample size. We calculated the number of viruses present in the study groups. To study the relation between disease severity and viral load, we compared the Ct-values in nasal washings in our study groups with Mann-Whitney U tests. We only included groups with more than six children based on the following power analysis: n= 2*(2.8*SD/Mean)^2, with an estimated mean of 25 and SD of 15 based on the number of patients needed to show a difference in Ct-values.

Ethics

This study was approved by the Medical Ethical Committee of the Erasmus MC, Rotterdam the Netherlands (MEC 2013-221). Informed consent was waived because this was a retrospective case-chart study. Data were stored anonymously and cannot be retraced to individual patients.

RESULTS

PICU-SARI patient characteristics

The selection of PICU-SARI patients is outlined in **Figure 1A** and details of excluded patients are listed in **S1 Table**. An overview of the epidemiological data is shown in **S1 Figure**. We identified 44 PICU-SARI patients with a median age of 9.6 months (LIQR 2 -UIQR 25, min 0.46 - max 152) and an equal gender distribution (males: 22/44, 50%) (**Table 1**). One patient was admitted twice because of SARI, with RSV or HRV as single causative agent detected, and with an admission interval of 23.5 months. Thirty-four patients had an underlying medical condition (34/44, 77%). The remaining ten patients were healthy until admission (10/44, 23%) (**Table 1**). The most common underlying medical condition was pre-term birth (10/34, 29%). Reasons for admission were: obstructive upper respiratory tract infection (URTI) (9/44, 20%), lower respiratory tract infection (LRTI) (15/44, 34%) and severe wheezing with respiratory distress (18/44, 41%). One patient was admitted with an apparent life-threatening event (ALTE (n=1)) with SARI and one patient with SARI related respiratory fatigue due to muscular disease (n=1) (2/44, 5%). Respiratory support consisted of supplemental oxygen (9/44, 20%), non–invasive respiratory support including non-rebreathing mask and optiflow was given in eight patients (8/44, 18%), whereas 27 patients required invasive respiratory support (27/44, 61%). For one patient additional ECMO was applied due to insufficient oxygenation (1/44, 2%). RSV was more frequently detected in boys (9/13, 69%), most patients were admitted with bronchiolitis (10/13, 77%), six had no underlying medical condition (6/13, 46%), and eight required invasive respiratory support (8/13, 62%). HRV was detected in six girls (6/11, 55%), most patients were admitted with LRTI (4/11, 36%) or wheezing (4/11, 36%), all patients had an underlying medical condition, and seven patients required invasive respiratory support (7/11, 64%). Overall, the median PICU-stay was 3 days (min 0-max 39), after which patients were either transferred from PICU to MC (n=14, 32%, after which n=8 (57%) were transferred to another hospital or discharged n=6 (43%)), transferred from PICU to another hospitals (n=22, 50%) or were discharged after PICU admission (n=8, 18%). Admission duration in other hospitals could not be analysed, as data was not available. The overall hospital stay for patients admitted to both PICU and MC was 7 days (min 3-max 30). Two patients died during PICU admission because of cardiorespiratory failure. One of these was a previously healthy child admitted for cardiorespiratory arrest upon influenza A virus infection, the other was a patient with spinal muscular atrophy (SMA) type-2, who developed respiratory failure due to PIV-3.

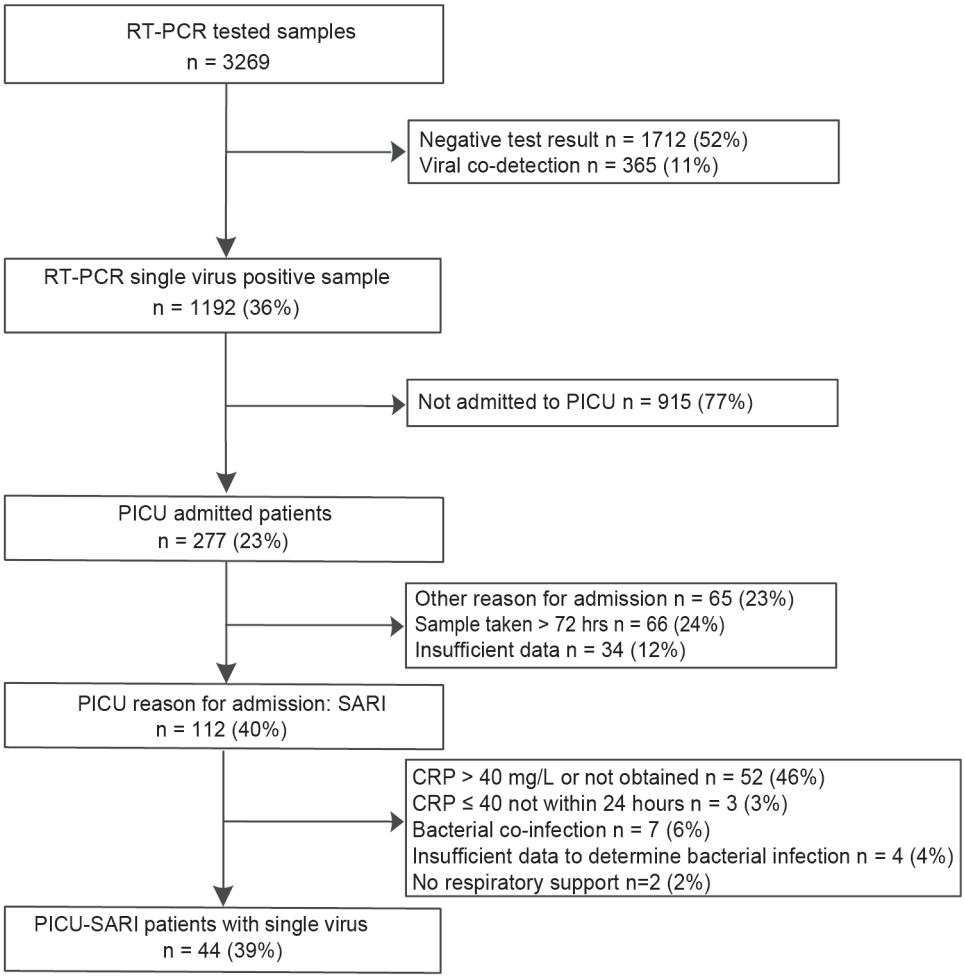


Figure 1A. Flowchart patient selection for patients admitted to the paediatric intensive care unit (PICU) with severe acute respiratory tract infections (SARIs) with one respiratory virus, in the absence of viral co-infections and typical bacterial co-infections in children admitted to Erasmus MC-Sophia between 2007 and 2012.

MC-ARTI patient characteristics

The selection of MC-ARTI patients is outlined in **Figure 1B** and details of excluded patients are listed in **S2 Table**. After using our predefined selection criteria 40 patients admitted to the MC with ARTI were identified. The median age of the MC-ARTI patients was 14 months (LIQR 7.6 -UIQR 30, min 0.7-max 199) with more males than females (22/40, 55%) (**Table 1**). One patient was admitted twice because of ARTI, with RSV or HMPV as single causative agent detected, and with an admission interval of 1.4 months.

Table 1. Baseline characteristics of children admitted to the paediatric intensive care unit (PICU) with severe acute respiratory tract infections (SARIs) or medium care (MC) with acute respiratory tract infections (ARTIs) associated with a single virus infection in the absence of viral co-infections and typical bacterial co-infections at Erasmus MC-Sophia from 2007-2012.

	PICU-SARI patients n = 44 (%)	**MC-ARTI patients n = 40 (%)**	p-values
Age			
Months	9.6 (1.8-24.7, 0.46-152)[a]	14.15 (7.6-30.1, 0.7-199)[a]	0.0447
Years	0.8 (0.15-2.1, 0.04-12.7)[a]	1.18 (0.63-2.5, 0.06-16.6)[a]	0.0442
Male	22 (50)	22 (55)	0.66
Reason for Admission			
Upper respiratory tract infections	9 (21)	9 (22.5)	
Lower respiratory tract infections	15 (34)	18 (45)	
Wheezing and oxygen need	18 (41)	11 (27.5)	
Others	2 (4)*	2 (5)**	
Medical history			
None	10 (23)	6 (15)	0.4 (F)
Pulmonary disease (including cystic fibrosis)	6 (14)	5 (12.5)	ns
Pre-term birth (gestational age < 37 weeks)	10 (23)	4 (10)	0.15 (F)
Anatomical malformations and syndromal	8 (18)	5 (12.5)	0.55 (F)
Cardiovascular	1 (2)	4 (10)	0.19 (F)
Oncology and immunology	-	2 (5)	-
Neuro-muscular, SGA and others	9 (21)	14 (35)	0.15 (F)
Specimens			
Nasal washing	34 (77)	39 (98)	0.006 (C)
Sputum	8 (18)	-	
Throat swab	2 (5)	-	
Nasal swab	-	1 (2)	
Respiratory support			
Supplemental	9 (20)	30 (75)	
Non-invasive	8 (18)	5 (12.5)	
Invasive	27 (61)	-	

Table 1. Continued

Extracorporeal oxygenation	1 (including invasive)	-	
None	-	5 (12.5)	
Length of stay			
PICU stay days	3 (0-39)[b]	0	0.0002
Hospital stay	0	5 (1-21)[b]	
Survival	42 (96)	39 (98)	0.61 (C)

[a] median, lower interquartile range – upper interquartile range, minimum-maximum; [b] median, minimum-maximum;

* apparent life-threatening event and respiratory fatigue due to muscular disease;

** exacerbation of cystic fibrosis and apparent life-threatening event; F = Fisher-exact-test C = Chi-square test.

Thirty-four patients had an underlying medical condition (34/40, 85%) including: pulmonary disease (5/34, 15%), a history of pre-term birth (4/34, 12%), and anatomical malformations and syndromal disorders (5/34, 15%) (**Table 1**). Reasons for admission were: URTI (9/40, 22.5%), LRTI (18/40, 45%) and wheezing (11/40, 27.5%). RSV was most frequently detected in boys (7/13, 54%), most patients were admitted with bronchiolitis (7/13, 54%), ten patients had underlying medical conditions (10/13, 77%), and all RSV infected patients' required supplemental or non-invasive respiratory support (12/13, 92% and 1/13, 8%). HRV was mostly detected in girls (7/12, 58%), the most common reason for admission was LRTI (7/12, 58%), ten patients had underlying medical conditions (10/12, 83%), and nine required supplemental oxygen (9/12, 75%), one received non-invasive respiratory support (1/12, 8%), and two HRV infected patients did not require respiratory support (2/12, 17%). In addition, one patient was admitted because of an exacerbation of cystic fibrosis with HRV as single pathogen detected (1/12, 8%) and one patient was admitted with an ALTE and ARTI with also a single HRV infection (1/12, 8%). The median hospitalisation stay of MC-ARTI patients was 5 days (1-21 days). In the MC-ARTI group one patient suffering from SMA-type 1 died with an HRV-infection.

Comparison of PICU-SARI patients with MC-ARTI patients

The median age of PICU-SARI patients was lower than for MC-ARTI patients (9.6 vs 14 months respectively; p=0.045). Specimens tested were mainly nasal washings in both groups (77% and 98%, p=0.006). In addition, CRP-levels were similar between both groups with a median of 6 and 7.5 mg/L respectively (p>0.05). As expected the number of bacterial sputum samples obtained was higher in PICU-SARI patients than in MC-ARTI patients (19/44; 43% vs 8/40; 20%, p=0.035). Overall, we found 23 patients with a single virus and a bacterial co-infection admitted to the PICU with SARI (**S3 Table**).

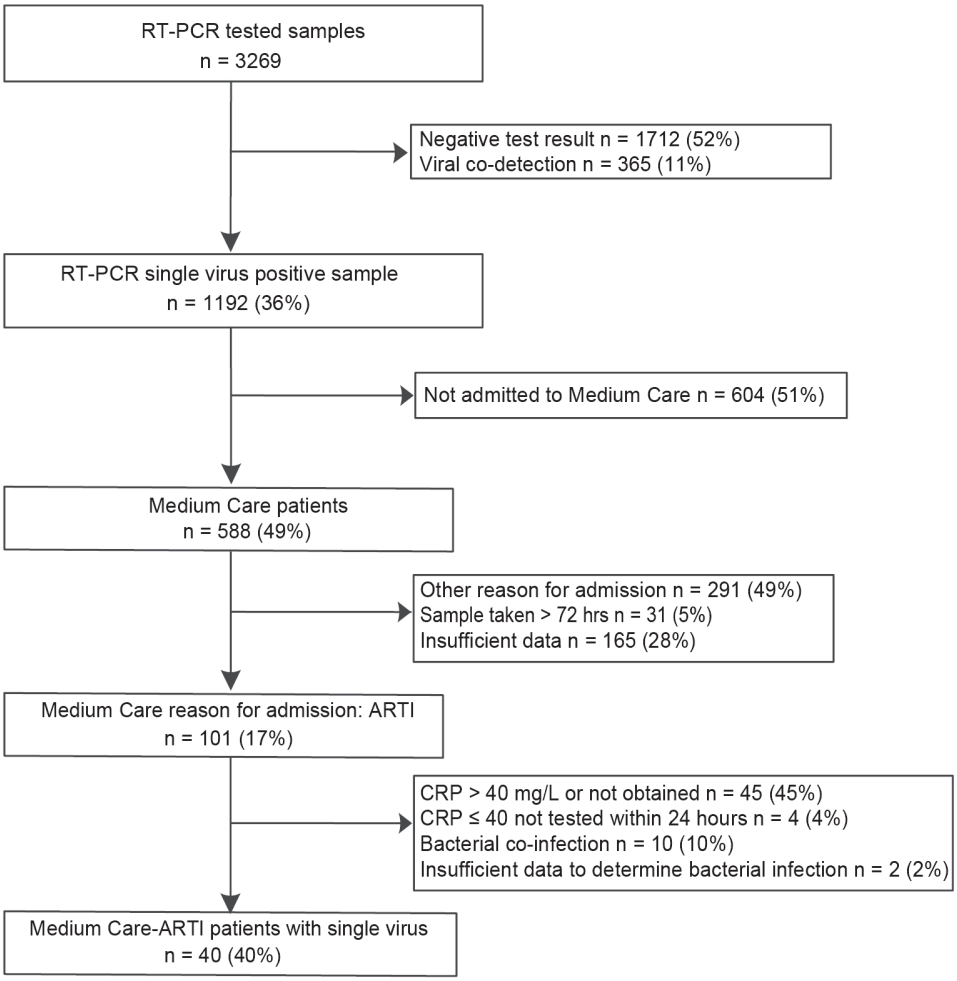


Figure 1B. Flowchart patient selection for patients admitted to medium care (MC) with acute respiratory tract infections (ARTI) with one respiratory virus, in the absence of viral co-infections and typical bacterial co-infections in children admitted to Erasmus MC-Sophia between 2007 and 2012.

Most bacterial co-infections were found in patients with RSV (8/23, 35%) and HRV (7/23, 30%) infections. Eleven patients admitted to the MC with ARTI had a proven bacterial co-infection (**S4 Table**), and PIV-3 was most frequently co-detected (3/11, 27%). Due to the limited number of proven bacterial co-infections, no associations between detected respiratory virus and bacterial infection could be evaluated. Twenty-three percent of PICU-SARI patients were previously healthy until admission compared to 15% of the MC-ARTI patients (10/44, 23% vs. 6/40, 15%, p=0.4) (**Table 1**).

Detection and distribution of respiratory viruses

Overall in the non selected PICU and MC patients HRV was very often detected and in a lower percentage in samples obtained from PICU patients (31%) than in samples of MC patients (42%) (p=0.002), whereas RSV (24% versus 15%, p=0.002) and ADV (10% versus 5%, p=0.007) were more often detected in samples obtained from PICU patients (**Table 2**). However, after applying our selection criteria for SARI and ARTI with a single virus only, these differences could no longer be found. Indeed RSV and HRV were represented roughly equally and predominated above all other viruses in both PICU-SARI patients and MC-ARTI patients (RSV: 13/44, 30% and 13/40, 33%, HRV: 11/44, 25% and 12/40, 30%). Besides HRV and RSV nine of the other viruses tested for were associated with SARI and seven of these viruses were also associated with ARTI (except for ADV and HCoV-NL63). Of the ten previously healthy PICU-SARI patients six were infected with RSV (6/10, 60%), three with HBoV (3/10, 30%), and one was infected with influenza A virus (1/10, 10%). Of the six previously healthy MC-ARTI patients, three patients were RSV infected (3/6, 50%), HRV was detected in two patients (2/6, 33%) and one patient tested positive for PIV-1 (1/6, 17%).

Ct-values of viral pathogens in respiratory tract samples of PICU-SARI patients compared to MC-ARTI patients

In an attempt to relate viral load to disease severity in PICU-SARI and MC-ARTI patients, we compared the total combined Ct-values in nasal washings, which revealed that the median Ct-values were not different between both groups (Ct values 22.2 vs 25.1 respectively; p=0.191, **Figure 2**). There was a difference between Ct-values of PICU-SARI patients compared to PICU-non-SARI patients (*p*=0.005). This was not found for MC-ARTI patients compared to MC-non-ARTI patients (p=0.0994). We also compared Ct-values of PICU-SARI patients to MC-ARTI patients with and without a medical history and we found no differences between the respective groups (p=0.289 and p=0.917, data not shown). Furthermore we compared PICU-SARI patients with invasive respiratory support with PICU-SARI patients and MC-ARTI patients with supplemental and non-invasive respiratory support and we found no difference between these two groups (p=0.0977, data not shown). Due to limited numbers we were not able to compare Ct-values for all individual viruses identified in this study, but based on our power calculation for both RSV and HRV infected patients, sufficient samples were available to compare Ct-values. No difference could be found in Ct-values between PICU-SARI patients and MC-ARTI patients with median Ct-values for RSV of 20.6 vs 21.4 (p=0.94) and HRV with 28.3 vs 26.4 (p=0.44) respectively (**Figure 4A** and **4B**).

Table 2. Viral pathogens detected with real time reverse transcriptase PCR in respiratory tract samples of patients admitted to the paediatric intensive care unit (PICU) with or without a severe acute respiratory tact infection (SARI) and medium care (MC) admitted patients with or without an acute respiratory tract infection (ARTI) at the Erasmus MC-Sophia over a 5-year period (2007-2012).

Viruses	PICU-SARI patients with a single virus and no bacterial co-infection n=44 (%)	All PICU admitted patients with a single virus n=277 (%)	MC-ARTI patients with a single virus and no bacterial co-infection n=40 (%)	All MC admitted patients with a single virus n=588 (%)	p-value between PICU-SARI patients and MC-ARTI patients	p-value between all PICU and all MC patients
Rhinovirus	11 (25)	86 (31)	12 (30)	247 (42)	0.6	0.002
Respiratory syncytial virus	13 (30)	65 (24)	13 (33)	86 (15)	0.8	0.002
Adenovirus	1 (2)	27 (10)	-	28 (5)	-	0.007
Human bocavirus	7 (16)	23 (8)	2 (5)	40 (7)	0.2	0.5
Influenza A virus	4 (9)	22 (8)	1 (3)	40 (7)	0.4	0.6
Human metapneumovirus	1 (2)	12 (4)	3 (8)	23 (4)	0.3	0.9
Parainfluenzavirus type 1	2 (5)	10 (4)	1 (2)	9 (2)	ns*	0.08
Human coronavirus OC43	1 (2)	10 (4)	2 (5)	16 (3)	0.6	0.5
Parainfluenzavirus type 3	1 (2)	7 (3)	5 (13)	36 (6)	0.1	0.03
Parainfluenzavirus type 4	1 (2)	6 (2)	1 (3)	13 (2)	ns	ns
Human coronavirus NL63	2 (5)	5 (2)	-	20 (3)	-	0.3
Influenza B virus	-	2 (1)	-	15 (3)	-	0.1
Parainfluenza virus type 2	-	1 (0.4)	-	7 (1)	-	0.5
Human coronavirus 229E	-	1 (0.4)	-	8 (1)	-	0.3

* ns, not significant.

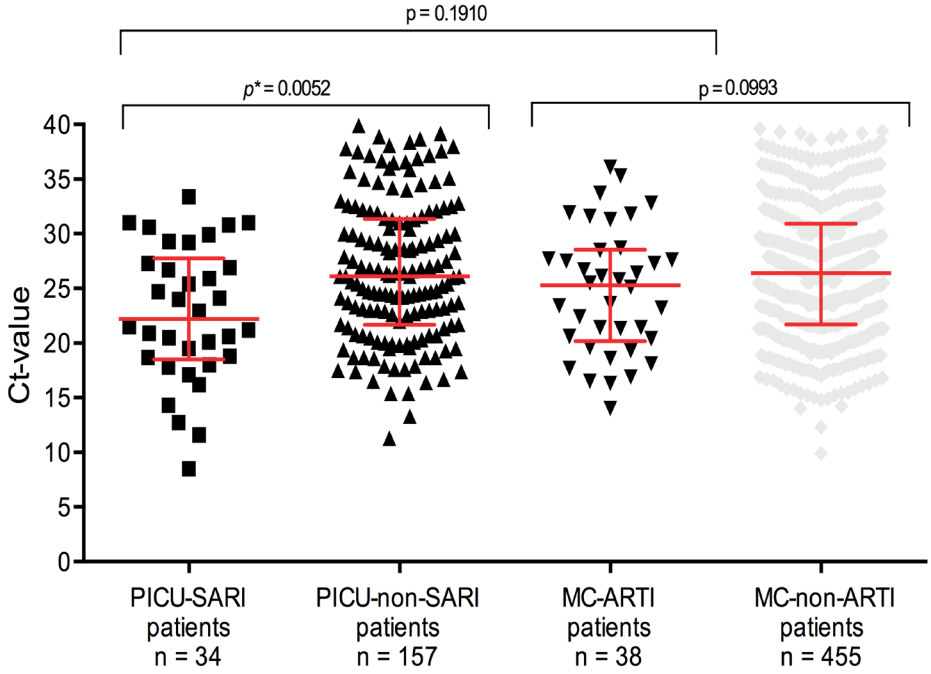


Figure 3. Cycle threshold (Ct) values of all single virus positive samples tested in nasal washings of patients admitted to the paediatric intensive care unit (PICU) with severe acute respiratory tract infections (SARIs) (PICU-SARI patients), all PICU admitted patients, patients admitted to medium care (MC) with acute respiratory tract infections (ARTIs) and all MC admitted patients between 2007 and 2012 (Note: Ct-values were not available for all samples, *with statistical significant difference).

DISCUSSION

In the present paper we have shown that in our tertiary care centre both SARI and ARTI may be caused by single viral pathogens, with RSV and HRV being the most frequent causes. In addition, nine others of the fifteen respiratory viruses tested for were identified as single agents that may be associated with SARI in previously healthy children as well as in children with a medical history. Except for ADV and HCoV-NL63, the remaining seven viruses were also associated with ARTI in previously healthy children as well as in children with a medical history. Our data does not support a relationship between viral load as reflected by surrogate marker Ct-values on the one hand and disease severity reflected by MC or PICU admission on the other. This is counter-intuitive and indeed various studies have indicated that higher viral loads are related to more severe disease as well as locally increased cytokine production (126,136,145,150–158). Other studies failed to replicate this finding (116,159–162). Our findings may have been

Figure 4A

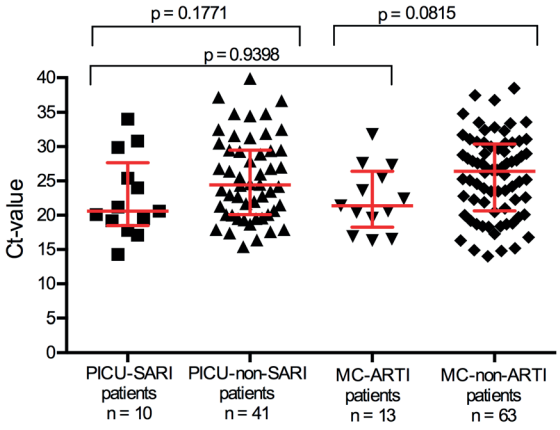


Figure 4B

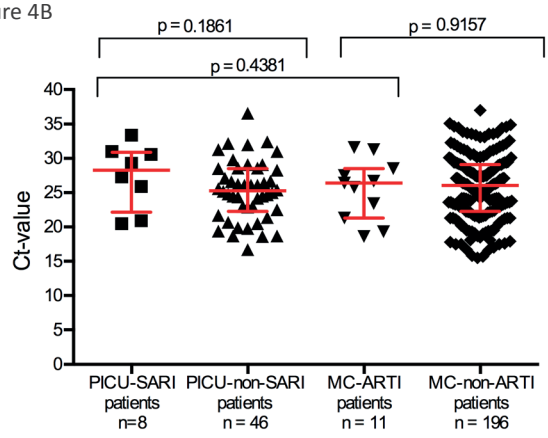


Figure 4A and 4B. Cycle threshold (Ct) values of positive samples for respiratory syncytial virus (RSV) (A) and human rhinovirus (HRV) (B) tested in nasal washings and compared between patients admitted to the paediatric intensive care unit (PICU) with severe acute respiratory tract infection (SARI) (PICU-SARI patients), all PICU admitted patients, patients admitted to medium care (MC) with acute respiratory tract infections (ARTIs) (MC-ARTI patients) and all MC admitted patients (Note: Ct-values were not available for all samples).

influenced by the selection of hospitalized and seriously ill children. Although Ct-values as presented in our study do not represent an absolute quantitation of viral loads, we did use these semi-quantitative values as a surrogate marker for viral load in our patient samples, as is not unusual in this type of studies (126,154). Moreover, viral load may be one of many factors that could be associated with disease severity. In this respect the

role of a deleterious or counter-productive immune response may be a factor related to disease severity. This has been subject of several studies into the pathogenesis of viral respiratory disease and a better understanding may lead to novel targets for treatment of critically ill patients (47,163–170).

In accordance with several other studies, RSV and HRV were the most frequently detected respiratory pathogens throughout the study (4,47,128,142). RSV is a well-established respiratory virus known to cause severe disease by itself, especially in younger children (4,47,128). For HRV this role as single pathogenic agent has been less well established as it is frequently detected in combination with other respiratory viruses, and has therefore frequently been considered an innocent bystander. We described eleven critically ill patients with HRV as single pathogen detected, supporting other studies that have described HRV as an agent that may cause more or less severe disease (142,171,172). Less common respiratory viruses such as HCoV and PIV 1-4 were detected, but additional analyses were not performed due to a lack of power.

Previous studies have demonstrated a higher susceptibility to several respiratory viruses in children with certain risk factors such as pre-term birth and broncho-pulmonary dysplasia (4,39). Indeed pre-term birth was also a marked factor related to hospitalization in our study. In agreement with other studies, RSV proved to be the most common pathogen detected in previously healthy children requiring PICU admission (4,127,128). It is interesting to note that HBoV and influenza A virus were also found in previously healthy PICU-SARI children. Previously we showed that also HBoV can cause SARI in paediatric patients (158). Two of our PICU-SARI patients died, which is comparable to previously published frequencies (3,4).

Our selection for a single virus only with an unlikely typical bacterial infection is largely dependent on CRP-levels, which are frequently tested in clinical settings and used to help differentiate between viral and bacterial infections (137,173). We are aware that the low CRP-levels (≤ 40 mg/L) tested within 24 hours after admission used in our study, may underestimate the occurrence of a bacterial infection or bacterial super-infection. Indeed we cannot fully exclude that some of our patients may have had secondary or pre-existing bacterial infections during hospitalization. We can also not fully exclude the presence of atypical bacteria such as *M. pneumoniae, C. pneumoniae* or *B. pertussis* as these were not routinely tested for. Still, considering the very low CRP used to include our patients, we feel that major bacterial involvement was unlikely at the time of hospital admittance (174).

Although this is a retrospective study from one tertiary care centre only, and bacterial cultures could not be performed for all patients for obvious reasons, we identified 44

PICU-SARI patients and 40 MC-ARTI patients infected with one single virus only from a total of 3269 patient samples over a 5-year period. The data generated shed new light on the aetiological role of several respiratory viruses: both SARI and ARTI can be caused by several single viral pathogens in the absence of viral and typical bacterial co-infections.

Acknowledgement

We thank Hans Kruining for his help with obtaining the data for the database.

S1 Table. Reasons for paediatric intensive care unit (PICU) admission of non-severe acute respiratory tract infection (SARI) patients (n=165) with respiratory tract samples tested for fifteen respiratory viruses at Erasmus MC-Sophia over a 5-year period (2007-2012).

	Reason for admission PICU-non-SARI patients												
Viruses detected	**ARTI sample obtained > 72 hours**	**Respiratory non-ARTI**	**Cardio-vascular disease**	**Neurology**	**Gastro-intestinal**	**Anatomical malformations**	**Sepsis like illness / severe systemic infection**	**Neutropenic fever / oncology**	**Others***	**Metabolic disorders**	**Skeletal-muscular disorders**	**Insufficient data available**	**Total**
Number of patients	n=53 (%)	n=13 (%)	n=19 (%)	n=16 (%)	n=7 (%)	n=7 (%)	n=6 (%)	n=3 (%)	n=3 (%)	n=3 (%)	n=1 (%)	n=34 (%)	165
Rhinovirus	18 (34)	4 (31)	9 (47)	4 (25)	5 (71)	1 (14)	2 (33)	1 (33)	3 (100)			13 (38)	60
Respiratory syncytial virus	12 (23)	4 (31)	2 (11)	2 (13)	1 (14)	4 (57)						5 (15)	30
Adenovirus	7 (13)		2 (11)	3 (19)		1 (14)	1 (17)	1 (33)		1 (33)		5 (15)	21
Human bocavirus	4 (8)	1 (8)		2 (13)		1 (14)	3 (50)					2 (6)	13
Influenza A virus	2 (4)	1 (8)	2 (11)							1 (33)	1 (100)	1 (3)	8
Human metapneumovirus				2 (13)				1 (33)				1 (3)	4
Parainfluenza virus type 1	3 (6)	2 (15)		1 (6)						1 (33)			7
Human coronavirus OC43					1 (14)							1 (3)	7
Parainfluenza virus type 3	2 (4)	1 (8)										2 (6)	5
Parainfluenza virus type 4	1 (2)		2 (11)									1 (3)	4
Human coronavirus NL63	4 (8)		1 (5)	1 (6)								2 (6)	3
Influenza B virus				1 (6)									1
Parainfluenza virus type 2												1 (3)	1
Human coronavirus 229E			1 (5)										1

PICU, paediatric intensive care unit; SARI, severe acute respiratory tract infection; ARTI, acute respiratory tract infection
*Apparent life threatening event (ALTE) in 2 patients, 1 trauma patient

S2 Table. Reasons for medium care (MC) admissions of non-acute respiratory tract infection (ARTI) patients (n=487) with respiratory tract samples tested for fifteen respiratory viruses at Erasmus MC-Sophia over a 5-year period (2007-2012).

	Reason for admission MC-non-ARTI patients																
Viruses detected	**ARTI sample obtained > 72 hours**	**Respiratory non-ARTI**	**Cardio-vascular disease**	**Neurology**	**Gastro-intestinal**	**Urology**	**Immunology**	**Skeletalmuscular disorders**	**Sepsis like illness / severe systemic infection**	**Neutropenic fever / oncology**	**Others***	**Metabolic disorders**	**Anatomical malformations**	**Transfer after PICU admission**	**Transfer after NICU admission**	**Insufficient data available**	Total
Number of patients	n=31 (%)	n=6 (%)	n=41 (%)	n=30 (%)	n=47 (%)	n=16 (%)	n=4 (%)	n=4 (%)	n=18 (%)	n=98 (%)	n=4 (%)	n=6 (%)	n=10 (%)	n=4 (%)	n=3 (%)	n=165 (%)	487
Rhinovirus	15 (48)	2 (33)	22 (54)	12 (40)	25 (53)	4 (25)	1 (25)		8 (44)	51 (52)	1 (25)	2 (33)	9 (90)	3 (75)	3 (100)	56 (34)	214
Respiratory syncytial virus	3 (10)	2 (33)		2 (7)	7 (15)	1 (6)		3 (75)	2 (11)	12 (12)	1 (25)	2 (33)	1 (10)			24 (15)	60
Adenovirus	1 (3)		3 (7)	2 (7)	2 (4)	1 (6)		1 (25)	2 (11)	1 (1)						11 (7)	24
Human bocavirus		1 (17)	3 (7)	1 (3)	7 (15)	3 (19)			2 (11)	8 (8)						11 (7)	36
Influenza A virus	2 (7)		2 (5)	2 (7)	1 (2)	3 (19)			2 (11)	5 (5)	1 (25)			1 (25)		16 (10)	35
Human metapneumovirus	1 (3)		2 (5)	2 (7)						6 (6)						7 (4)	18
Parainfluenza virus type 1				1 (3)		1 (6)										5 (3)	7
Human coronavirus OC43			1 (2)							2 (2)		1 (17)				6 (4)	10
Parainfluenza virus type 3	6 (19)		3 (7)	1 (3)	3 (6)	2 (13)				2 (2)	1 (25)					8 (5)	26
Parainfluenza virus type 4	1 (3)	1 (17)	1 (2)	1 (3)						2 (2)		1 (17)				4 (2)	11
Human coronavirus NL63			2 (5)	1 (3)	1 (2)		1 (25)		2 (11)	1 (1)						10 (6)	18
Influenza B virus			1 (2)	4 (13)		1 (6)	2 (50)			4 (4)						2 (1)	14
Parainfluenza virus type 2	1 (3)			1 (3)						1 (1)						4 (2)	7
Human coronavirus 229E	1 (3)		1 (2)		1 (2)					3 (3)						1 (1)	7

MC, medium care; ARTI, acute respiratory tract infection

*1 multitrauma patient; 1 patients with suspected child battering; 2 patients admitted for evaluation failure to thrive

S3 Table. Bacterial co-infections in single respiratory virus positive patients admitted to the paediatric intensive care unit (PICU) with severe acute respiratory tract infection (SARI) at Erasmus MC-Sophia over a 5-year period (2007-2012).

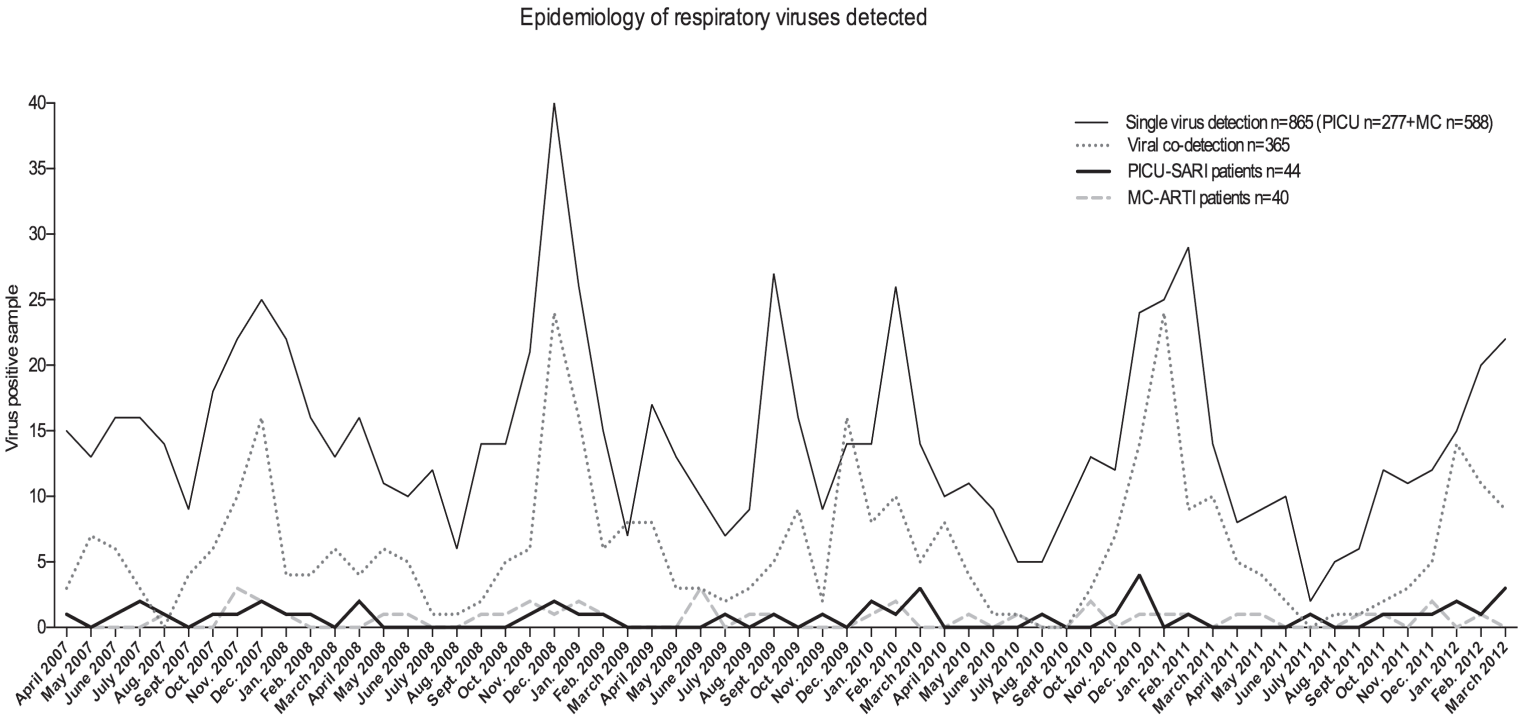
Virus detected	Ct-value	Sputum sample obtained <48 hours after admission	Sputum test result	Bloodculture sample obtained <48 hours after admission	Blood-culture test result	Other samples tested for bacteria
Respiratory syncytial virus	26	Yes	negative	Yes	negative	NASW^: PCR *M. pneumoniae*
Respiratory syncytial virus	20	Yes	*H. influenzae*, *S. pneumoniae*, *S. aureus*	Yes	negative	
Respiratory syncytial virus	27	Yes	*H. influenzae*, *S. aureus*	No		
Respiratory syncytial virus	22	Yes	*M. catarrhalis*	No		
Respiratory syncytial virus	19	Yes	*E. coli*	Yes	negative	
Influenza A virus	27	Yes	> 10 bacteria per ocular field in gram stain	No		
Influenza A virus	35	No (336)	negative	yes	*S. aureus*	BAL**: *S. aureus*
Rhinovirus	18	Yes	*E. coli*	No		
Rhinovirus	24	Yes	*S. pneumoniae*	Yes	*S. pneumoniae*	
Rhinovirus	22	Yes	*P. auruginosa*, *Corynebacterium sp.*	No		
Parainfluenza virus type 3	33	Yes	*S. pneumoniae*, *M. catarrhalis*	No	negative	
Human bocavirus	39	Yes	*K. pneumoniae*, *E.coli*	Yes	negative	
Adenovirus	21	Yes	*P. auruginosa*	No		
Human metapneumovirus	20	Yes	*S. aureus*	No		
Respiratory syncytial virus	24	Yes	*P. auruginosa*, *S. pyogenes*	No		
Respiratory syncytial virus	23	Yes	*H. influenzae*	Yes	negative	
Respiratory syncytial virus	22	Yes	*P. auruginosa*	No		
Rhinovirus	23	Yes	*S. pneumoniae*, *S. aureus*	Yes	negative	
Rhinovirus	29	Yes	*P. aeruginosa*, *M. catarrhalis*	No		
Adenovirus	32	Yes	*P. aeruginosa*	No		
Human metapneumovirus	21	No (72)	negative	Yes	*Coagulase negative S. aureus*	
Rhinovirus	19	Yes	*M. catarrhalis*		No	
Rhinovirus	29	Yes	*S. aureus*		No	

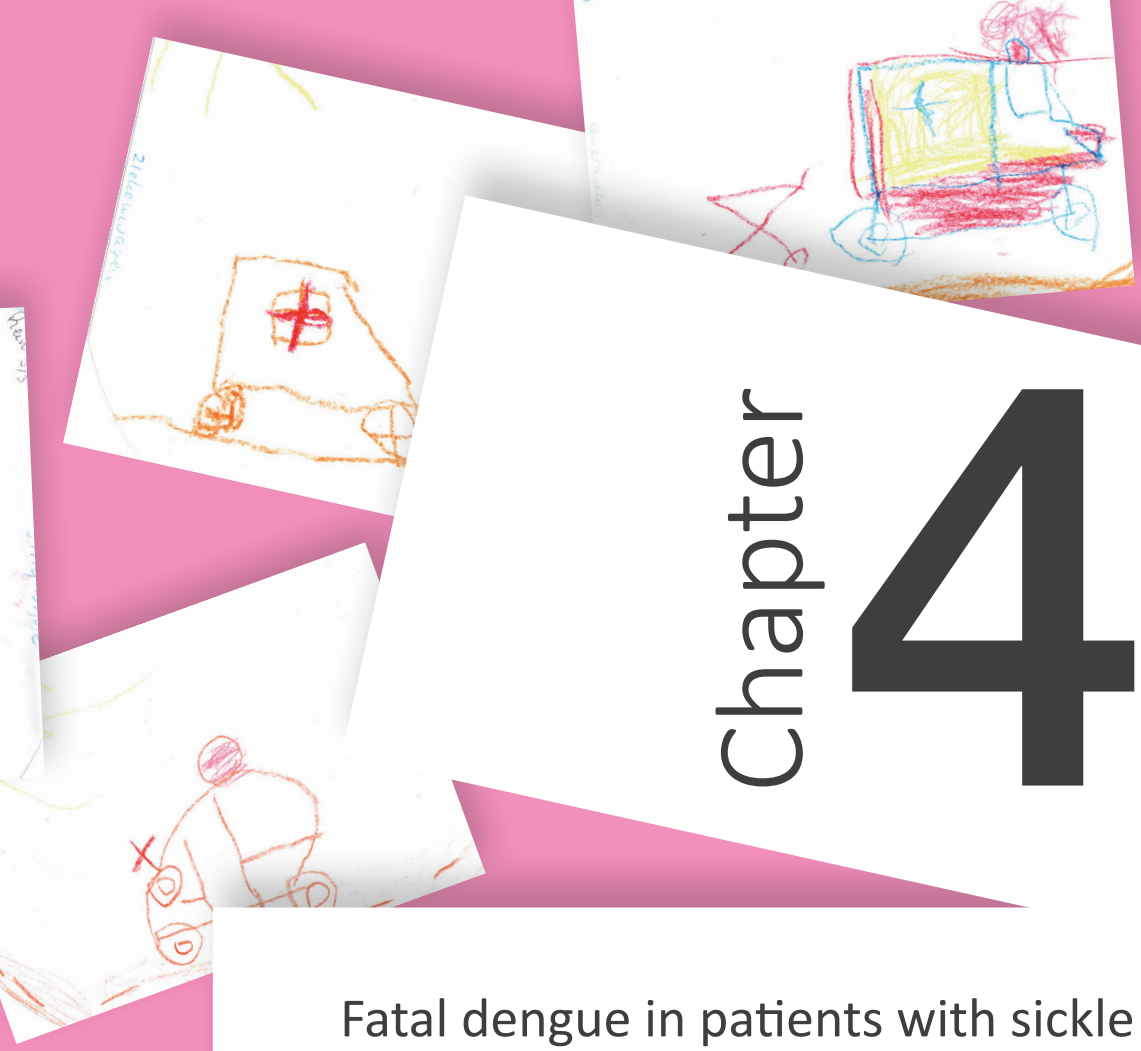
S4 Table. Bacterial co-infections in single respiratory virus positive patients admitted to medium care (MC) with acute respiratory tract infection (ARTI) at Erasmus MC-Sophia over a 5-year period (2007-2012).

Virus detected	Ct-value	Sputum sample obtained <48 hours after admission	Sputum test result	Bloodculture sample obtained < 48 hours after admission	Bloodculture test result	Other samples tested for bacteria
Parainfluenza virus type 3	37	Yes	*P. aeruginosa*	No		
Rhinovirus	30	Yes	*P. aeruginosa*	No		
Parainfluenza virus type 3	N.A.*	Yes	*S. pneumoniae*, *H. influenzae*	No		
Parainfluenza virus type 3	25	Yes	*S. aureus*, *E. coli*	No		
Human coronavirus OC43	28	Yes	*Aspergillus sp.*, *S. maltophilia*, *P. auruginosa*	No		
Human coronavirus OC43	24	Yes	*M. catarrhalis*, *H. influenzae*, *E.coli*	No		
Respiratory syncytial virus	29	Yes	*S. aureus*	No		
Rhinovirus	33	Yes	*M. catarrhalis*	Yes	negative	
Influenza A virus	32	Yes	*H. influenzae*	No		Throatswab: *C. albicans*
Parainfluenza virus type 4	22	Yes	*P. auruginosa*	No		
Human metapneumovirus	37	Yes	*P. auruginosa*	No		

*N.A., not available

S1 Figure. Epidemiology of respiratory virused detected at Erasmus MC-Sophia over a 5-year period (2007-2012).





Chapter 4

Fatal dengue in patients with sickle cell disease and sickle cell anemia in Curaçao: two case reports.

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PLOS Neglected Tropical Diseases 2013; 7(8):e2203

Presentation of Cases

Case 1. A 10-year-old girl presented to our clinic with a history of four days fever, malaise and a generalized tonic-clonic convulsion. She had been diagnosed with SCD type HbSC before. At presentation she was alert, felt nauseous and complained of headache and painful eyes. Temperature was 38.0°C, heart rate (HR) 135/min, blood pressure (BP) 94/62 mmHg, respiratory rate (RR) 20/min; liver and spleen were 1 cm palpable, abdominal tenderness was present in the right upper quadrant. No further abnormalities were reported on physical examination. Initial lab results revealed a moderate increase of CRP, anemia with thrombocytopenia, increased liver enzymes, and decreased albumin and hematocrit (Ht) (**Table S1**). On day two, during defervescence she became hypotensive and was treated with further hyperhydration and inotropic agents. Blood products were transfused to correct anemia and thrombocytopenia. Acute hemorrhage, hemolysis and splenic sequestration were considered, but could not be confirmed with abdominal ultrasound. However, pleural effusion and ascites were seen. On day three she lost vision. A brain CT-scan revealed no abnormalities. Lumbar puncture was deferred and antibiotic treatment was adjusted to cover possible meningitis. Hereafter she developed acute respiratory distress syndrome (ARDS) requiring mechanical ventilation. Dengue serology was positive for IgM (titer: 3.26) and IgG (titer: 3.26) and RT-PCR showed dengue serotype 2 (DENV-2). Her clinical situation deteriorated with an increased hemorrhagic tendency, and blood, clotting products, and clotting factors were administered. In time multi organ dysfunction syndrome (MODS) developed and deteriorated. Blood cultures remained negative for bacteria, but Candida was cultured and antifungals were initiated. Despite these efforts the patient died 28 days after admission.

Case 2. A 19-year-old female presented to our clinic with a history of fever, headache, retro-orbital pain, and muscle ache for two days, hematemesis and epistaxis starting on the day of admission. She had arrived a few weeks earlier in Curaçao where she was born and raised till the age of 7. Her medical history revealed SCA. On examination she was alert, with a body temperature of 37°C, BP 120/80 mm Hg, HR 100/min and RR 15-20 / min. She had abdominal tenderness in the right upper quadrant. No other abnormalities on physical examination were found. Initial lab results revealed moderate increase of CRP, anemia with thrombocytopenia and increased liver enzymes. NS1-antigen tested positive and dengue serology was negative for IgM (titer 0.41) and positive for IgG (5.71), RT-PCR showed DENV-2. Shortly after admittance she became hypotensive, with distension of her abdomen and was admitted to the intensive care unit. Profuse abdominal bleeding was suspected with marked decrease of hemoglobin, low platelets, severe disrupted coagulation parameters and intra- and retro-abdominal free fluid with fibrin strands on ultrasound. She was intubated on a seizure. Although in the course of time shock and bleeding stabilized, she continued to develop MODS. In addition, both

pupils became less reactive to light until fully non reactive. CT-brain imaging could not be performed. On day 4 she died upon cardiac arrest.

Case Discussion

Dengue and Curaçao

During the past decades dengue has been reported to occur in Curaçao. Until now fatal cases were never documented. However the 2010-2011 outbreak with 1822 serologically confirmed cases was associated with four deaths (personal communication). Two of these four patients, were previously diagnosed with sickle cell disease (SCD) (combined heterozygosity for the abnormal hemoglobin S and normal Hb C; HbSC) and sickle cell anemia (SCA homozygosity for abnormal hemoglobin S; HbSS). Although chronic diseases such as SCD are considered to be a risk factor for the development of severe dengue, few cases of children or adolescents have been reported (175).

Short Literature Review

We gave a detailed description of two fatal dengue cases with underlying SCD that occurred in Curaçao where SCD is not uncommon (176). These cases indicate that SCD may predispose for severe manifestations of dengue infection. In the Americas all four serotypes of DENV circulate as well as in Asia where fatal dengue is frequently

Table 1. Summary of literature review

Author, year of publication	Country	Number of Patients	Gender/ age (years)	SCD type	**Dengue virus serotype**	**Outcome**
Present case 1	Curaçao	1	F/10	HbSC	DENV-2	Died
Present case 2	Curaçao	1	F/19	HbSS	DENV-2	Died
Limonta et al., 2009	Cuba	2	M/34 M/25	HbSC HbSA	DENV-3 (s) DENV-3 (s)	Died Died
Andrianarisoa et al., 2007	Madagascar	ND	ND	ND	ND	ND
Ware et al., 1999	Jamaica	1	F/19	HbSS	ND	Died
Teruel-Lopez et al., 1991**	Venezuela	ND	ND	ND	ND	ND
Bravo et al., 1987	Cuba	4 4	Adults Children	HbSS HbSS	DENV-2 (ND) DENV-2 (ND)	Died Died
Gentilini et al., 1964 *	Haiti	ND	ND	ND	ND	ND

M, male; F, female; *, no abstract available; ND, no data available; DENV, dengue virus serotype; (s), secondary; HbSC, heterozygote sickle cell disease; HbSS, homozygote sickle cell anemia; HbAS, sickle cell trait.** review describing Bravo et al article.

reported. Hitherto no cases have been found in Asia in SCD patients, probably due to the relatively low prevalence of SCD (177). Furthermore reports describing patients with this combination of diseases are scarce, little detailed and restricted to a few fatal cases and apart form the study from Cuba concern adult patients only (see **Table 1**) (178–184).

Presented Cases and Treatment Difficulties

The clinical findings of the two cases described here are difficult to interpret because of the apparent complementary vascular endothelial damage in SCD, SCA and severe dengue infection. Interesting are the essential similarities between these cases: shock, plasma leakage, hemorrhage and MODS (175). The onset of vaso-occlusion with SCD and SCA is often to be triggered by inflammation, as is the case in a dengue infection (185). A dengue review reports that severe bleeding seldom happens in children (186). Still our cases proved otherwise in this distinct risk group. An additional challenge was that treatment is basically different for both diseases. In SCD and SCA it is preferred to have a low Ht to reduce the risk of vaso-occlusion whereas anemia as a result of dengue may necessitate transfusion. The WHO reports an increase of Ht as a sign of plasma leakage and a decrease may be due to hemorrhage. Both cases show a continuing decrease of Ht, associated with hemorrhage, but plasma leakage was predominating as well suggesting not to use this marker in patients with SCD and SCA. Iatrogenic interstitial fluid overload in patients with SCD, SCA and dengue infection because of pre-existing vascular endothelial damage is not an imaginary hazard. Whether the clinical manifestations would have occurred due to a secondary dengue infection only, despite SCD or SCA remains a matter for debate.

This report is based on two clinical cases of SCD, SCA and severe dengue during the 2010-2011 epidemic in Curaçao. There is little information on how to treat these patients.

In populations with a high prevalence of SCD, SCA and dengue activity clinicians should be aware of the possible pitfalls in clinical management.

Chapter 5

Diagnostic performance of influenza viruses and RSV rapid antigen detection tests in children in tertiary care

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ABSTRACT

Background: Rapid antigen detection tests (RADTs) are increasingly used to detect influenza viruses and respiratory syncytial virus (RSV). However, their sensitivity and specificity are a matter of debate, challenging their clinical usefulness.

Objectives: Comparing diagnostic performances of BinaxNow Influenza AB® (BNI) and BinaxNow RSV® (BNR), to those of real-time reverse transcriptase PCR (RT-PCR), virus isolation and direct immunofluorescence (D-IF) in paediatric patients.

Study design: Between November 2005 and September 2013, 521 nasal washings from symptomatic children (age <5 years) attending our tertiary care centre were tested, with a combination of the respective assays using RT-PCR as gold standard.

Results: Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of BNI were 69% (confidence interval [CI] [51-83]), 96% [94-97], 55% [39-70] and 98% [96-99] respectively. Of eleven false-negative samples, RT-PCR Ct-values were higher than all RT-PCR positive test results (27 vs 22, p=0.012). Of twenty false-positive samples, none were culture positive and two tested positive in D-IF.

Sensitivity, specificity, PPV and NPV for BNR were 79% [73-85], 98% [96-99], 97% [93-99] and 88% [84-91]. Of the 42 false-negative samples the median Ct-value was higher than that of all RT-PCR positive samples (31 vs 23, p<0.0001). Five false-positive samples were detected. Three of these tested positive for RSV in virus isolation and D-IF.

Conclusions*:* RADTs have a high specificity with BNR being superior to BNI. However*,* their relative low sensitivity limits their usefulness for clinical decision making in a tertiary care paediatric hospital.

BACKGROUND

Influenza viruses and respiratory syncytial viruses (RSV) cause acute respiratory tract infections (ARTIs) in children, being a leading cause of hospitalization (4,127,187). Identification of both viruses is important for disease management, as the presence of these infections may require specific treatment (i.e. oseltamivir) and hospital containment measures. The current gold standard for detection of these viruses is real-time reverse transcriptase PCR (RT-PCR) (40). This is however not performed in all hospitals, as it requires a molecular diagnostic laboratory with specialized personnel and equipment. Instead, rapid antigen detection tests (RADTs) are used as these assays are easier and cheaper to perform and less time-consuming (43–45). The performance of these tests depends on factors like time between disease onset and sampling, quality and type of specimen and epidemiological parameters (188). Diagnostic value and clinical usefulness of RADTs for influenza diagnosis vary greatly (43–45,189–192). This prompted us to evaluate the diagnostic performance of the routinely used RADTs (manufactured by Alere BinaxNOW®) for these two viruses as used in our tertiary care paediatric hospital.

OBJECTIVES

Comparing diagnostic performances of two RADTs, BinaxNow Influenza AB® (BNI) and BinaxNow RSV® (BNR), with those of RT-PCR in samples of paediatric patients attending our tertiary care centre with ARTIs for a period of almost eight consecutive years. Discrepant data were subsequently compared with those of virus isolation and direct immunofluorescence (D-IF) assays.

STUDY DESIGN

This study was conducted from November 2005 through September 2013, we identified paediatric patients between 0-5 years who attended Erasmus MC-Sophia's emergency department, out-patient-clinic and those who were hospitalized in this period. To analyse the performance of the BNI and BNR compared to RT-PCR we selected 521 nasal washings of 489 patients with a median age of 4 months (minimum 0.03 - maximum 58 months, lower interquartile range 1.6 - upper interquartile range 9.8) and 55% (268/489) were male. Nasal washings were obtained during routine clinical practice in symptomatic children and were tested immediately after sampling by trained laboratory personnel using all four diagnostic methods. Multiple samples from the same patient were included in our analysis. Therefore patients are referred to as cases. Data regarding gender, age and hospital admission were obtained from the electronic patient files.

Ethics

Data collection and analyses were conducted on anonymized samples, which does not require further medical ethics review as consented by our Medical ethical board (MEC-2015-306).

Tests

RT-PCR gold standard

All nasal washings were tested for the presence of selected viruses by means of RT-PCR with primers and probes sets used in the routine setting of our department (42). In short, RNA and DNA were extracted using MagnaPureLC (Roche Diagnostics, Almere, the Netherlands) and the total nucleic acid isolation kit. The extractions were internally controlled by addition of a known concentration of phocine distemper virus (PDV) and phocine herpes virus (PHV). Uni-plex RT-PCR was used to detect RSV-A, RSV-B, human rhinovirus (HRV), parainfluenza virus (PIV) type 3 (PIV-3), adenovirus (ADV), and human bocavirus (HBoV). Duplex reactions were performed combining influenza A virus and PDV, influenza B virus and human coronavirus (HCoV) OC43 (HCoVOC43), human metapneumovirus (HMPV) and PIV-2, HCoV229E and PIV-4, and HCoVNL63 and PIV-1. A cycle threshold value (Ct-value) of <40 was defined positive for any virus. RT-PCRs were developed in-house for influenza viruses and RSV-A and validated (42). RSV-B primers and probes were used as reported by Dewhurst-Maridor et al. (193).

Rapid antigen detection tests (RADTs)

Alere BinaxNOW® Influenza A and B (BNI) and Alere BinaxNOW® RSV (BNR) (Scarborough, Maine, USA) are commercially available in vitro immunochromatographic assays for the qualitative detection with monoclonal antibodies directed against influenza A and B virus nucleoproteins and RSV fusion protein antigen, respectively. Nasal washings were obtained using standard protocols and rapid antigen testing was performed as described by the manufacturer. For our analyses the test results of BNI influenza A and influenza B were combined into a single influenza BNI dataset as influenza B was not encountered frequently with only four influenza B BNI positive samples, two of which were influenza B RT-PCR positive.

Virus isolation assay

Virus isolation assays were always performed in combination with D-IF. Madin-Darby Canine Kidney (MDCK) cell line (NBL-2) (ATCC® CCL-34™) and the human cell line HEp-2 (ATCC® CCL-23™) were used to isolate influenza viruses and RSV respectively. Virus cultures were regularly checked for cytopathic effect by light microscopy. Immunofluorescence with fluorescein isothiocyanate (FITC) labeled monoclonal antibodies was used to confirm the presence of influenza virus or RSV (194).

Direct immunofluorescence (D-IF) assays in clinical specimens

Cells were isolated from nasal washings, dried on microscope slides, and fixed with acetone. Subsequently, cells were stained with FITC conjugated monoclonal antibodies against influenza A virus, influenza B virus or RSV (IMAGEN™ Influenza A and B and IMAGEN™ RSV, Hampshire, United Kingdom). Specimens were incubated with FITC-conjugated antibodies for 15 minutes at 37°C, subsequently excess reagent was washed off with phosphate buffered saline. The stained area was then mounted and viewed by fluorescent microscopy.

Comparison between tests

The focus of our study was to compare data obtained with two RADTs, BNI and BNR with those obtained by RT-PCR as gold standard. We defined false-negative tests as those for which the rapid test was negative and the gold standard RT-PCR positive; a false-positive test result was defined if the rapid test tested positive and the gold standard RT-PCR tested negative. We compared the available Ct-values in all respective categories of samples and analysed whether there was an association between Ct-values and RADTs results and hospitalization. For influenza all Ct-values were available, for RSV Ct-values were available for 183/204 (90%) of the performed tests. Missing Ct-values were from samples tested in 2005 and 2006 when routine input of Ct-values in our laboratory system was not yet performed and digital documentation was not available. Finally, false-negative and false-positive test results were compared to test results obtained with the other virus detection methods: virus isolation and D-IF assays.

Statistical analyses

The main outcomes of this study were the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the BNI and BNR rapid test results compared to RT-PCR during the total study period and during viral season (October 1st through March 31st). Ct-values were compared with Mann-Whitney U tests.

RESULTS

Sensitivity and specificity of BNI

Of 521 nasal washings both influenza RT-PCR and BNI data were available. Most were obtained between September and March (supplemental data **Figure 1** and **Figure 2**). Of these, 35 cases tested positive with RT-PCR (35/521, 7%, median Ct-value 22 [range] [17–39]) whereas 44 tested positive in the BNI (44/521, 8%). Of the 35 RT-PCR positive cases 24 also tested positive in the BNI (24/35, 69%, median Ct-value 21 [17-31]). The eleven RT-PCR positive (median Ct-value 27 [18-39]) and BNI negative cases were considered false-

Table 1. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of rapid antigen detection test BinaxNOW influenza AB® and BinaxNOW RSV® compared to gold standard RT-PCR tested in nasal washings of children between 0-5 years at Erasmus MC-Sophia from 2005-2013.

Rapid antigen detection tests	Sensitivity (%) [95% confidence interval; CI] n	Specificity (%) [95% CI] n	PPV (%) [95% CI] n	NPV (%) [95% CI] n
BinaxNow Influenza AB®	68.6 [51-83]	95.8 [94-97]	54.5 [39-70]	97.7 [96-99]
vs RT-PCR n=521	24/35	466/486	24/44	466/477
BinaxNow RSV®	79.4 [73-85]	98.4 [96-99]	97 [93-99]	88 [84-91]
vs RT-PCR n=514	162/204	305/310	162/167	305/347
Samples obtained in respiratory virus season				
BinaxNow Influenza AB®	66.7 [48-82]	95.3 [93-97]	53.7 [37-69]	97.2 [95-99]
vs RT-PCR n=436	22/33	384/403	22/41	384/395
BinaxNow RSV®	80 [74-85]	98 [96-100]	98 [94-99]	85 [80-89]
vs RT-PCR n=428	160/200	224/228	160/164	224/264

negative cases (11/521, 2%). Of the 486 RT-PCR negative cases, 20 were BNI positive, and were therefore considered false-positive cases (20/521, 4%). Considering RT-PCR as the gold standard, it may be concluded that the BNI has a relatively low sensitivity of 69% (confidence interval (CI): [51-83]) (24/35), a high specificity of 96% [CI: 94-97] (466/486), a low PPV of 55% [CI: 39-70] (24/44) and a high NPV of 98% [CI: 96-99] (466/477) (**Table 1**). We also calculated these parameters only with samples obtained in the period from October 1st through March 31st, when respiratory viruses are more prevalent in the Netherlands. Sensitivity and specificity decreased with 2% and 1% respectively (69% to 67% and 96% to 95%). PPV and NPV both decreased with 1% from 55% to 54% and 98% to 97% respectively.

Sensitivity and specificity of BNR

Of 514 nasal washings both RSV RT-PCR and BNR data were available. Of these, 204 cases were RSV RT-PCR positive (204/514, 40%) with Ct-values available for 183 samples ranging from 14-39 (median Ct-value 23) and 167 were BNR positive (167/514, 32%) (**Table 1**). Hundred sixty-two samples were RT-PCR positive and BNR positive (162/514, 32%, median Ct-value 21 [14-35], no Ct-value available n=15). Forty-two cases were considered false- negative (42/514, 8%, median Ct-value 31 [22-39], no Ct-value available n=6). Of the 310 RT-PCR negative cases, five were BNR positive and considered false-positive cases (5/514, 1%). The overall test performance of BNR was relatively high with a sensitivity of 79% [CI: 73-85] (162/204), specificity of 98% [CI: 96-99] (305/310), PPV of 97% [CI: 93-99] (162/167) and NPV of 88% [CI: 84-91] (305/347). We also calculated these parameters

during the respiratory virus season (October-March) and sensitivity increased with 1% (79% to 80%), but the specificity remained the same (98%). PPV increased with 1% (from 97% to 98%) and NPV decreased with 3% from 88% to 85% respectively.

Discordant samples

False-negative rapid antigen detection tests

From the eleven false-negative BNI cases, influenza virus was successfully isolated in six cases (6/11, 55%, median Ct-value 25 [17-27]), three of which were also influenza D-IF positive. By means of RT-PCR, virus isolation or D-IF 7/11 (63%) samples tested positive for another virus, most frequently RSV (n=3) or adenovirus ADV (n=3) (**supplemental Table 1a**).

BNR results were considered false-negative in 42 cases, in 25 (60%) of those RSV was cultured successfully. In these 25 cases the Ct-values ranged from 22-39 with a median of 32. In addition, in 20/42 (48%) cases, RSV D-IF tested positive (median Ct-value 29 [22-37]). Co-infections were found in 16/42 (38%) cases and most often HRV (n=7). In four cases BNI tested positive for influenza, which could be confirmed for three samples by RT-PCR (**supplemental Table 1b**).

False-positive rapid antigen detection tests

Of the 20 influenza false-positive cases, six tested positive in BNR (6/20, 30%). Moreover, six cases tested RSV RT-PCR positive (6/20, 30%) of which five were also BNR positive. In eight samples another respiratory virus than influenza virus or RSV was detected with RT-PCR (8/20, 40%). For two samples the D-IF was positive for influenza, in accordance with the BNI, but for both virus isolation did not yield influenza virus (**supplemental Table 2a**).

For BNR only five false-positive cases were found. Three of these were RSV positive in virus isolation and D-IF (3/5, 60%). One case tested negative in all methods except for BNI (**supplemental Table 2b**). The sensitivity (80%), specificity (99%) and PPV (99%) increased if we considered the three RSV positive virus isolations as true-positive cases, resulting in only two false-positive cases (2/514, 0,4%).

Test results and hospitalization

Of all 521 patients tested for BNI and BNR 361 patients (361/521, 69%) were hospitalized. Hospitalization rates were 16/24 (67%) and 115/162 (71%) for true-positive BNI and BNR cases respectively. False-negative test results did not seem to have a major impact on hospitalization with hospitalization rates of 7/11 (64% vs 69%, p=1) and 25/42 (60% vs 71%, p=0.1912) for BNI and BNR test results respectively **(Table 2**).

Comparing the Ct-values of the respective categories, BNI and BNR cases that were false-negative displayed overall higher Ct-values (p=0.012 vs p<0.0001) (**Figure 1A** and

Table 2. BinaxNow Influenza AB® (BNI) and BinaxNOW RSV® (BNR) test results in relation to hospitalization of children between 0-5 years at Erasmus MC-Sophia from 2005-2013.

BNI	true-positive (%)	false-negative (%)	false-positive (%)
Admitted	16/24 (67)	7/11 (64)	16/20 (80)
Not admitted	8/24 (33)	4/11 (36)	2/20 (10)
Unknown	-	-	2/20 (10)
BNR			
Admitted	115/162 (71)	25/42 (60)	5/5 (100)
Not admitted	47/162 (29)	17/42 (40)	-

Figure 1B). However, no differences were found in Ct-values of hospitalized and non-hospitalized patients within the respective case groups ($p>0.5$ for both BNI and BNR) (**Figure 2A** and **Figure 2B**). The BNI test result did not differentiate for severe disease with three of the false-negative cases admitted to the paediatric intensive care unit (PICU), but also three false-positive cases. For BNR six patients were admitted to the PICU (6/42, 14%) despite a false-negative test result. None of the five BNR false-positive tested patients were admitted to the PICU (0/5).

DISCUSSION

The present study evaluated the diagnostic performance of BNI and BNR RADTs in a large number of symptomatic paediatric patients between 0-5 years attending our tertiary care paediatric hospital during almost eight consecutive years. By testing fresh nasal washings with RT-PCR, we found a relatively low sensitivity and PPV for BNI. The overall test performance of BNR scored higher for all these aspects. Both BNI and BNR false-negative cases displayed a significantly higher Ct-value compared to all RT-PCR positive and true-positive tested cases.

The accuracy of rapid tests is generally less than that of RT-PCR and virus isolation assays. However, RADTs are valuable as a point-of-care test for their ease of use, fast results and laboratory independence (188). These advantages, and especially the high specificity are important for their use as surveillance tools for influenza outbreaks as recommended by the World Health Organization (188). Indeed for surveillance purpose a high specificity is of importance, which we found to be the case in our study. However, for clinical management this is not sufficient. In theory, the decision to start antiviral therapy and to refrain from unnecessary further diagnostic testing and antibiotic use may be based on RADT (195–197). In addition, rapid test results may result in more effective isolation and containment measures (44). For this purpose assay sensitivity is of utmost importance. Therefore, we conclude that the relatively low sensitivity of the BNI in our tertiary care

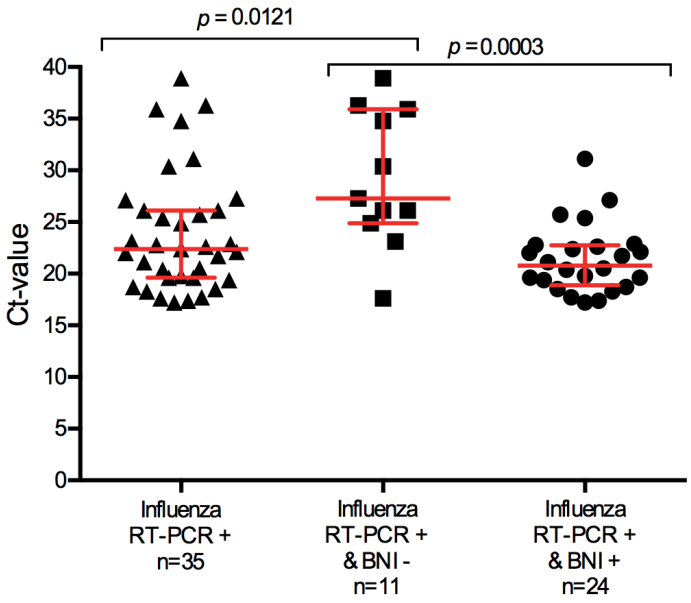


Figure 1A. Ct-values compared for BinaxNOW influenza AB® (BNI) rapid test positive results and rapid test negative results for influenza in children between 0-5 years at Erasmus MC-Sophia from 2005-2013.

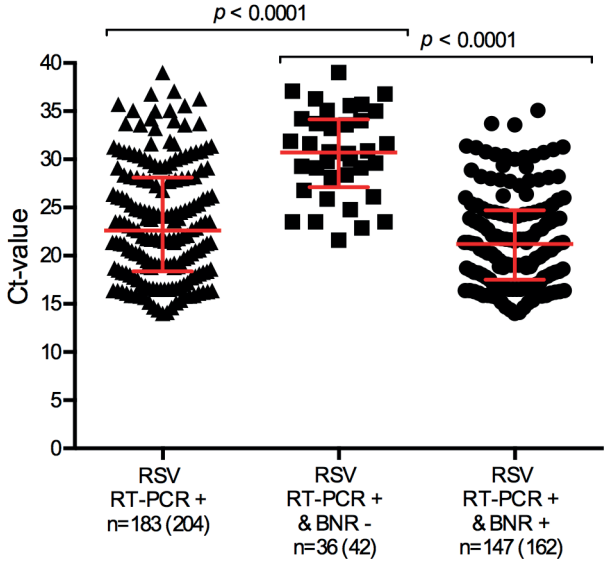


Figure 1B. Ct-values compared for BinaxNOW RSV® (BNR) rapid test positive results and rapid test negative results for RSV in children between 0-5 years at Erasmus MC-Sophia from 2005-2013. The number between ( ) refers to all RT-PCR positive results, for 21 samples no Ct-values were available.

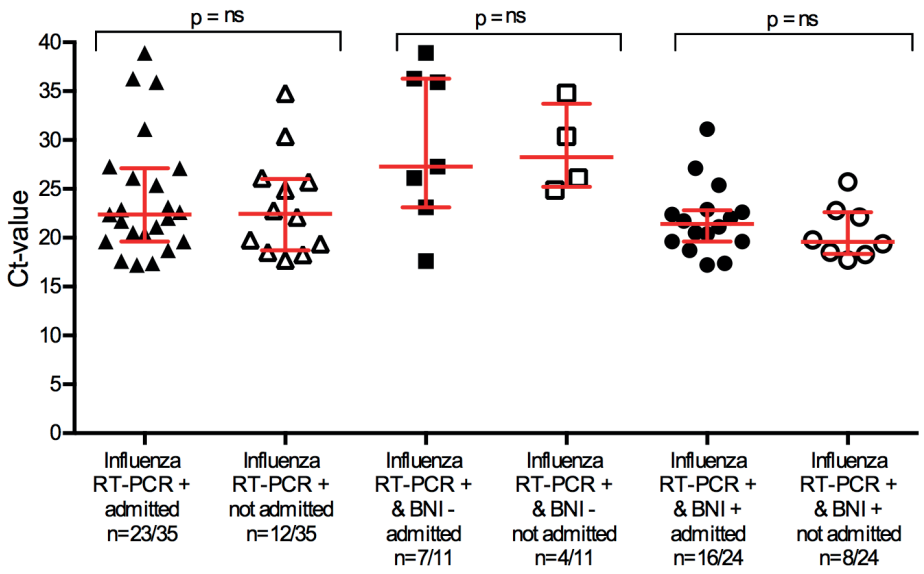


Figure 2A. Ct-values in relation to BinaxNOW influenza AB® (BNI) rapid test results and hospitalization of children between 0-5 years at Erasmus MC-Sophia from 2005-2013. ns= no statistical significant difference.

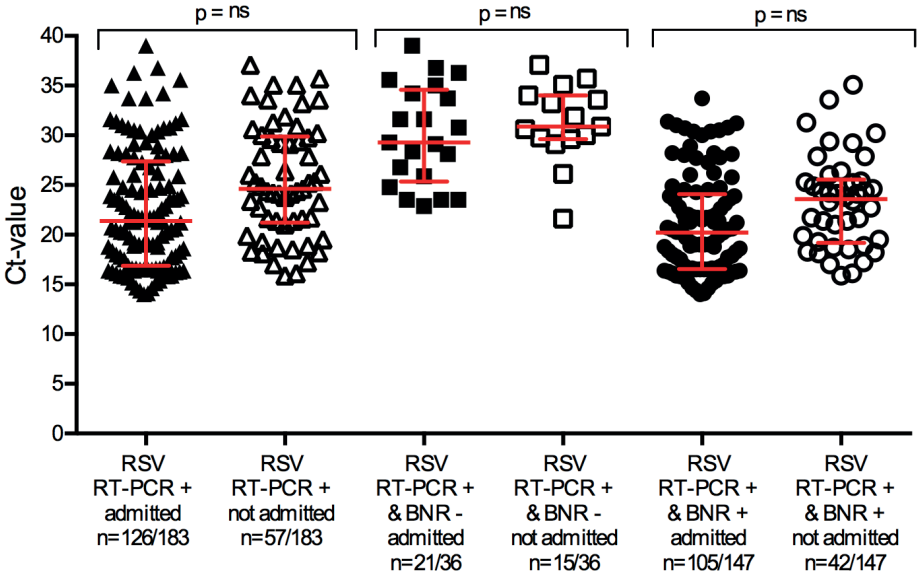


Figure 2B. Ct-values in relation to BinaxNOW RSV® (BNR) rapid test results and hospitalization of children between 0-5 years at Erasmus MC-Sophia from 2005-2013. ns= no statistical significant difference.

centre is worrisome. Of note, BNR test performance proved to be better compared to the BNI, although false-negative cases were detected. Based on our results we stopped using BNI and will use rapid PCR-based tests for detection of influenza virus and RSV.

We considered the clinical implications of a false-negative and false-positive test result in relation to hospitalization and found no significant differences between these groups and RT-PCR positive and BNI positive tested cases, indicating that the clinical observation is still pivotal in admission decision-making as suggested by current guidelines. In the present study we were not able to study effects of testing on treatment since antiviral medication was not routinely used in our hospital before 2009. Data on isolation and containment measures were not available.

Although the retrospective nature of our study has inherent limitations, we were able to include more than 500 samples of patients over time. Since, our study spanned a considerably longer period of time than previous studies, we were able to test the clinical feasibility of RADTs "in a real life" clinical setting and for different circulating virus subtypes during eight consecutive seasons. Overall, most studies reported a high specificity and high NPV versus a low sensitivity and low PPV for BNI, which is largely in agreement with our results (43–45,189–192). We did find a relatively high sensitivity of 79% and an even higher PPV (97%) for BNR, which is in agreement with data obtained in other studies (43,198,199).

During the last decade, RT-PCR has become the gold standard for detecting the presence of respiratory viruses (40). The downside of RT-PCR is the relatively long time (6-24 hours) between sample collection and availability of test results (200). This makes current RT-PCR formats less useful for admission decision-making and calls for faster methods. Indeed, new rapid point-of-care PCRs are being developed and implemented with a shorter turnaround time (201–206). Studies comparing their performances with those of RT-PCR would allow us to judge their potential for clinical decision-making.

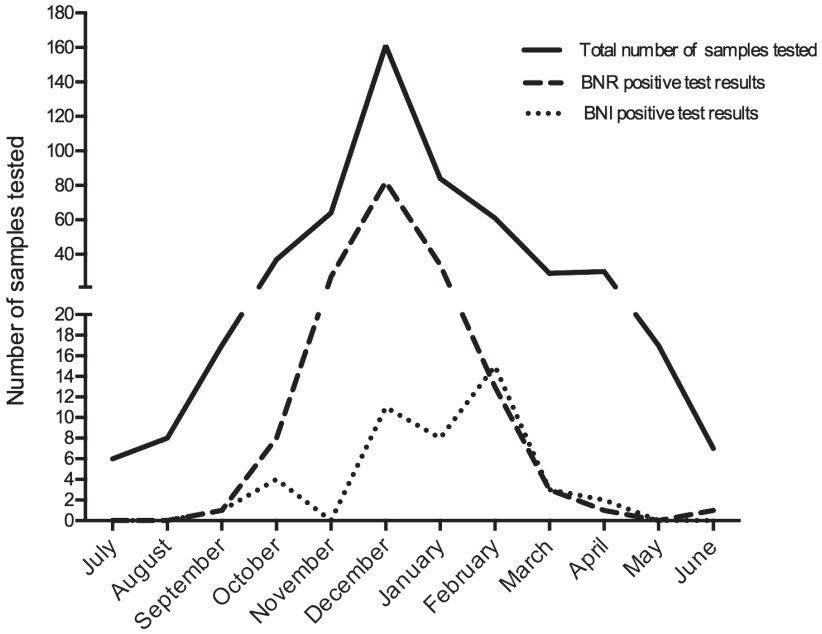
In conclusion, we evaluated the performance of the RADTs BNI and BNR in a tertiary care paediatric hospital setting over eight consecutive years. We showed that sensitivity and PPV of BNI were relatively low (69% and 55%), whereas those of BNR were higher (79% and 97%) when compared to the respective gold standard RT-PCR. False-negative samples consistently displayed high Ct-values, although this did not influence whether patients were hospitalized or not. Given the relatively low sensitivity and PPV of the BNI we strongly advocate a restricted use of BNI or similar rapid influenza antigen detection assays in a tertiary paediatric care setting. In contrast, the higher sensitivity and PPV of the BNR rendered this rapid test more useful, albeit still less sensitive than RT-PCR.

Ethical approval: Data collection and analyses were conducted on anonymized samples, which does not require further medical ethics review as consented by our Medical ethical board (MEC-2015-306).

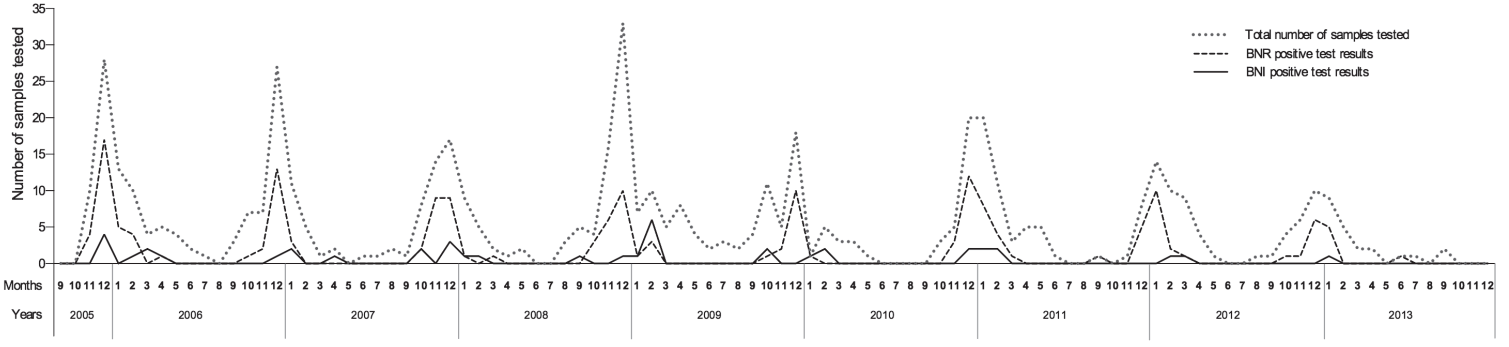
Acknowledgements: We thank Hans Kruining for his help with obtaining the data for the database.

SUPPLEMENTAL DATA

Supplemental Figure 1. Number of all BinaxNOW influenza AB® (BNI) and BinaxNOW RSV® (BNR) rapid tests performed and all positive test results for BNI and BNR in children between 0-5 years at Erasmus MC-Sophia from 2005 – 2013 in months.



Supplemental Figure 2. Number of all BinaxNOW influenza AB® (BNI) and BinaxNOW RSV® (BNR) rapid tests performed and all positive test results for BNI and BNR in children between 0-5 years at Erasmus MC-Sophia from 2005 – 2013 in months per years.



Supplemental Table 1a BinaxNOW influenza AB® (BNI) false-negative samples (n=11) compared to BinaxNOW RSV® (BNR), RT-PCR, virus isolation and direct immunofluorescence (D-IF) tested in nasal washings obtained from symptomatic children between 0-5 years at Erasmus MC-Sophia from 2005-2013. In addition co-infections are reported.

BNI false-negative samples	BNR	RT-PCR (Ct-value)	virus isolation	D-IF
1	negative	influenza (30)+RSV* (26)	negative	RSV
2	positive	influenza (35)	negative	RSV
3	positive	influenza (36)+HRV* (no Ct)	RSV	RSV
4	negative	influenza (25)	influenza	negative
5	negative	influenza (36)+ADV* (37)	negative	negative
6	negative	influenza (27)+ADV (26)	influenza	negative
7	negative	influenza (26)	influenza	influenza
8	negative	influenza (39)	negative	picornavirus
9	negative	influenza (18)+ADV (no Ct)	influenza	influenza
10	negative	influenza (26)	influenza	negative
11	negative	influenza (23)	influenza	influenza

* RSV: respiratory syncytial virus; ADV: adenovirus; HRV: human rhinovirus

Supplemental Table 1b BinaxNOW RSV® (BNR) false-negative samples (n=42) compared to BinaxNOW influenza AB® (BNI), RT-PCR, virus isolation and direct immunofluorescence (D-IF) tested in nasal washings obtained from symptomatic children between 0-5 years at Erasmus MC-Sophia from 2005-2013. In addition co-infections are reported.

BNR false-negative samples	BNI	RT-PCR (Ct-value)	virus isolation	D-IF
1	negative	RSV* (26)+influenza (30)	negative	RSV
2	positive	RSV (24)	negative	RSV
3	positive	RSV (31)+influenza (19)	influenza	negative
4	positive	RSV (32)+influenza (20)	influenza	influenza
5	positive	RSV (31)+influenza (19)	influenza	influenza
6	negative	RSV (30)	negative	negative
7	negative	RSV (34)+HRV* (29)	RSV	RSV
8	negative	RSV (30)	negative	RSV
9	negative	RSV (35)+HRV (29)	RSV	RSV
10	negative	RSV (33)+ADV* (38)	RSV	RSV
11	negative	RSV (22)	RSV	RSV
12	negative	RSV (no Ct)	negative	negative
13	negative	RSV (34)+ADV (35)	RSV	RSV
14	negative	RSV (no Ct)	RSV	negative

Supplemental Table 1b Continued

15	negative	RSV (37)+HRV (no Ct)	RSV	picornavirus
16	negative	RSV (36)+HRV (18)	negative	picornavirus
17	negative	RSV (29)	negative	negative
18	negative	RSV (30)	RSV	RSV
19	negative	RSV (no Ct)	RSV	RSV
20	negative	RSV (35)	negative	picornavirus
21	negative	RSV (25)	RSV	negative
22	negative	RSV (34)+HRV (no Ct)+HBoV (no Ct)	negative	negative
23	negative	RSV (32)	negative	negative
24	negative	RSV (37)	RSV	RSV
25	negative	RSV (23)	RSV	RSV
26	negative	RSV (32)+ADV (36)	RSV	negative
27	negative	RSV (39)	RSV	negative
28	negative	RSV (28)	negative	negative
29	negative	RSV (31)	RSV	RSV
30	negative	RSV (36)+ADV (36)	RSV	negative
31	negative	RSV (27)+HRV (23)+ HCoV229E* (27)	RSV	RSV
32	negative	RSV (no Ct)	RSV	RSV
33	negative	RSV (28)	RSV	RSV
34	negative	RSV (29)+HRV (34)	RSV	picornavirus
35	negative	RSV (29)	RSV	RSV
36	negative	RSV (24)	negative	RSV
37	negative	RSV (24)	RSV positive	negative
38	negative	RSV (no Ct)	negative	negative
39	negative	RSV (no Ct)	RSV	RSV
40	negative	RSV (36)+HCoVOC43 (28)	RSV	negative
41	negative	RSV (26)	negative	RSV
42	negative	RSV (34)	RSV	negative

* RSV: respiratory syncytial virus; ADV: adenovirus; HRV: human rhinovirus; HMPV: human metapneumovirus; HBoV: human bocavirus; HCoV: human coronavirus; No Ct: no Ct-value available

Supplemental Table 2a BinaxNOW influenza AB® (BNI) false-positive samples (n=20) compared to BinaxNOW RSV® (BNR), RT-PCR, virus isolation and direct immunofluorescence (D-IF) tested in nasal washings obtained from symptomatic children between 0-5 years at Erasmus MC-Sophia from 2005-2013. In addition co-infections are reported.

BNI false-positive samples	BNR	RT-PCR (Ct-value)	virus isolation	D-IF
1	positive	negative	negative	negative
2	negative	RSV* (24)	negative	RSV
3	positive	RSV (18)	RSV	RSV
4	positive	RSV (16)	RSV	RSV
5	positive	RSV (no Ct)	RSV	RSV
6	positive	RSV (21)	RSV	RSV
7	positive	RSV (19)	RSV	RSV
8	negative	negative	negative	picornavirus
9	negative	HRV* (32)+HBoV* (34)	negative	influenza
10	negative	negative	negative	negative
11	negative	HMPV* (38)	negative	negative
12	negative	HRV (no Ct)	picornavirus	picornavirus
13	negative	HMPV (29)	negative	negative
14	negative	negative	negative	negative
15	negative	negative	negative	influenza
16	negative	HCoVNL63* (39)	negative	negative
17	negative	negative	negative	negative
18	negative	HCoVOC43 (27)	negative	negative
19	negative	HCoVOC43 (19)	negative	negative
20	negative	HRV (24)+HCoVNL63 (19)	negative	negative

* RSV: respiratory syncytial virus; HRV: human rhinovirus; HMPV: human metapneumovirus; HBoV: human bocavirus; HCoV: human coronavirus; No Ct: no Ct-value available

Supplemental Table 2b BinaxNOW RSV® (BNR) false-positive samples (n=5) compared to BinaxNOW influenza AB® (BNI), RT-PCR, virus isolation and direct immunofluorescence (D-IF) tested in nasal washings obtained from symptomatic children between 0-5 years at Erasmus MC-Sophia from 2005-2013. In addition co-infections are reported.

BNR false-positive samples	BNI	RT-PCR (Ct-value)	virus isolation	D-IF
1	positive	negative	negative	negative
2	negative	negative	RSV*	RSV
3	negative	negative	RSV	RSV
4	negative	negative	RSV	RSV
5	negative	negative	negative	negative

* RSV: respiratory syncytial virus

Chapter 6

Safety and immunogenicity of an MVA-based influenza A/H5N1 vaccine. A randomized phase I/IIa clinical trial.

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ABSTRACT

Background: Modified Vaccinia virus Ankara (MVA) is a promising viral vector platform for H5N1 influenza vaccine development. Preclinical evaluation of MVA-based H5N1 vaccines showed their immunogenicity and safety in various animal models, warranting clinical evaluation.

Methods: In this randomized double-blind phase I/IIa study young healthy volunteers were immunized once or twice with a normal dose (10^8 plaque forming units (pfu)) or a tenfold lower dose of either the MVA-H5-sfMR (vector encoding the hemagglutinin gene of influenza A/Vietnam/1194/04 virus (H5N1 subtype) or MVA-F6-sfMR (empty vector) vaccine. Healthy volunteers that received the MVA-H5-sfMR vaccine were eligible for a boost immunization one year after the first immunization. Primary safety endpoints were local and/or systemic reactions. Secondary outcomes were hemagglutination inhibition (HI) and virus-neutralization (VN) antibody titers in the sera from the healthy volunteers. The trial is registered at the Dutch Trial Register (www.trialregister.nl) (NTR registration number: NTR3401).

Findings: 79 of the 80 healthy volunteers that were enrolled completed the study. No serious adverse events occurred. The majority of the healthy volunteers experienced one or more local and systemic reactions. Healthy volunteers that received the tenfold lower dose were prone to develop less systemic reactions. The MVA-H5-sfMR 10^8 pfu vaccine induced significantly higher antibody responses after one and two immunizations. 27 of the 39 eligible healthy volunteers were enrolled in the boost immunization study. The results indicated that a single shot MVA-H5-sfMR 10^8 pfu prime immunization resulted in higher antibody responses upon boost immunization than two shots with MVA-H5-sfMR at a ten-fold lower dose.

Interpretation: This study illustrates that the MVA-based pandemic H5N1 vaccine was well-tolerated and immunogenic and underlines that vaccine candidates arising from the MVA platform hold great promise for the future.

INTRODUCTION

Avian influenza viruses are continuously introduced into the human population with variable impact for infected individuals and the population at large. First reports of human infection with highly pathogenic avian influenza (HPAI) viruses of the H5N1 subtype date back to 1997 (207). Since their re-occurrence in 2003, H5N1 viruses have infected at least 650 humans and 386 of these cases were fatal (9). To facilitate fast-track vaccine development for these viruses the European Medicines Agency (EMA) has developed a pandemic vaccine mock-up dossier for Europe (208). The influenza A (H1N1) 2009 pandemic highlighted that in spite of the possibility for fast-track licensing of conventional vaccines, the delay between onset of the pandemic and arrival of vaccines on the market proved to be unacceptable (209). Consequently the production and distribution time of vaccines in the early pandemic period still is an unsolved problem that should be taken into account in the development of candidate vaccines against future, possibly emerging H5N1 pandemic viruses (210).

The immunogenicity of H5N1 vaccine formulations based on traditional vaccine platforms is low compared to that of seasonal influenza vaccines, resulting in the need to use multiple-shot immunization regimens or the addition of an adjuvant. The limited immunogenicity is not only associated with the relative naivety of the human population towards these avian influenza viruses, but also results from an intrinsic limited immunogenicity of the H5 hemagglutinin (HA). This phenomenon was identified for various avian influenza-virus derived HA's, like H5, H7, and H9 (211). Couch et al have assessed the immunogenicity of various avian influenza vaccines and found that the secondary structures of the vaccine antigens may affect their immunogenicity (211). It is difficult to ensure consistency of the appropriate morphology when the vaccine is produced with traditional methods of virus inactivation and breakdown through treatment with detergents. Furthermore, Couch et al illustrated that in comparison with seasonal influenza virus HA's, the conformation of avian influenza HA's and their uptake and processing by immune cells is similar and does not result in limited immunogenicity. This indicates that avian influenza vaccine development could profit considerably from novel vaccine production platforms such as those based on recombinant proteins, virosomes and viral or bacterial vectors (212–215). An additional challenge for H5N1 vaccine development is the explosive antigenic diversification of avian H5N1 viruses in the past decades, which has resulted in the emergence of a plethora of virus clades, subclades and lineages (216).

Consequently a future HA-based pandemic H5N1 vaccine should be suitable for fast and large-scale production and preferably present HA in its native multimeric form.

Viral vectors such as adenoviruses and poxviruses have proven to be potent antigen-presentation platforms that comply largely with these requirements (217–219). Poxviruses and more specifically Modified Vaccinia virus Ankara (MVA) are of special interest. The latter is replication-deficient in mammalian cells which is a clear advantage from a safety perspective. This will eventually allow for large-scale immunization campaigns that may also include the classical high-risk groups for influenza. Preclinical evaluation of MVA-based H5N1 vaccines showed their immunogenicity and safety in various animal models, warranting clinical evaluation (220–225).

Here we present data from the first-in-man phase I/IIa clinical trial conducted with the MVA-HA-based H5N1 vaccine MVA-H5-sfMR assessing its safety and immunogenicity. This is the first ever demonstration that a MVA-based H5N1 candidate vaccine is safe and immunogenic in humans.

MATERIALS & METHODS

Study Design

The phase 1/2a study was randomized and double-blind performed in a single center: Erasmus Medical Center, Rotterdam, the Netherlands (226). The primary study objective was assessment of safety. The immunogenicity of the vaccines was assessed as a secondary objective. Our working hypothesis was that a recombinant MVA-based H5 vaccine is safe and immunogenic in humans.

Both the healthy volunteers and the physicians who did the examinations and administered the vaccine were blinded for the vaccine. Eighty young adult volunteers were recruited (both male and female). When they met the inclusion and exclusion criteria (as described in the study protocol (226)) and provided written informed consent the healthy volunteers were randomly assigned to one of the eight study arms (n=10 per arm). The arms of the study were based on the number of immunizations (one or two), the immunization dose (10^7 or 10^8 pfu) and the vaccine (MVA-H5-sfMR or MVA-F6-sfMR) (see **Table 1**)(see **Appendix** for details on vaccine generation and characterization). Sample size was calculated based on the mean antibody titers obtained in the preclinical study with a prime and boost immunization with an MVA-H5 vaccine in macaques (221).

At the first visit (week 0) blood was drawn (max 34ml) and for pregnancy was excluded within 15-30 minutes prior to the vaccine administration among women who participated. Subsequently, the healthy volunteers received one immunization, administered as a solution of 0.5ml through intramuscular injection in the deltoid muscle. Healthy volunteers were observed for one hour at the trial unit and a blood sample was taken.

Then they were sent home with an ear thermometer and diary card for them to keep track of possible side-effects during seven subsequent days. In week 4 a blood sample was drawn from all healthy volunteers followed by a second immunization for the healthy volunteers in study arms 5-8. After an observation period of one hour a blood sample was taken again they were asked to complete a diary card for the next seven days. Eight and twenty weeks after their first immunization all healthy volunteers returned for their close-out visit at which the last blood sample was taken. The study design was reviewed and approved by the Central Committee on Research involving Human Subjects (CCMO) in the Netherlands. The trial is registered at the Dutch Trial Register (www.trialregister.nl) (NTR registration number: NTR3401).

Safety Assessment

The safety of the vaccine candidates was assessed using multiple tests and scores. Each subject underwent a short physical examination prior to the immunization. Heart rate, blood pressure and body temperature were registered, the injection site was examined and questions were asked on possible pre-existing conditions. This physical examination was repeated 1 hour after immunization and an additional examination was performed if necessary upon indication during the 1 hour observation period post immunization. Before and 1 hour after immunization blood was drawn to measure clinical chemical and haematological parameters in order to detect acute effects that occurred upon immunization. Four weeks after the first immunization all healthy volunteers again underwent the short physical examination and were asked by a trial physician if they had experienced any adverse reactions during and after the first week post immunization. Subsequently blood was drawn to measure the parameters as described above. Only the healthy volunteers that received a second immunization, determined by randomisation, had an additional examination and blood sampling 1 hour after immunization.

To assess reactogenicity of the vaccine, the healthy volunteers received a diary card to be completed during the first seven days post immunization. In addition they received an earthermometer to measure their body temperature twice a day (morning and evening).

Booster immunization strategy

In order to assess the possibility to boost of the H5-specific immune response, healthy volunteers that received the MVA-H5-sfMR vaccine during the main study were approached for a follow-up study (if they approved of this by informed consent). Healthy volunteers that agreed to a booster immunization received a single shot of MVA-H5-sfMR with the same dose that they received originally. Immunizations were performed 1 year after the first MVA-H5-sfMR immunization. Prior to the booster blood was drawn to determine baseline influenza virus H5-specific and MVA-IgG specific antibody responses.

Healthy volunteers received a diary card to be completed during the first seven days post immunizations. Four weeks after their visit the healthy volunteers returned and blood was drawn to determine the boost-effect of the MVA-H5-sfMR immunization.

Detection of influenza virus H5-specific antibodies

Serum was obtained from blood that was collected and centrifuged in coagulation tubes (Greiner Bio-one, Alphen a/d Rijn, the Netherlands). Sera were treated with a receptor-destroying enzyme (cholera filtrate) and then heat-inactivated at 56°C. Subsequently the sera were pretreated with horse erythrocytes after which the sera were ready to be tested for the presence of anti-H5 antibodies. This was tested in the hemagglutination inhibition assay (HI) with an adapted protocol using 1% horse erythrocytes and four HA-units of either the homologous influenza H5N1 virus A/VN/1194/04 (clade 1) or the antigenically distinct strain: A/Indonesia5/05 (A/IND/5/05) (clade 2.1) (227). For serology, viruses were used from which the multi basic cleavage site in the HA, associated with high virulence, was deleted by reverse genetics. The use of these reverse genetics (RG) viruses in the HI assay was validated and the obtained antibody titers were comparable with those against the wild type strains (data not shown).

The sera were also tested for the presence of virus-neutralizing antibodies using a micro virus neutralization (VN) assay with the RG viruses described above. The VN assay was performed as described previously (228). Sera from MVA-H5-SFMR immunized New Zealand White Rabbits and Cynomolgus Macaques were used as a positive controls in both the HI and VN assays. For calculation purposes serum samples with an antibody titer of <10 were arbitrarily assigned a titer of 5. Seroconversion was arbitrarily defined as a post-vaccination titer of ≥20 or a fourfold rise in the antibody titer when the previous titer was >10. Antibody titers of ≥40 were arbitrarily considered to be seroprotective.

Detection of MVA-specific antibodies

Serum samples were tested for the presence of MVA-specific immunoglobulin G (IgG) antibodies. To this end, Baby Hamster Kidney-21 (BHK-21) cells (permissive for MVA virus infection) were infected with MVA-F6-sfMR at an MOI of 10 and incubated for 6-8 hours. In parallel, serum samples were preincubated with uninfected BHK-21 cells. After the incubation period serum was added to either uninfected or infected BHK-21 cells in triplicates. The cells were incubated for 16 hours at 4◦C and then washed and subsequently incubated with a FITC-labelled Rabbit anti-human IgG antibody preparation (DAKO, Glostrup, Denmark). Cells were analysed by flow cytometry using a FACS Canto (BD Biosciences, Breda, The Netherlands). The fold-increase in FITC-positive cells was calculated by dividing the values from the sera incubated with the infected cells by that from the corresponding sera incubated with the uninfected cells.

Randomization and masking

Study subjects were assigned to one of the eight study groups based on a computer-generated randomization list with 10 blocks of 8 randomized study arms. The randomization code was kept by the hospital pharmacist till the end of the study period. Based on the list a generic label was generated by the hospital pharmacy that masked the content of the vaccine vial and only indicated a reference to the respective recipient. A trial nurse would check the content of the vial and would fill the syringe from the vial and provide this with the masked label to the trial physician.

Statistics

To assess statistical significance of the differences between groups the Mann-Whitney U test was performed using IBM SPSS Statistics 22. For the analysis of the HI and VN antibody titers the log2 values of the titers were used.

Role of the funding source

The European Research Council (ERC) that provided funding for this study (project FLUPLAN (250136)) had no role in the design of the study or data interpretation. AO designed the protocol together with JK, GS and GR and had final responsibility for the study.

RESULTS

Study demographics

Within 2 months after recruitment start (December 2012), eighty healthy young adults, 18-28 years of age (mean= 21.9 ± 2.0), were enrolled in the study. Females comprised 61% of these volunteers. All healthy volunteers were randomly assigned to one of the eight arms of the study. The male-females ratio's per study arm are indicated in **Table 1**. Study immunizations began in February 2013 and the last study visits took place in July 2013. Of the eighty healthy volunteers enrolled, 79 completed the study. Thirty-nine of these healthy volunteers received one or two immunizations with the MVA-H5-sfMR vaccine and thus were eligible for inclusion in a booster immunization study that was performed one year later (January 2014). Twenty-seven healthy volunteers were enrolled in this follow-up study and received a booster immunization.

Safety

No serious adverse events occurred during either the main or the follow-up study. Eleven adverse events were registered during the study period (indicated for the respective group and study period in **Tables 2-4**). One subject (group 6) experienced

Table 1. Immunisation dosing schedules in young healthy adults

	Single immunization				Two immunizations			
	Group 1, MVA-H5-sfMR 10^8 pfu	Group 2, MVA-H5-sfMR 10^7 pfu	Group 3, MVA-F6-sfMR 10^8 pfu	Group 4, MVA-F6-sfMR 10^7 pfu	Group 5, MVA-H5-sfMR 10^8 pfu	Group 6, MVA-H5-sfMR 10^7 pfu	Group 7, MVA-F6-sfMR 10^8 pfu	Group 8, MVA-F6-sfMR 10^7 pfu
Number of participants at each visit after first immunisation								
Week 0, visit 1 (men, women)	10* (4, 6)	10* (6, 4)	10* (6, 4)	10* (3, 7)	10* (3, 7)	10* (2, 8)	10* (4, 6)	10* (2, 8)
Week 4, visit 2 (men, women)	9† (4, 5)	10 (6, 4)	10 (6, 4)	10 (3, 7)	10 (3, 7)	10 (2, 8)	10 (4, 6)	10 (2, 8)
Week 8, visit 3 (men, women)	9 (4, 5)	10 (6, 4)	10 (6, 4)	10 (3, 7)	10 (3, 7)	10 (2, 8)	10 (4, 6)	10 (2, 8)
Week 20, visit 4 (men, women)	9 (4, 5)	10 (6, 4)	10 (6, 4)	10 (3, 7)	10 (3, 7)	10 (2, 8)	10 (4, 6)	10 (2, 8)
Number of participants at each visit after booster immunisation								
Week 0, visit 1 (men, women)	5* (3, 2)	7* (4, 3)	NA	NA	6* (1, 5)	9* (2, 7)	NA	NA
Week 4, visit 2 (men, women)	5 (3, 2)	7 (4, 3)	NA	NA	6 (1, 5)	9 (2, 7)	NA	NA

MVA=modified vaccinia virus Ankara. MVA-H5-sfMR=vector encoding the haemagglutinin gene of influenza A/Vietnam/1194/2004 virus H5N1 subtype. MVA-F6-sfMR=empty vector. pfu=plaque-forming units. NA=not applicable because only individuals who were immunised with MVA-H5-sfMR in the first part of the study were eligible for a booster immunisation. *Individuals who received an immunisation at the timepoint. †One individual discontinued after visit 1 because of time constraints and side-effects after the first immunisation.

severe headache and lightheadedness during the first hour after vaccination and received paracetamol. This event may have been related to the vaccination. No further acute effects were observed during the observation period nor during the subsequent physical examination. The ten other adverse events occurred in the four weeks after immunization. One subject in group 8 reported erythema nodosum, which was unlikely but possibly related to vaccination. Five healthy volunteers (group 1, 8) reported respiratory illness and/or sore throat during the first week post immunization, which is most likely unrelated to vaccination. Their respiratory illness was accompanied by flu-like symptoms. One subject in group 5 reported a local reaction at the injection site (evening post immunization) characterized by red spots (1-2 cm in diameter). The other three healthy volunteers (group 2, 6, 7) suffered from other conditions unlikely to have a causal relation to the vaccination. Blood samples of all healthy volunteers were analyzed for the before mentioned biochemical and hematological parameters. The majority of the values were within the standard ranges (specified per sex). The deviations that were observed were either pre-existing or isolated deviations that could be attributed to the healthy volunteer's background and/or lifestyle. One subject had an elevated CK after vaccination which could have a causal relation to the vaccination, but this did not result in clinical symptoms and was not accompanied by any other biochemical deviation. Diary cards filled in for a period of seven days after each immunization recorded local and systemic reactions after the first immunization (**Table 2**), the second immunization (**Table 3**) and the booster immunization (**Table 4**). The majority of the healthy volunteers reported one or more local and systemic reactions, occurring within 72 hours post immunization and the majority of the reported reactions resolved within 6 days post immunization. Focusing on the local reactions, pain at the injection site was reported most and was graded as mild-moderate by most healthy volunteers and only a minority reported the pain as severe. Itch, swelling and redness were only reported in a few cases distributed over the different groups. No difference in incidence and severity of the local reactions were experienced after the first, second or booster immunization. Most healthy volunteers experiencing systemic reactions reported them to be mild to moderate. There was no clear difference in incidence and severity of the systemic reactions after one, two or the booster immunization. There appeared however to be a dose-response effect, as the 10^7 pfu dose of either the MVA-H5-sfMR or MVA-F6-sfMR vaccines resulted in less reported systemic reactions than the10^8 pfu dose. Overall the MVA-H5-sfMR and MVA-F6-sfMR vaccines were well tolerated.

Table 2. Local and systemic reaction after the first immunisation

	Group 1, MVA-H5-sfMR 10^8 pfu (n=9)	Group 2, MVA-H5-sfMR 10^7 pfu (n=10)	Group 3, MVA-F6-sfMR 10^8 pfu (n=10)	Group 4, MVA-F6-sfMR 10^7 pfu (n=10)	Group 5, MVA-H5-sfMR 10^8 pfu (n=10)	Group 6, MVA-H5-sfMR 10^7 pfu (n=10)	Group 7, MVA-F6-sfMR 10^8 pfu (n=10)	Group 8, MVA-F6-sfMR 10^7 pfu (n=10)
Local reactions	7 (78%)	7 (70%)	8 (80%)	4 (40%)	9 (90%)	7 (70%)	4 (40%)	2 (20%)
Itch								
None	8 (89%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)	8 (80%)	10 (100%)	9 (90%)
Mild	1 (11%)	-	-	-	-	2 (20%)	-	1 (10%)
Moderate	-	-	-	-	-	-	-	-
Severe	-	-	-	-	-	-	-	-
Pain				7 (70%)				
None	2 (22%)	4 (40%)	2 (20%)	3 (30%)	1 (10%)	3 (30%)	5 (50%)	8 (80%)
Mild	2 (22%)	6 (60%)	4 (40%)	-	3 (30%)	5 (50%)	4 (40%)	2 (20%)
Moderate	1 (11%)	-	3 (30%)	-	3 (30%)	2 (20%)	1 (10%)	-
Severe*	4 (45%), 1.5 days (1.0-2.0)	-	1 (10%), 1 days (1.0-1.0)		3 (30%), 1.7 days (1.0-3.0)	-	-	-
Redness								
None	6 (67%)	7 (70%)	10 (100%)	8 (80%)	9 (90%)	10 (100%)	10 (100%)	10 (100%)
Mild	2 (22%)	3 (30%)	-	2 (20%)	1 (10%)	-	-	-
Moderate	1 (11%)	-	-	-	-	-	-	-
Severe	-	-	-	-	-	-	-	-
Diameter (mm)	8.0 (8.0-8.0)	2.5 (2.0-3.0)	-	7.5 (5.0-10.0)	2.0 (2.0-2.0)	-	-	-
Swelling								
None	9 (100%)	9 (90%)	9 (90%)	9 (90%)	9 (90%)	10 (100%)	9 (90%)	10 (100%)
Mild	-	1 (10%)	1 (10%)	1 (10%)	1 (10%)	-	1 (10%)	-
Moderate	-	-	-	-	-	-	-	-
Severe	-	-	-	-	-	-	-	-
Systemic reactions	8 (89%)†	9 (90%)	10 (100%)	6 (60%)‡	10 (100%)	8 (80%)	9 (90%)	7 (70%)‡
Rise in body temperature (>37.5°C)								
Number	5 (56%)	4 (40%)	7 (70%)	5 (50%)	7 (70%)	3 (30%)	4 (40%)	4 (40%)
Temperature range	37.6-38.2	37.6-38.5	37.6-39.1	37.6-38.3	37.6-39.9	37.6-39.0	37.8-39.0	37.8-39.0
Days	1.2	3.5	2.4	2.0	1.3	2.0	1.5	1.5
Headache								
None	3 (33.3%)	6 (60%)	3 (30%)	7 (70%)	1 (10%)	7 (70%)	6 (60%)	7 (70%)
Mild	3 (33.3%)	2 (20%)	3 (30%)	1 (10%)	4 (40%)	-	2 (20%)	1 (10%)
Moderate	3 (33.3%)	2 (20%)	3 (30%)	2 (20%)	3 (30%)	2 (20%)	2 (20%)	1 (10%)
Severe*	-	-	1 (10%), 1 days (1.0-1.0)	-	2 (20%), 1 days (1.0-1.0)	1 (10%), 2 days (2.0-2.0)	-	1 (10%)

Table 2. Continued

Myalgia				5 (50%)				
None	3 (33%)	4 (40%)	1 (10%)	5 (50%)	-	2 (20%)	3 (30%)	5 (50%)
Mild	2 (22%)	4 (40%)	4 (40%)	-	1 (10%)	5 (50%)	-	4 (40%)
Moderate	1 (11%)	2 (20%)	3 (30%)	-	6 (60%)	3 (30%)	6 (60%)	1 (10%)
Severe*	3 (33%), 1 days (1.0-1.0)	-	2 (20%), 2 days (2.0-2.0)		3 (30%), 2 days (1.0-3.0)	-	1 (10%), 1 days (1.0-1.0)	-
Arthralgia				10 (100%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)
None	8 (89%)	10 (100%)	7 (70%)	-	-	-	-	-
Mild	-	-	-	-	-	-	-	-
Moderate	-	-	2 (20%)	-	-	-	-	-
Severe*	1 (11%), 1 days (1.0-1.0)	-	1 (10%), 2 days (2.0-2.0)					
Chills								
None	4 (45%)	9 (90%)	5 (50%)	10 (100%)	4 (40%)	8 (80%)	3 (30%)	7 (70%)
Mild	-	-	1 (10%)	-	3 (30%)	2 (20%)	3 (30%)	2 (20%)
Moderate	2 (22%)	-	1 (10%)	-	2 (20%)	-	3 (30%)	1 (10%)
Severe*	3 (33%), 1 days (1.0-1.0)	1 (10%)	3 (30%), 1 days (1.0-1.0)	-	1 (10%), 1 days (1.0-1.0)	-	11 (10%), 2 days (2.0-2.0)	-
Malaise				9 (90%)				8 (80%)
None	2 (22%)	9 (90%)	2 (20%)	-	4 (40%)	7 (70%)	5 (50%)	1 (10%)
Mild	3 (33%)	-	1 (10%)	1 (10%)	2 (20%)	1 (10%)	-	1 (10%)
Moderate	3 (33%)	-	4 (40%)	-	3 (30%)	1 (10%)	4 (40%)	-
Severe	1 (11%), 1 days (1.0-1.0)	1 (10%), 3 days (3.0-3.0)	3 (30%), 1 days (1.0-1.0)		1 (10%), 1 days (1.0-1.0)	1 (10%) 1 days (1.0-1.0)	1 (10%), 1 days (1.0-1.0)	
Fatigue				9 (90%)	5 (50%)		4 (40%)	8 (80%)
None	6 (67%)	6 (60%)	6 (60%)	-	3 (30%)	5 (50%)	4 (40%)	1 (10%)
Mild	1 (11%)	1 (10%)	-	1 (10%)	2 (20%)	2 (20%)	2 (20%)	1 (10%)
Moderate	-	1 (10%)	3 (30%)	-	-	2 (20%)	-	-
Severe	2 (22%), 1 days (1.0-1.0)	2 (20%), 1.5 days (1.0-2.0)	1 (10%), 1 days (1.0-1.0)			1 (10%), 1 days (1.0-1.0)		

Data are number (%) or mean (range), unless otherwise indicated. MVA=modified vaccinia virus Ankara. MVA-H5-sfMR=vector encoding the haemagglutinin gene of influenza A/Vietnam/1194/2004 virus H5N1 subtype. MVA-F6-sfMR=empty vector. pfu=plaque-forming units. NA=not applicable because only individuals who were immunised with MVA-H5-sfMR in the first part of the study were eligible for a booster immunisation. *Mean number of days which the reaction was reported as severe. †One individual reported respiratory illness that started on the evening after the immunisation. ‡One individual reported having a sore throat for at least 1 day in the week after the immunisation.

Table 3. Local and systemic reactions after the second immunisation

	Group 5, MVA-H5-sfMR 10^8 pfu (n=10)	Group 6, MVA-H5-sfMR 10^7 pfu (n=10)	Group 7, MVA-F6-sfMR 10^8 pfu (n=10)	Group 8, MVA-F6-sfMR 10^7 pfu (n=10)
Local reactions	8 (80%)	8 (80%)	4 (40%)	7 (70%)
Itch				
None	9 (90%)	7 (70%)	9 (90%)	9 (90%)
Mild	-	2 (20%)	1 (10%)	1 (10%)
Moderate	-	-	-	-
Severe	-	-	-	-
Pain				
None	4 (40%)	4 (40%)	6 (60%)	4 (40%)
Mild	-	3 (30%)	2 (20%)	6 (60%)
Moderate	3 (30%)	3 (30%)	2 (20%)	-
Severe§	3 (30%), 1 days (1.0-3.0)	-	-	-
Redness				
None	7 (70%)	9 (90%)	7 (70%)	8 (80%)
Mild	3 (30%)	1 (10%)	3 (30%)	2 (20%)
Moderate	-	-	-	-
Severe	-	-	-	-
Diameter (mm)	2.0 (1.0-3.0)	2.0 (2.0-2.0)	3.0 (3.0-3.0)	2.0 (1.0-2.0)
Swelling				
None	5 (50%)	9 (90%)	7 (70%)	8 (80%)
Mild	3 (30%)	1 (10%)	3 (30%)	2 (20%)
Moderate	2 (20%)	-	-	-
Severe	-	-	-	-
Systemic reactions	10 (100%)	9 (90%)	8 (80%)†	5 (50%)‡
Rise in body temperature (>37.5°C)				
Number	4 (40%)	4 (40%)	3 (30%)	4 (40%)
Temperature range	37.6-39.1	37.6-38.3	37.6-38.4	37.6-37.9
Days	2.0	2.3	2.3	2.5
Headache				
None	-	6 (60%)	6 (60%)	8 (80%)
Mild	6 (60%)	2 (20%)	2 (20%)	2 (20%)
Moderate	3 (30%)	1 (10%)	1 (10%)	-
Severe§	1 (10%), 1 days (1.0-1.0)	1 (10%), 1 days (1.0-1.0)	1 (10%), 1 days (1.0-1.0)	-

Table 3. Continued

Myalgia				
None	3 (30%)	2 (20%)	2 (20%)	5 (50%)
Mild	4 (40%)	4 (40%)	4 (40%)	4 (40%)
Moderate	1 (10%)	4 (40%)	3 (30%)	1 (10%)
Severe§	2 (20%), 1 days	-	1 (10%), 1 days	-
Arthralgia				
None	9 (90%)	10 (100%)	9 (90%)	10 (100%)
Mild	-	-	1 (10%)	-
Moderate	-	-	-	-
Severe§	1 (10%), 1 days (1.0-1.0)	-	-	-
Chills				
None	7 (70%)	10 (100%)	8 (80%)	10 (100%)
Mild	-	-	1 (10%)	-
Moderate	2 (20%)	-	1 (10%)	-
Severe§	1 (10%), 1 days (1.0-1.0)	-	-	-
Malaise				
None	6 (60%)	7 (70%)	8 (80%)	9 (90%)
Mild	2 (20%)	1 (10%)	1 (10%)	1 (10%)
Moderate	1 (10%)	1 (10%)	-	-
Severe§	1 (10%), 1 days (1.0-1.0)	1 (10%) 1 days (1.0-1.0)	1 (10%), 1 days (1.0-1.0)	-
Fatigue				
None	4 (40%)	5 (50%)	7 (70%)	9 (90%)
Mild	2 (20%)	2 (20%)	1 (10%)	1 (10%)
Moderate	3 (30%)	1 (10%)	2 (20%)	-
Severe§	1 (10%), 6 days (6.0-6.0)	2 (20%), 1 days (1.0-1.0)	-	-

Data are number (%) or mean (range), unless otherwise indicated. MVA=modified vaccinia virus Ankara. pfu=plaque-forming units. *One individual reported red spots (diameter 1-2 cm) around the injection site on the evening after immunisation. †One individual reported having a sore throat for at least 1 day in the week after immunisation. ‡One individual reported sore throat and respiratory illness that started on day 1 after immunsation. §Mean number of days for which the reaction was reported as severe.

Table 4. Local and systemic reactions after booster immunisation

	Group 1, MVA-H5-sfMR 10^8 pfu (n=5)	Group 2, MVA-H5-sfMR 10^7 pfu (n=7)	Group 5, MVA-H5-sfMR 10^8 pfu (n=6)	Group 6, MVA-H5-sfMR 10^7 pfu (n=9)
Local reactions	4 (80%)	5 (71%)	6 (100%)	8 (89%)
Itch				
None	5 (100%)	6 (86%)	4 (67%)	7 (78%)
Mild	-	1 (14%)	2 (33%)	1 (11%)
Moderate	-	-	-	1 (11%)
Severe	-	-	-	-
Pain	2 (40%)	3 (43%)	-	2 (22%)
None	2 (40%)	2 (29%)	1 (17%)	4 (45%)
Mild	1 (20%)	1 (14%)	2 (33%)	3 (33%)
Moderate	-	1 (14), 1 days	3 (50%), 1.3 days	-
Severe†		(1.0-1.0	(1.0-2.0)	
Redness				
None	3 (60%)	6 (86%)	2 (33%)	8 (89%)
Mild	2 (40%)	1 (14%)	4 (67%)	9 (11%)
Moderate	-	-	-	-
Severe	-	-	-	-
Diameter (mm)	10.0 (10.0-10.0)	3.0 (3.0-3.0)	2.7 (1.0-3.0)	8.0 (2.0-8.0)
Swelling				
None	4 (80%)	6 (86%)	2 (33%)	7 (78%)
Mild	1 (20%)	1 (14%)	4 (67%)	2 (22%)
Moderate	-	-	-	-
Severe	-	-	-	-
Systemic reactions				
Rise in body temperature (>37.5°C)	5 (100%)	5 (71%)	3 (50%)	4 (44%)
Number	37.7-38.5	37.6-38.2	37.6-38.2	37.6-39
Temperature range	1.6	3.2	2.7	1.3
Days				
Headache				
None	2 (40%)	5 (71%)	-	2 (22%)
Mild	2 (40%)	2 (29%)	1 (17%)	3 (33%)
Moderate	1 (20%)	-	4 (67%)	3 (33%)
Severe†	-	-	1 (17%), 1 days	1 (11%), 1 days
			(1.0-1.0)	(1.0-1.0)

Table 4. Continued

Myalgia				
None	-	1 (14%)	1 (17%)	3 (33%)
Mild	3 (60%)	1 (14%)	-	3 (33%)
Moderate	2 (40%)	5 (72%)	2 (33%)	2 (22%)
Severe†	-	-	3 (50%), 1 days (1.0-1.0)	1 (11%), 1 days (1.0-1.0)
Arthralgia				
None	3 (60%)	6 (86%)	4 (67%)	7 (78%)
Mild	2 (40%)	-	1 (17%)	1 (11%)
Moderate	-	1 (14%)	1 (17%)	1 (11%)
Severe†	-	-	-	-
Chills				
None	1 (20%)	5 (72%)	4 (67%)	7 (78%)
Mild	-	1 (14%)	-	-
Moderate	2 (40%)	1 (14%)	2 (33%)	2 (22%)
Severe†	2 (40%), 1 days (1.0-1.0)	-	-	-
Malaise				
None	1 (20%)	3 (43%)	2 (33%)	5 (56%)
Mild	-	3 (43%)	-	2 (22%)
Moderate	3 (60%)	1 (14%)	3 (50%)	1 (11%)
Severe†	1 (20%), 1 days (1.0-1.0)	-	1 (17%), 1 days (1.0-1.0)	1 (11%), 1 days (1.0-1.0)
Fatigue				
None	1 (20%)	5 (72%)	3 (50%)	5 (56%)
Mild	1 (20%)	1 (14%)	2 (33%)	4 (44%)
Moderate	2 (40%)	1 (14%)	-	-
Severe†	1 (20%), 1 days (1.0-1.0)	-	-	-

Data are number (%) or mean (range), unless otherwise indicated. MVA=modified vaccinia virus Ankara. MVA-H5-sfMR=vector encoding the haemagglutinin gene of influenza A/Vietnam/1194/2004 virus H5N1 subtype. pfu=plaque-forming units. * One individual reported flu-like symptoms before the immunisation on the diary card.

†Mean number of days which the reaction was reported as severe.

H5-specific immune responses

Pre- and post-immunization sera were tested for the presence of H5 specific antibodies in the HI and VN assay against homologous H5N1 influenza A/VN/1194/04 virus (**Table 5**) and heterologous H5N1 influenza A/IND/5/05 virus (**Table 6**). Four weeks after the first immunization antibodies were detectable against both A/H5N1 viruses and titers were higher in healthy volunteers that received the 10^8 pfu dose of MVA-H5-sfMR, groups 1 and 5 respectively. The A/VN/1194/04-specific HI GMT of the latter was significantly higher than that in groups 2 and 6, in which healthy volunteers had received the tenfold lower dose of the MVA-H5-sfMR vaccine. A second immunization (groups 5 and 6) further boosted the antibody response as measured at 8 weeks after the first immunization. HI GMT also rose for group 1, despite the lack of a second immunization, and were significantly higher than that of group 2. HI GMT of group 5 was significantly higher than that of groups 1, 2 and 6. Over time antibody titers waned as measured on time point 20 weeks, however titers for group 5 remained significantly higher than those of groups 1, 2 and 6. In the main study the maximal seroconversion rates (% of healthy volunteers with at least a four-fold increase in titer) and seroprotection rates (% of healthy volunteers with a titer of ≥40) were reached after two immunizations (at 8 weeks) in group 5, 100% and 80% respectively. The HI data were confirmed by those obtained with the VN assay though VN GMT generally were lower in the latter.

The extra immunization in the follow-up study resulted in a substantial boost of the antibody responses against the homologous A/VN/1194/04 (**Figure 1**) and heterologous A/IND/5/05 strain (**Figure 2**). Highest GMTs were measured for group 1 with a 100% seroconversion and 100% seroprotection rate (**Appendix 2**). The titers in this group were significantly higher than in group 6 in the HI assay against the A/VN/1194/04 strain and significantly higher than group 2 in the VN assay against the A/IND/5/05 strain. Also for group 2 100% seroconversion and 100% seroprotection were reached with the extra immunization.

Sera were not only tested against the homologous H5N1 influenza A/Vietnam/1194/04 virus but also against viruses from antigenically distinct clades: H5N1 influenza A/Turkey/Turkey/1/2005 virus (clade 2.2) and the transmissible variant of the H5N1 influenza A/Indonesia/5/2005 virus (clade 2.1) as described recently (21,229). The boosting effect was also confirmed for the antibody responses against these viruses (see **Appendix 3**). Of the healthy volunteers immunized twice with MVA-F6-sfMR 10e8 pfu, three out of ten seroconverted in the HI assay (two had seroprotective antibodies titers) with a GMT of 10.1 (SD=2.8). One of ten healthy volunteers immunized twice with the 10-fold lower dose seroconverted (GMT 5.7 (SD=1.6)).

All subjects had antibody titers of <10 (arbitrarily assigned a value of 5 for calculation purposes) at the beginning of the study (0 weeks post immunization) in the

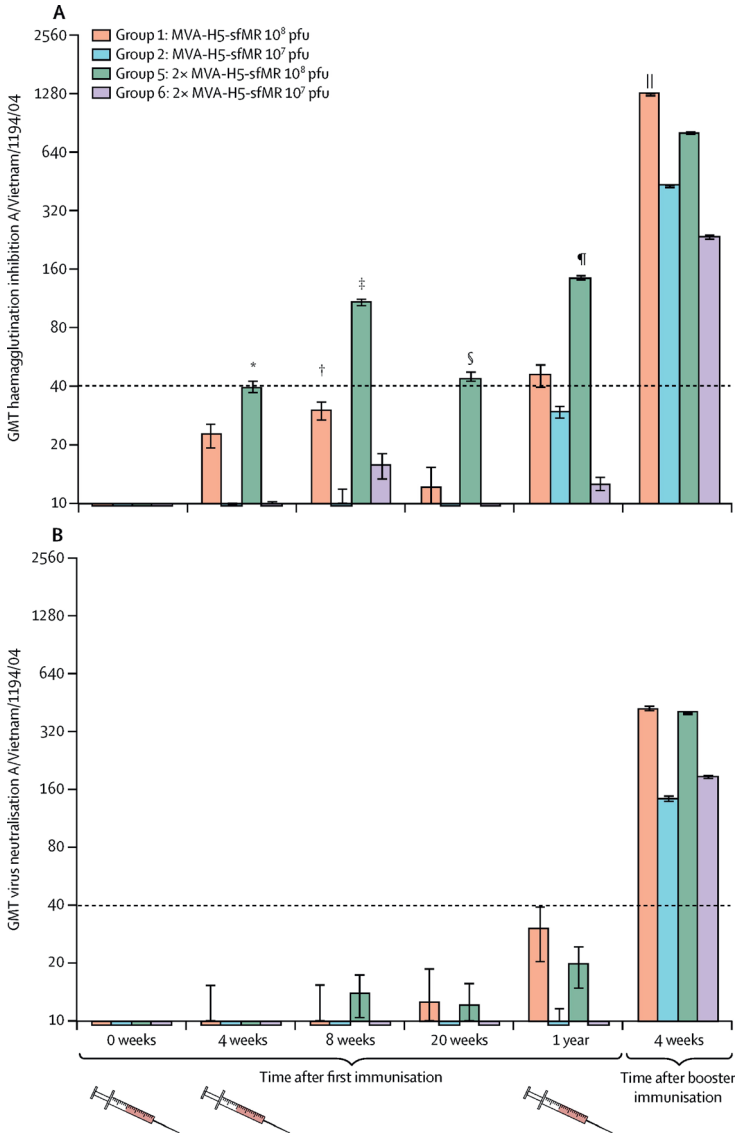

Figure 1. Mean HI (A) and VN (B) antibody titers against influenza A/Vietnam/1194/04 virus of the healthy volunteers that received a boost immunization after one year.

To assess statistical significance of the differences between groups the Mann-Whitney U test was performed. For the analysis of the HI and VN antibody titers the log2 values were used of the titers of 0 weeks to 20 weeks for the complete groups (thus including the healthy volunteers that did not receive a booster immunization). For the 1 year and 4 weeks post boost immunization the statistics logically were performed with the boosted individuals only. *Antibody titers were significantly higher than in groups 2 and 6. ** Antibody titers were significantly higher than in group 6. *** Antibody titers were significantly higher than in groups 1, 2 and 6. **** Antibody titers were significantly higher than in group 6.

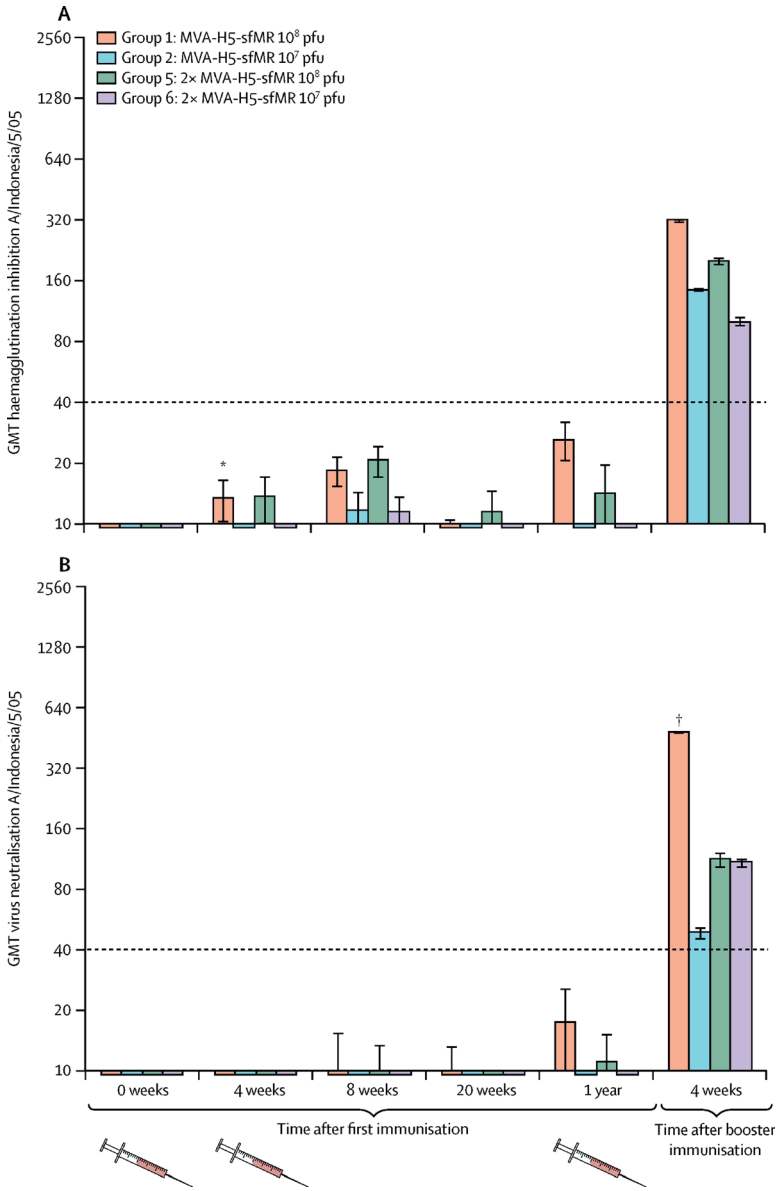

Figure 2. Mean HI (A) and VN (B) antibody titers against influenza A/Indonesia/5/05 virus of the healthy volunteers that received a boost immunization after one year.

To assess statistical significance of the differences between groups the Mann-Whitney U test was performed. For the analysis of the HI and VN antibody titers the log2 values were used of the titers of 0 weeks to 20 weeks for the complete groups (thus including the healthy volunteers that did not receive a booster immunization). For the 1 year and 4 weeks post boost immunization the statistics logically were performed with the boosted individuals only. *Antibody titers were significantly higher than in group 2 (MVA-H5-sfMR 10^7).

Table 5. Cumulative seroconversion and seroprotection against homologous H5N1 virus influenza A/Vietnam/1194/2004 (clade 1)

			Time after 1st immunization					
			4wks		8wks		20wks	
Group			HI	VN	HI	VN	HI	VN
1	Single shot MVA-H5-sfMR 10^8 pfu	Seroconversion*	56% (5)	11% (1	78% (7)	11% (1)	78% (7)	33% (3)
		Seroprotection	56% (5)	11% (1)	44% (4)	11% (1)	44% (4)	11% (1)
		GMT (SD)	22.6 (4.6)	9.3 (6.3)	**30.2 (3.8)**[2]	9.3 (6.3)	12.2 (3.7)	12.6 (6.3)
2	Single shot MVA-H5-sfMR 10^7 pfu	Seroconversion	30% (3)	10% (1)	40% (4)	10% (1)	40% (4)	10% (1)
		Seroprotection	10% (1)	-	-	-	-	-
		GMT (SD)	8.5 (2.4)	5.7 (1.6)	9.2 (2.3)	5.0 (1.0)	6.2 (1.6)	5.0 (1.0)
5	Two shot MVA-H5-sfMR 10^8 pfu	Seroconversion	80% (8)	10% (1)	100% (10)	50% (5)	100% (10)	40% (4)
		Seroprotection	60% (6)	-	80% (8)	30% (3)	70% (7)	20% (2)
		GMT (SD)	**39.3 (4.1)**[1]	5.7 (1.6)	**108.1 (2.4)**[3]	14.1 (3.4)	**43.9 (3.4)**[3]	12.3 (3.7)
6	Two shot MVA-H5-sfMR 10^7 pfu	Seroconversion	20% (2)	10% (1)	50% (5)	10% (1)	50% (5)	20% (2)
		Seroprotection	20% (2)	-	30% (3)	-	-	-
		GMT (SD)	8.1 (2.4)	5.7 (1.6)	15.8 (3.2)	5.0 (1.0)	6.9 (2.0)	5.7 (1.6)

Hemagglutination Inhibition (HI) and Virus Neutralization (VN) assay. *Seroconversion was arbitrarily defined as a post-vaccination titer of ≥20 or a fourfold rise in the antibody titer when the previous titer was >10. Seroconversion is cumulative to 20 weeks. A subject that was seroconverted on 4 weeks after the first immunization is accounted as seroconverted on 8 weeks and 20 weeks also. The numbers in parenthesis after the percentages are the absolute number of subjects for seroconversion and seroprotection. [1]Antibody titers were significantly higher than that in groups 2 and 6. [2]Antibody titers were significantly higher than that in group 2. [3] Antibody titers were significantly higher than than in groups 1, 2 and 6.

All subjects had antibody titers of <10 (arbitrarily assigned a value of 5 for calculation purposes) at the beginning of the study (0 weeks post immunization) in the Hemagglutination Inhibition (HI) and Virus Neutralization (VN) assay. *Seroconversion was arbitrarily defined as a post-vaccination titer of ≥20 or a fourfold rise in the antibody titer when the previous titer was >10. Seroconversion is cumulative to 20 weeks. A subject that was seroconverted on 4 weeks after the first immunization is accounted as seroconverted on 8 weeks and 20 weeks also. The numbers in parenthesis after the percentages are the absolute numbers of subjects for seroconversion and seroprotection. [1]Antibody titers were significantly higher than that in group 2.

Table 6. Cumulative seroconversion and seroprotection against heterologous H5N1 virus influenza A/Indonesia/5/2005 (clade 2.1)

			Time after 1st immunization					
			4 weeks		8 weeks		20 weeks	
Group			HI	VN	HI	VN	HI	VN
	Single shot	Seroconversion*	33% (3)	11% (1)	56% (5)	11% (1)	56% (5)	22% (2)
1	MVA-H5-sfMR	Seroprotection	22% (2)	-	33% (3)	11% (1)	11% (1)	11% (1)
	10^8 pfu	GMT (SD)	**13.6 (3.2)**[1]	5.8 (1.6)	18.5 (3.1)	9.3 (6.3)	7.3 (3.2)	9.3 (4.1)
	Single shot	Seroconversion	10% (1)	-	50% (5)	-	-	-
2	MVA-H5-sfMR	Seroprotection	-	-	10% (1)	-	-	-
	10^7 pfu	GMT (SD)	5.7 (1.6)	-	11.8 (2.7)	-	-	-
	Two shot	Seroconversion	40% (4)	10% (1)	50% (5)	20% (2)	50% (5)	20% (2)
5	MVA-H5-sfMR	Seroprotection	30% (3)	10% (1)	40% (4)	20% (2)	20% (2)	10% (1)
	10^8 pfu	GMT (SD)	13.7 (3.6)	6.2 (1.9)	20.8 (3.7)	9.3 (4.1)	11.5 (3.4)	7.1 (2.1)
	Two shot	Seroconversion	30% (3)	-	50% (5)	10% (1)	-	10% (1)
6	MVA-H5-sfMR	Seroprotection	-	-	20% (2)	-	-	-
	10^7 pfu	GMT (SD)	7.6 (2.0)	-	11.5 (2.3)	5.7 (1.6)	-	5.0 (1.0)

MVA-specific IgG responses

MVA-specific IgG responses are expressed as fold increase in mean fluorescence (FIMF) (**Figure 3**). Four weeks after the first immunization differences between the different groups were minimal and the mean responses ranged from 1.62 to 2.1 FIMF. The second immunization for groups 5-8 induced significantly higher (p values <0.05) MVA-specific IgG responses at time point 8 weeks when compared to groups 1-4 that did not receive a second immunization (exception: the difference between group 3 and 6 was not significant). The responses waned for all groups between 8 and 20 weeks and at the latter time point MVA-specific IgG response in Groups 5, 7 and 8 were significantly higher (p values <0.05) than in Group 2. Group 7 also had a significantly higher response than Group 6 (p=0.04). The boost immunization for groups 1, 2, 5 and 6 resulted in a rise in MVA-specific IgG responses as measured four weeks after the booster. Differences between the groups were minimal.

DISCUSSION

An influenza A/H5N1 candidate vaccine based on the replication deficient viral vector MVA was safe and immunogenic in volunteers. The reactogenicity of MVA-H5-sfMR in young healthy adults was mild to moderate and apart from the limited number of adverse events, local and systemic reactions are acceptable and are in line with reports

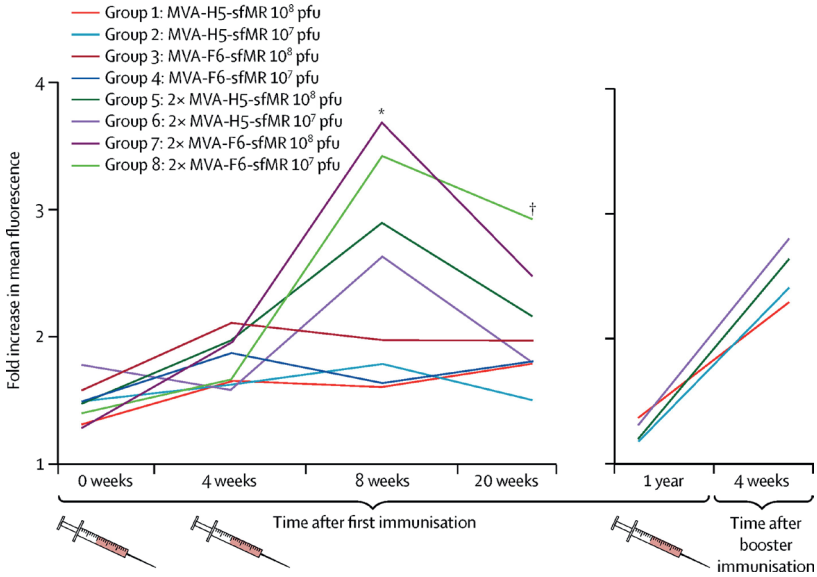

Figure 3. MVA specific antibody responses after the first, second and booster immunization.
MVA-specific IgG antibodies were measured in serum by incubating it with uninfected or MVA infected BHK-21 cells and subsequently analyzing these cells by labeling them with an anti-human IgG antibody and measuring the mean fluorescence of the cells on the FACS. MVA-specific IgG antibody responses are expressed as fold increase in mean fluorescence, calculated by dividing the values from the sera incubated with the infected cells by that from the corresponding sera incubated with the uninfected cells.
To assess statistical significance of the differences between groups the Mann-Whitney U test was performed. For the analysis of the MVA-specific IgG antibody responses values were used of 0 weeks to 20 weeks for the complete groups (thus including the healthy volunteers that did not receive a booster immunization). For the 1 year and 4 weeks post boost immunization the statistics logically were performed with the boosted individuals only.
*The MVA-specific IgG response in groups 5, 6, 7 and 8 was significantly higher (p values <0.05) than in groups 1, 2, 3 and 4 (exception: the difference between group 3 and 6 was not significant).
**The MVA-specific IgG response in groups 5, 7 and 8 was significant higher (p values <0.05) than in group 2. Group 7 also had a significant higher response than Group 6 (p=0.04).

of clinical evaluations of other MVA-based vaccines (for review see Gilbert) (230). The ten-fold lower dose of MVA-H5-sfMR was slightly less reactogenic than the 10^8 pfu dose, but its immunogenicity was lower. The latter induced significantly higher antibody responses, especially after two immunizations. One immunization with a dose of 10^8 pfu of MVA-H5-sfMR primed for the highest antibody responses upon boost immunization given after one year. Such a regimen would be of interest considering a pre-pandemic immunization scenario to establish priming for a H5N1-specific antibody response in a naïve population that could be boosted once a pandemic H5N1 virus would emerge.

Of interest, the antibodies induced after MVA-H5-sfMR immunization cross-reacted with influenza A/H5N1 viruses of antigenically distinct clades of H5N1 viruses, which suggests that this vaccine based on the clade 1 virus A/Vietnam/1194/04 would afford some level of protection to H5N1 viruses belonging to other clades as was demonstrated previously in mice and non-human primates (220–222).

Currently, circulating H5N1 viruses do not spread efficiently from human-to-human. A handful of mutations has been identified that are associated with gain-of-function and would allow these viruses to spread from human-to-human. Most of the mutations have been identified in avian influenza viruses currently circulating in birds and it has been shown that they may accumulate upon mammalian passage of these viruses (229). This possible scenario underscores the need for the development of effective vaccines. Therefore, numerous attempts have been made to develop such vaccines. Only with high doses of conventional vaccine preparations or the use of adjuvants appreciable H5N1 virus specific antibody responses could be induced (214). Here we demonstrate that the MVA-H5-sfMR replication-deficient vector vaccine candidate was immunogenic and that especially a booster vaccination given after one year, resulted in high antibody titers. The boosting effect observed here is reminiscent of the booster effect observed with an adjuvanted heterologous H5 vaccine (231).

To compare the immunogenicity of this vaccine with conventional H5N1 inactivated (un) adjuvanted vaccine candidates head to head clinical trials are necessary.

A limitation of the use of vector-based influenza vaccines may be interference of vector-specific immunity induced by natural infections or vaccination that could affect the immunogenicity of subsequent immunizations with vaccines based on the same vector (232). The MVA-based platform does apparently not suffer from this possible drawback as was shown previously (233). In the present study we also showed that the MVA-H5-sfMR vaccine elicits strong anamnestic antibody responses to the influenza virus HA upon second and third immunizations despite the induction of anti-vector immunity upon the first immunization.

The boostability of the transgene encoded antigen-specific antibody response can probably be attributed to the administration of a relatively high dose of replication deficient MVA, resulting in an incomplete round of replication and co-incited expression of the transgene. The entry of MVA into the cells that eventually express and present the antigen, can apparently not be blocked by pre-existing MVA specific antibodies. This is not surprising, as antibody-mediated in vitro neutralization of poxviruses is notoriously inefficient and its mechanistic correlation to in vivo protection remains still unclear (234). T cell mediated anti-viral immunity requires at least one round of infection, gene

expression and antigen presentation before becoming active and efficient recombinant antigen synthesis is possible even in the presence of strong MVA-specific CD8+ T cell responses (235). This aspect favors the use of this vector over replication competent viral vectors, like adenoviruses, that may suffer from pre-existing immunity against naturally occurring adenoviruses or vaccination induced vector neutralizing antibodies. The use of such vectors may have to rely on multiple serotypes and/or genetically modified variants, which could complicate practical and regulatory acceptance of the vaccine. Thus, our data show that MVA vector-specific immunity cannot abrogate the ability of the MVA-H5 vaccine to induce strong H5-specific booster responses. However, it is still unknown to what extent the priming of an H5-specific response could be modulated by anti-vaccinia immunity. This first-in-man study included only vaccinia naïve individuals and the capacity of MVA-H5 to elicit primary H5-specific immunity in healthy volunteers that were vaccinated against smallpox remains to be studied. Such follow-up studies would also inform about a potential influence of anti-vaccinia immunity on vaccine reactogenicity.

Although that this was only a single center trial, both the safety and immunogenicity data observed in humans with the MVA-H5-sfMR candidate vaccine are in accordance with previous results that we obtained with MVA-H5 candidate vaccines in mice and non-human primates (220–222). In addition, the MVA-influenza platform could be employed for the development of vaccines against emerging influenza viruses of subtypes other than H5N1. This way a library could be established consisting of recombinant MVA vaccines, each with a prototype HA gene (e.g. H5, H7, H9, H10) that can be used as a seed virus for fast up scaling of production on a suitable platform such as CEFs which are a well-established cell substrate that allows for large scale production of recombinant MVA viruses in a short time.

In conclusion, the present study demonstrates the favorable safety and immunogenicity profile of the MVA-based H5N1 vaccine and underlines that vaccine candidates arising from this platform hold great promise for the future.

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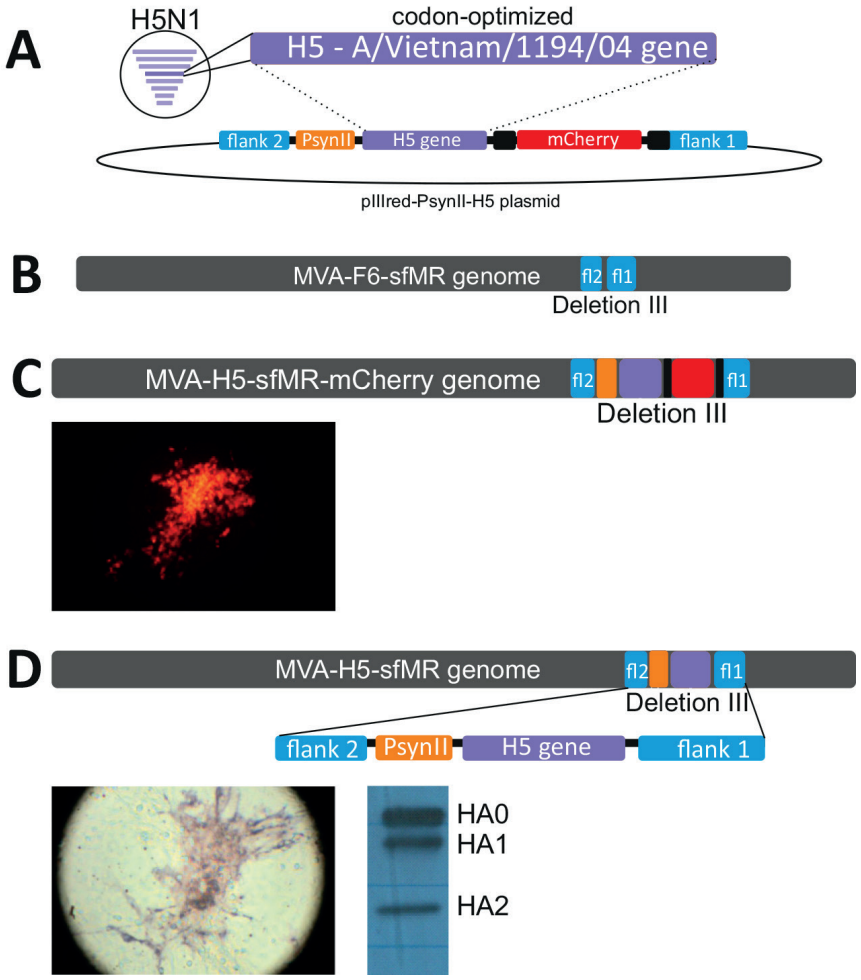


Appendix 1. MVA-H5-sfMR construction.

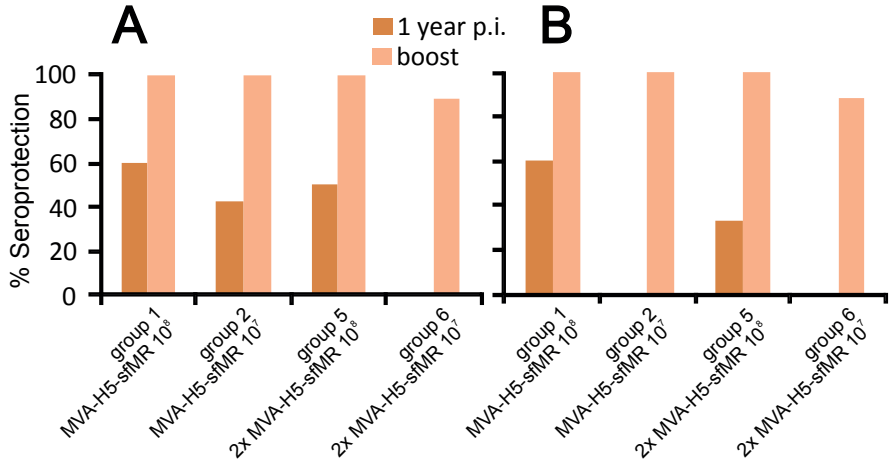
A shows the pIIIred-PsynII-H5 plasmid that was a result of cloning of the PsynII promoter and codon-optimized HA gene from influenza A/Vietnam/1194/04 virus in the pIIIred plasmid. B, MVA-F6-sfMR genome with the flank 2 region on the 5'end and the flank 1 region on the 3'end of the deletion III site. C, MVA-H5-sfMR-mCherry genome, the recombinant virus before the mCherry gene is looped out by homologous recombination. The picture shows a focus of red fluorescent cells as result of infection with MVA-H5-sfMR co-producing the marker protein mCherry.

D depicts the genome of the MVA-H5-sfMR recombinant virus that is evaluated as H5N1 vaccine candidate in the phase I/IIa clinical trial described here. The immunocytochemistry picture shows a plaque resulting of MVA-H5-sfMR infection in CEF cells. The staining shows H5 antigen expression, for this purpose an H5-specific mouse monoclonal antibody (IgG2a) was used. The Western blot lane depicts the influenza virus A/Vietnam/1194/04 HA0 protein (and cleavage products HA1 and HA2) in cell lysates of MVA-H5-sfMR infected baby hamster kidney cells (BHK-21 cells). The same mouse monoclonal antibody was used as for the immunocytochemistry staining.

Appendix 2. Seroconversion and seroprotection against homologous and heterologous H5N1 virus influenza viruses after boost immunization

Group		Time after 1st immunization		Time after Boost immunization			
		1 year		4 weeks			
				A/Vietnam/1194/04		A/Indonesia/5/05	
				HI	VN	HI	VN
1 Single shot MVA-H5-sfMR 10^8 pfu	Seroconversion*	Immunization	N=5	100% (5)	100% (5)	100% (5)	100% (5)
	Seroprotection			100% (5)	100% (5)	100% (5)	100% (5)
2 Single shot MVA-H5-sfMR 10^7 pfu	Seroconversion		N=7	100% (7)	100% (7)	100% (7)	86% (6)
	Seroprotection			100% (7)	86% (6)	100% (7)	86% (6)
5 Two shot MVA-H5-sfMR 10^8 pfu	Seroconversion	Immunization	N=6	100% (6)	100% (6)	83% (5)	83% (5)
	Seroprotection			100% (6)	100% (6)	83% (5)	67% (4)
6 Two shot MVA-H5-sfMR 10^7 pfu	Seroconversion		N=9	89% (8)	100% (9)	89% (8)	78% (7)
	Seroprotection			89% (8)	100% (9)	89% (8)	67% (6)

* Seroconversion is cumulative to 20 weeks. A subject that was seroconverted on 4 weeks after the first immunization is accounted as seroconverted on 8 weeks and 20 weeks also. The numbers in parenthesis after the percentages are the absolute numbers of subjects for seroconversion and seroprotection.



Appendix 3. Seroprotection rates against H5N1 viruses from antigenically distinct clades Seroprotection (antibody titers of >40 against: H5N1 influenza A/Turkey/Turkey/1/2005 virus (clade 2.2) and the transmissible variant of the H5N1 influenza A/Indonesia/5/2005 virus (clade 2.1) are described here for the individuals in groups 1,2,5 and 6 after boost immunization.

Chapter 7

Summarizing Discussion

SUMMARIZING DISCUSSION

The present thesis addresses the impact of acute viral infections in paediatric patients admitted to intensive care. Special attention is given to disease symptoms and signs, impact of underlying disease, and options for diagnosis and prevention of recently discovered respiratory virus infections, re-emerging dengue, and emerging avian influenza virus (H5N1). In this chapter the main findings are reviewed and discussed in the light of current clinical practice and future perspectives for improvement of clinical care.

Detection of previously unknown viruses has rapidly increased with the advent of random amplification and next-generation sequencing (NGS) technologies. Many recently discovered human respiratory viruses arose from the animal world and have, since crossing the species barrier to humans, adapted to the new species, eventually resulting in efficient human-to-human infection (62,236,237). Although new viruses are usually detected in individuals with clinical disease, upon detection, it is usually not clear to what extent the newly discovered virus is indeed associated with clinical manifestations in different age groups. The discovery of human metapneumovirus (HMPV, in 2001) was the first in a row to shed new light on new causes of bronchiolitis in paediatric patients, which until then was predominantly thought to be associated with the well-established viral pathogen RSV (4,27,47). Human coronavirus NL63 (HCoV-NL63) was discovered in 2004 and has since been related to acute respiratory tract infections (ARTI) (28,238,239). In 2005 human bocavirus (HBoV) was described in patients with respiratory complaints, while follow-up studies reported that HBoV was also associated with asymptomatic infections (113,240,241). Currently, four HBoV genotypes (HBoV 1-4) have been identified in humans known to cause mostly mild respiratory or gastro-intestinal disease (114).

In an attempt to assess the pathogenic potential of the recently discovered respiratory viruses in children, we studied paediatric patients with severe respiratory illness for the presence of these recently discovered respiratory viruses (**Chapter 2** and **Chapter 3**). We first studied paediatric patients who had tested positive for HBoV by RT-PCR and had been admitted to paediatric intensive care unit (PICU) excluding those with viral and bacterial co-infections. Pivotal to our approach was the addition of NGS data to the clinical and conventional laboratory data. NGS is a novel molecular method that among many other applications is currently also used to discover viruses. A major advantage of this method is that it bypasses the need to culture a virus, as not all viruses can be isolated *in vitro*. HBoV is a clear example of a virus that could not be isolated *in vitro* at the moment of discovery, and would probably not have been detected without NGS

technologies (113). In addition, NGS assays may even be expected to eventually replace RT-PCR based diagnostic tools in the future, because of their ability to also detect modified known viruses (33,134). In our study we used NGS besides our routine RT-PCR, since RT-PCR uses more or less fixed primer sets to detect viral pathogens. Thus the aim was to identify all viruses putatively present in the samples. We showed that HBoV without any detectable co-infection could cause severe acute respiratory tract infection (SARI) in paediatric patients requiring intensive care (**Chapter 2**). Moreover, we analysed the viral load of HBoV in these patients in which only HBoV could be detected and found significantly lower Ct-values (inversely related to viral loads) in these severely ill patients, compared to paediatric patients with viral co-infections. Therefore we concluded that HBoV in the absence of other detectable pathogens can cause SARI in children. Based on the abundant HBoV detection in children with either respiratory or gastro-intestinal complaints worldwide, the development of intervention strategies may have to be considered (118,241–247). Antiviral treatment of severely ill patients, with or without underlying disease, could reduce length of intensive care stay, hospital stay and improve patient outcome and would therefore be indicated if available. HBoV can now be cultured, which gives us the opportunity to start *in vitro* tests on possible antiviral treatment strategies (248–250).

The next step could be to consider vaccination against HBoV, although this should be subject to a thorough evaluation according to stringent scrutiny by industry and advisory bodies like Health Councils (251). The name HBoV (human **boca**virus) is derived from **bo**vine parvovirus and minute virus of **ca**nines, which are genetically closely related to HBoV and members of the parvovirus family: *Parvoviridae* (113). Bocaviruses can cause severe disease in different mammalian species, including humans and dogs (133,252). Dogs and cats are currently being vaccinated against another member of this family: canine parvovirus (CPV) and cats against feline parvovirus (FPV), which both may cause severe and even fatal disease in the respective species (253,254). The availability of registered safe and effective veterinary vaccines for members of this family, indicates that also the development of vaccines against HBoV might be feasible.

As a follow up to our HBoV study we also addressed the causative role of other recently discovered respiratory viruses, such as HMPV and HCoV-NL63 in children with severe respiratory disease admitted to PICU or medium care (MC), and compared their presence with those of longer known respiratory viruses such as RSV, human rhinovirus (HRV) and parainfluenza viruses (PIV) (**Chapter 3** and **Figure 1**). Essential to our approach again was to the best of our possibilities; exclude known viral and bacterial co-infections. Virus infections were only considered to be associated with a single virus when other respiratory viruses tested negative with RT-PCR (fifteen viruses tested for). Bacterial co-infections

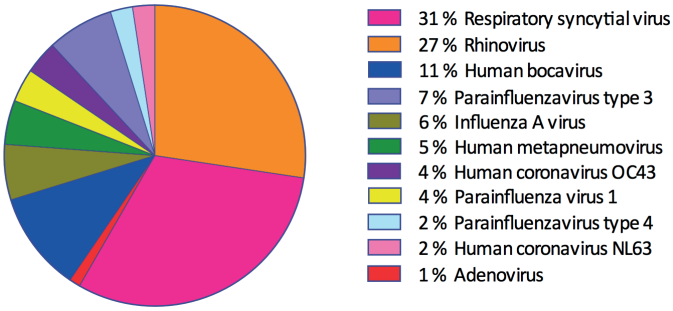


Figure 1. Single respiratory virus infections in paediatric patients admitted to intensive care and medium care at the Erasmus MC-Sophia over a 5-year period (2007-2012).

were considered absent or unlikely based on low C-reactive protein (CRP) levels (CRP ≤ 40 mg/L), as CRP-levels are frequently used in clinical settings to help differentiate between viral infections and bacterial infections (137,173). In addition, we included bacterial test results performed on sputum, whenever available. These studies showed that the 'big six' of respiratory viruses in paediatric patients admitted to intensive care or medium care are RSV, HRV, influenza A virus, HBoV, PIV-3 and HMPV. However the longer known respiratory viruses (RSV and HRV) are still the leading causes of severe disease, although newly discovered viruses like HBoV and HMPV were also detected after exclusion of viral co-infections and bacterial co-infections, although numbers of HMPV detection were relatively low in our study compared to other studies (4,19,20,39). Overall, both our and other studies emphasize the importance of respiratory viruses in paediatric patients. In the pneumococci vaccination era, it is becoming clear that respiratory viruses appear to be at least as important as bacterial pathogens as causes of severe disease of previously healthy children and children with underlying medical conditions (2,4,39,47,127,128,187). In **Chapter 3** we reported on 68 patients with underlying disorders admitted to PICU and MC. Current guidelines advice to vaccinate children with underlying disorders against influenza and to passively immunize premature infants (gestational age < 32 weeks) against RSV (46,255). When new intervention strategies become available for other respiratory viruses, these patients should be considered for prevention or therapy with priority.

This thesis also deals with the possible role of emerging and re-emerging viruses as a cause of severe disease in paediatric patients with underlying disorders. Therefore we studied the clinical impact of dengue virus (DENV) infection in patients with underlying disorders during the dengue outbreak in Curaçao that took place during the rainy season from 2010 to 2011. In total 1822 serologically confirmed cases were reported and four deaths. In these studies we identified two patients with DENV infection and underlying

sickle cell disease or sickle cell anaemia. **Chapter 4** describes the case histories of these patients. Both patients died, despite intensive treatment, which led us to hypothesize that extensive endothelial cell damage caused by DENV infection and sickled erythrocytes characteristic of sickle cell disease is a fatal combination. Although DENV prevention and control today solely depends on effective vector control measures (11), patients with underlying sickle cell disease or sickle cell anaemia should probably be considered in the future among the high-risk groups for vaccination or specific treatment.

Controlling paediatric respiratory viral infections from primary through tertiary care is dependent on currently available intervention strategies, which include prevention, early detection and treatment. Although the most cost-effective way to prevent respiratory viral infections would be the implementation of vaccination strategies, currently only for influenza preventive vaccines are available and their universal paediatric use beyond well-defined risk groups is still a matter of debate (256–258). In spite of decades of major research efforts, no effective vaccines against RSV and HRV are available, whereas vaccine research into the newly discovered viruses is still in its infancy (59,80,259). Therefore to prevent nosocomial infections in the respective hospital settings, intervention strategies for respiratory viral infections are largely limited to targeted hygienic and epidemiological measures. As recently reported by Kulkarni et al. spontaneously breathing and ventilated infants infected with RSV spread large amounts of RSV in aerosols, which remain airborne and infectious over significant periods of time and could be inhaled into the lower respiratory airways by other patients admitted to the ward and cause infection (260). This is a serious risk especially for immune-compromised and other high-risk paediatric patients.

The number of immune-compromised patients and patients with other underlying disorders tends to increase due to advances in medical treatment options. Therefore rapid and reliable diagnostic facilities are of utmost importance to identify respiratory viral infections as soon as they emerge in the hospital setting. Currently, several diagnostic methods are available for this purpose, including: RT-PCR, viral culture, direct immunofluorescence (D-IF), and rapid antigen detection tests (RADTs). In this thesis we focused on the evaluation of the value for the tertiary hospital care setting of commercially available RADTs for influenza A virus and RSV as an alternative to the gold standard RT-PCR based methods (**Chapter 5**). We studied the diagnostic performance of a influenza A virus rapid test in our tertiary care paediatric hospital over a period of almost 8-years that included the influenza H1N1 pandemic that started in 2009. However the test results were rather disappointing as we showed that sensitivity and positive predictive value (PPV) of the assay evaluated were relatively low (69% and 55% respectively), when compared with the gold standard RT-PCR. Therefore we would not

encourage the use of such first generation RADTs in a tertiary care setting, and either use newer generations RADTs with better performances or only gold standard RT-PCR methods. In contrast, the higher sensitivity and PPV of a RADT for RSV rendered this rapid test more useful, albeit still less sensitive than RT-PCR in our tertiary care setting. The downside of gold standard RT-PCR is the relatively long time (6-24 hours) between sample collection and availability of test results (200). This makes current RT-PCR formats less useful for admission decision-making and calls for the availability of faster methods. Indeed, currently new rapid point-of-care PCRs are being developed and implemented with a shorter turnaround time (201–206).

Although respiratory viruses can be detected with advanced diagnostic methods, clinicians are still largely empty-handed when it comes to specific antiviral treatment options. Treatment of respiratory virus infections in the paediatric setting is largely limited to symptomatic treatment as -with the exception of influenza viruses- for these virus infections limited or no antiviral treatment options are available (46,59,261). Currently, several antivirals are being tested for RSV and other respiratory virus infections, including: immunoglobulins, siRNA-interference, fusion inhibitors and small molecules (46).

Finally, an important aspect of respiratory virus infections is the threat by, as well as preparedness for a newly emerging pandemic influenza virus. Considering the paediatric population not only as a potential high-risk group, but also a reservoir for viral spread, it is highly recommended to pay special attention to this group in pandemic preparedness programs, as was practised e.g. in The Netherlands during the last influenza pandemic that started in 2009 (262,263). In the context of pandemic preparedness a clinical trial phase 1/2A study was successfully conducted to test a vaccine-candidate based on the Modified Vaccinia virus Ankara (MVA) vector platform expressing the HA of avian influenza virus (H5N1), in young adult volunteers as a proof of principle (**Chapter 6**). The data clearly highlighted that priming the population with a MVA that expresses the HA of the influenza virus (H5N1) from Vietnam (VN/1194/04) not only provides protective antibody responses against the homologous virus strain, but upon booster immunization also against a distant influenza virus (H5N1) from Indonesia (IND/5/05). This illustrates that this vaccination strategy may be useful to heterologously prime the human population with a vaccine derived from a distantly related virus of the same subtype, at times of an influenza pandemic threat.

Future perspectives & concluding remarks

Viruses are here to stay and will continue to cause disease in paediatric patients. Despite advanced diagnostic methods, about 10-20% of patients with acute respiratory disease remain undiagnosed, even when using state-of-the-art technology. This probably leaves the door open for the discovery of new pathogens (4).

Moreover, although the complex interplay between viral and bacterial co-infections has been studied widely, the exact basis of their mutual interactions within the host is still far from understood (264,265). Using state-of-the-art technologies, new insights into the bacterome and virome as well as their interplay with the host will surface and help us better understand this interaction between both groups of pathogens within and with the human host. Antibiotic resistance is a worldwide problem, and better studying and managing the interplay between bacterial infections and viral infections in the host could probably reduce antibiotic administration considerably. The need for antibiotics may therefore be expected to further be reduced with the future development and use of novel generations of antivirals and antiviral vaccines.

Virus infections may also be involved in the development of certain forms of chronic disease, causing activation or altering of host immunity. The role of previous virus infections in the pathogenesis of complex diseases such as type 1 diabetes, asthma and inflammatory bowel disease will further have to be elucidated and may lead to a better understanding of their pathogeneses and therefore to novel interventions for these diseases.

Worldwide eradication programs encouraged by WHO that target vaccine preventable diseases have been applauded but also raise new concerns. With our current knowledge of the human-animal interface, it is not unlikely that animal viruses related to the eradicated viruses, have a tendency to cross the animal-human interface when cross-reactive immunity is waning in the population. Perhaps the most significant example is the re-emergence of monkey- and cowpox virus infections in humans after smallpox eradication and subsequent abolishment of smallpox vaccination (266). A similar threat may come from animal morbilliviruses after the envisaged eradication of measles from humans (267,268). Therefore active surveillance programs in animals and humans alike, as well as the development of vaccination strategies for such post-eradication events should be considered essential elements of future virus eradication strategies.

Finally, intervention programmes for respiratory and other viral threats should be developed in ‘peace-time’, based on the development of more efficient, and most importantly, more rapid vaccine development and production platforms for rapid ‘war-time’ response to future influenza and other epidemic and pandemic viral threats (269).

Chapter 8

Summaries

ENGLISH SUMMARY

The present thesis describes studies on different aspects of acute respiratory tract infections (ARTIs) caused by viruses in paediatric patients admitted to intensive care. Special attention is given to disease symptoms and signs, impact of underlying disease, and options for diagnosis and prevention of recently discovered respiratory virus infections, re-emerging dengue, and emerging avian influenza virus (H5N1). In this chapter the main findings are summarized.

Chapter 1 gives an overview of virus infections in paediatric patients, starting by giving an overview of ARTIs caused by respiratory viruses and proceeding to discuss the diagnostic and prevention methods of these viruses. In addition newly discovered viruses, emerging and re-emerging virus infections are described in more detail.

Virus discovery

Detection of previously unknown viruses has rapidly increased with the advent of random amplification and next-generation sequencing (NGS) technologies (62,236,237). Although new viruses are usually detected in individuals with clinical disease upon detection, as described for avian influenza virus (H5N1), severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle-East respiratory syndrome coronavirus (MERS-CoV) for example, it is usually not clear to what extent the newly discovered virus is indeed associated with clinical manifestations in different age groups (30,31). The discovery of human metapneumovirus (HMPV) in 2001 was the first in a row to shed new light on new causes of bronchiolitis in paediatric patients, which until then was predominantly thought to be associated with the well-established viral pathogen respiratory syncytial virus (RSV) (4,27,47). Human coronavirus NL63 (HCoV-NL63) was discovered in 2004 and since has been related to ARTI (28,238,239). In 2005 human bocavirus (HBoV) was described in patients with respiratory complaints, while follow-up studies reported that HBoV was also associated with other disease manifestations and asymptomatic infections (113,240,241).

Respiratory viruses

The present thesis describes studies on different aspects of ARTIs caused by respiratory viruses in paediatric patients. It is not uncommon for a child under the age of five to suffer up to six febrile ARTI episodes per year (12–14). Moreover, ARTIs are the most important reason for emergency department visits and hospitalization of young children and are often caused by respiratory viruses (15–18).

In an attempt to assess the pathogenic potential of the recently discovered respiratory viruses in children, we studied paediatric patients with severe respiratory illness for the

presence of these recently discovered respiratory viruses (**Chapter 2** and **Chapter 3**). We first studied HBoV real-time reverse transcriptase polymerase chain reactions (RT-PCR) positive samples from paediatric patients admitted to paediatric intensive care unit (PICU) excluding those with viral and bacterial co-infections. Pivotal to our approach was the addition of next-generation sequencing (NGS) data to the clinical and conventional laboratory data. NGS is a novel molecular method that among many other applications is currently also used to discover viruses. In our study we used NGS besides our routine RT-PCR, since RT-PCR uses more or less fixed primer sets to detect viral pathogens. Thus the aim was to identify all viruses putatively present in the samples. We showed that HBoV without any detectable co-infection could cause severe acute respiratory tract infection (SARI) in paediatric patients requiring intensive care (**Chapter 2**). Therefore we concluded that HBoV in the absence of other detectable pathogens could cause severe respiratory disease in children.

As a follow up to our HBoV study we also addressed the causative role of other recently discovered respiratory viruses, such as HMPV and HCoV-NL63 in children with severe respiratory disease admitted to PICU or medium care (MC). We compared their presence with those of longer known respiratory viruses such as RSV, human rhinovirus (HRV), and parainfluenza viruses (PIVs) (**Chapter 3**). Essential to our approach again was to the best of our possibilities, exclude known viral and bacterial co-infections. Severe respiratory tract infection was only considered to be associated with a single virus when other respiratory viruses tested negative with RT-PCR (fifteen viruses tested for). Bacterial co-infections were considered absent or unlikely based on low C-reactive protein (CRP) levels (CRP ≤ 40 mg/L), as CRP-levels are frequently used in clinical settings to help differentiate between viral infections and bacterial infections (137,173). In addition, we included bacterial test results performed on sputum, whenever available. Finally, we identified 40 patients admitted to MC and 44 patients admitted to PICU with a single respiratory virus infection. This study showed that the 'big six' of respiratory viruses in paediatric patients admitted to PICU or MC are RSV, HRV, influenza A virus, HBoV, PIV-3 and HMPV. Overall, our studies emphasize the importance of these respiratory viruses as sole causative agents of SARIs in children.

Controlling paediatric respiratory viral infections from primary through tertiary care is dependent on currently available intervention strategies, which include prevention, early detection and treatment. Although the most cost-effective way to prevent respiratory viral infections would be the implementation of vaccination strategies, currently only preventive vaccines are available for influenza and their universal paediatric use beyond well-defined risk groups remains a matter of debate (256–258). Moreover, passive immunisation against RSV is currently being used for high-risk infants

only. In spite of decades of major research efforts, no effective vaccines against RSV and HRV are available, whereas vaccine research into the newly discovered viruses is still in its infancy (59,80,259). Epidemiological studies, such as presented in **Chapter 2** and **Chapter 3**, describing the prevalence of different respiratory viruses in specific patients groups provide valuable information for when specific vaccines and antiviral treatments become available.

Emerging and re-emerging viruses

This thesis also deals with the possible role of emerging and re-emerging viruses as a cause of severe disease in paediatric patients with underlying disorders. Dengue virus (DENV) is a re-emerging virus that circulates in tropical and subtropical areas around the world and is transmitted by the bite of infected mosquitoes of the *Aedes* species. There are four DENV serotypes (1-4). Yearly, 390 million people are at risk to become infected with DENV, many of them are children. Seventy-five per cent do not develop clinical signs or symptoms, 25% do develop symptoms, of which a small percentage develop severe DENV infection. Several risk factors have been determined for patients to develop severe DENV infection, such as a previous infection with a different DENV serotype or underlying disorders. We studied the clinical impact of DENV infection in patients with underlying disorders during the dengue outbreak in Curaçao that took place during the rainy season from 2010 to 2011. In total 1822 serologically confirmed cases were reported and four deaths. In these studies we identified two patients with DENV infection and underlying sickle cell disease or sickle cell anaemia. **Chapter 4** describes the case histories of these patients. Both patients died, despite intensive treatment, which has not been described before in Curaçao. Recently, the first dengue vaccine, Dengvaxia (CYD-TDV) by Sanofi Pasteur, was registered in several countries for use in individuals between 9-45 years of age living in endemic areas (11,106,109). Soon recommendations of the WHO will follow concerning immunization with this new dengue vaccine. Patients with underlying sickle cell disease or sickle cell anaemia should probably be considered among the high-risk groups for vaccination or specific treatment when these become available.

Virus diagnostics

Currently, several diagnostic methods are available to detect respiratory viruses, including: RT-PCR, viral culture, direct immunofluorescence (D-IF), and rapid antigen detection tests (RADTs). Identification of respiratory viruses is important for disease management, as the presence of these infections may require specific treatment (i.e. oseltamivir) and hospital containment measures. Gold standard RT-PCR is not performed in all hospitals, as it requires a molecular diagnostic laboratory with specialized personnel and equipment. Instead, RADTs are often used as these assays are easier and cheaper to perform and less time-consuming (15-30 minutes vs 6-24 hours) (43–45). In

the present thesis we evaluated the diagnostic performance of commercially available RADTs BinaxNOW® for influenza A&B viruses and RSV in our tertiary care paediatric hospital over a period of almost 8-years (**Chapter 5**). We analysed the results obtained with fresh nasal washings from children between 0-5 years who visited our emergency department or were admitted to our hospital. The test results for influenza A virus were rather disappointing with a sensitivity and positive predictive value (PPV) of 69% and 55% respectively, when compared with RT-PCR. In contrast, the higher sensitivity and PPV of a RADT for RSV rendered this rapid test more useful, albeit still less sensitive than RT-PCR in our tertiary care setting (79% and 97% respectively). Based on our results we stopped using both rapid tests and currently use rapid PCR-based tests for detection of influenza virus and RSV.

Prevention

An important aspect of respiratory virus infections is the threat by, as well as preparedness for newly emerging pandemic influenza viruses. In **Chapter 6** we describe a clinical trial phase 1/2A study in 79 young adult volunteers, that was successfully conducted to test a vaccine-candidate based on the Modified Vaccinia virus Ankara (MVA) vector platform expressing the haemagglutinin (HA) gene of avian influenza virus (H5N1). The safety of the use of an MVA-vector was shown, and the data clearly highlighted that priming the population with a MVA that expresses the HA of the influenza virus (H5N1) from Vietnam (VN/1194/04) not only provides protective antibody responses against the homologous virus strain, but upon booster immunization of 27 volunteers, also against a distant influenza virus (H5N1) from Indonesia (IND/5/05). This illustrates that this vaccination strategy may be useful to heterologously prime the human population with a vaccine derived from a distantly related virus of the same subtype, at times of an influenza pandemic threat.

The last chapter of this thesis (**Chapter 7**) comprises an overview of its findings, together with a general discussion and future perspectives.

In conclusion this thesis addresses the disease burden of more recently discovered respiratory viruses and their role in severe disease in paediatric patients. Moreover, the re-emerging DENV is described in relation to patients with underlying disease. In addition, we describe the diagnostic performance of rapid antigen detection tests for RSV and influenza viruses in our hospital. Finally, a study with a new prevention method against the emerging avian influenza virus (H5N1), making use of a vectored candidate vaccine, is described.

NEDERLANDSE SAMENVATTING

Dit proefschrift beschrijft studies naar verschillende aspecten van ernstige virale luchtweginfecties in kinderen. Het voorkomen van reeds langer bekende respiratoire virussen zoals respiratory syncytial virus (RSV) en influenza virus (griepvirus) wordt vergeleken met dat van meer recent ontdekte virussen zoals human metapneumovirus (HMPV) en humaan bocavirus (HBoV). Er wordt vooral aandacht besteed aan klachten die geassocieerd kunnen zijn met deze respiratoire virusinfecties, het eventuele onderliggend lijden van deze patiënten en naar de mogelijkheden van diagnostiek om deze respiratoire virussen aan te tonen. Daarnaast worden twee fatale gevallen van dengue beschreven in patiënten met onderliggend lijden. Het proefschrift sluit af met een studie naar een preventiemethode voor het opkomende aviair influenza virus (H5N1) (vogelgriep).

De introductie, beschreven in **hoofdstuk 1**, geeft een overzicht van virale infectieziekten bij kinderen. Er wordt ingegaan op acute bovenste en onderste luchtweginfecties veroorzaakt door respiratoire virussen. Daarnaast worden de huidige diagnostiek en preventiemethoden voor respiratoire virussen beschreven. Tevens is er in dit hoofdstuk aandacht voor opkomende virusinfecties zoals coronavirussen (o.a. SARS- en MERS coronavirussen) en virussen overgedragen door muggen zoals denguevirus, Chikungunya virus en Zika virus.

Recent ontdekte virussen

Met de opkomst van geavanceerde detectiemethoden voor virussen op basis van het genetisch materiaal van het virus, is recent een aantal nieuwe virussen ontdekt (62,236,237). Vaak worden deze nieuwe virussen gevonden bij patiënten met ernstige klachten, zoals patiënten met aviaire influenza, SARS en MERS, maar voor sommige virussen is het niet altijd direct duidelijk of deze klachten ook daadwerkelijk veroorzaakt worden door het nieuw ontdekte virus en wat de rol van deze virussen in de gehele populatie is (30,31). In 2001 werd HMPV ontdekt bij jonge kinderen met een ernstige bronchiolitis (27). Dit ziektebeeld wordt meestal veroorzaakt door RSV, maar bleek nu ook door HMPV te kunnen worden veroorzaakt (4,27,47). In 2004 werd het human coronavirus NL63 (HCoV-NL63) ontdekt. Dit virus wordt sindsdien vaak gevonden bij patiënten met luchtweginfecties (28,238,239). HBoV werd voor het eerst beschreven in 2005 (113). Dit virus werd gevonden in patiënten met respiratoire klachten, maar vervolgstudies beschreven ook HBoV geïnfecteerde patiënten met andere, of zelfs in het geheel geen klachten (113,240,241).

Respiratoire virussen

In dit proefschrift wordt aandacht besteed aan ziektebeelden veroorzaakt door respiratoire virussen. Kinderen jonger dan vijf jaar kunnen tot wel zes keer per jaar last hebben van een acute luchtweginfectie (12–14). Mede daardoor zijn acute luchtwegproblemen bij kinderen jonger dan 1 jaar de meest voorkomende reden van huisarts-consultatie en ziekenhuis- opname (15–18). Virussen zijn meestal de oorzaak van acute luchtwegproblemen, maar welke van de recent ontdekte virussen al dan niet ernstige klachten kunnen veroorzaken bij paediatrische patiënten is op dit moment nog niet duidelijk. In een groot deel van dit proefschrift ligt de nadruk op onderzoek naar de rol van deze recent ontdekte respiratoire virussen.

Een voorbeeld van een recent ontdekt virus waarvan nog niet duidelijk is of het daadwerkelijk ernstige ziekte kan veroorzaken is HBoV. Onderzoek naar dit virus wordt beschreven in **hoofdstuk 2**. Wij identificeerden ernstig zieke patiënten over een periode van vijf jaar die positieve testresultaten te zien gaven in de HBoV RT-PCR. RT-PCR is momenteel de gouden standaard om respiratoire virussen aan te tonen met specifieke primers om een bepaald gedeelte van het genetische materiaal van het virus te amplificeren. Deze methode wordt meer en meer gebruikt voor virusdiagnostiek. De geselecteerde patiënten waren opgenomen op de Intensive Care unit (IC) met ernstige luchtweginfecties omdat zij respiratoir-insufficiënt waren en beademd moesten worden. Wij ontwikkelden een algoritme waarmee bacteriële en virale co-infecties zoveel mogelijk werden uitgesloten. Op basis hiervan bleek dat HBoV kinderen inderdaad ernstig ziek kan maken in afwezigheid van bacteriële en virale co-infecties. Wij gebruikten hiervoor onder andere next-generation sequencing, een methode om bekende, onbekende en veranderde virussen op basis van hun genetische materiaal aan te tonen. Dit deden we als aanvulling op de reeds uitgevoerde RT-PCR testen om vijftien bekende virussen op te sporen.

Als een vervolg op deze studie hebben we ook de rol bestudeerd van andere recent ontdekte virussen zoals HMPV en HCoV-NL63 bij het ontstaan van ernstige ziekte bij kinderen in afwezigheid van bacteriële en virale co-infecties (**hoofdstuk 3**). Hierbij vergeleken wij het voorkomen van deze virussen met die van de reeds langer bekende virussen zoals RSV, humaan rhinovirus (HRV) en parainfluenza virussen (PIV). We hebben twee patiëntengroepen met elkaar vergeleken, de eerste groep was opgenomen op de medium care (MC) en de tweede groep patiënten op de IC. Door middel van het nauwkeurig bestuderen van de medische dossiers, virologische, bacteriologische en andere laboratoriumuitslagen, vonden wij uiteindelijk 40 patiënten opgenomen op de MC en 44 patiënten opgenomen op de IC met als enige verwekker één respiratoir virus. Een bacteriële co-infectie als additionele reden van de ernstige ziekte was

onwaarschijnlijk op basis van een laag C-reactief proteïne (CRP ≤ 40 mg/L) en, voor zover getest, een negatieve bacteriologische uitslag. CRP is een veel gebruikte bloedwaarde om de aanwezigheid van een bacteriële infectie aan te tonen (137,173). Deze studie laat zien dat RSV, HRV, influenza A virus, HBoV, PIV-3 en HMPV de meest voorkomende respiratoire virussen zijn bij kinderen opgenomen op de MC en IC. Samenvattend laten beide studies zien dat naast de reeds langer bekende respiratoire virussen, recent ontdekte virussen zoals HBoV, HMPV en HCoV-NL63 ook ernstige respiratoire ziekte kunnen veroorzaken bij kinderen.

Ondanks vele jaren onderzoek naar geschikte preventiemethoden en antivirale therapieën zijn deze tot dusver niet beschikbaar voor de meeste respiratoire virussen. Hoewel vaccineren de meest kostenefficiënte manier is om virale luchtweginfecties te voorkomen is er momenteel alleen een effectief vaccin beschikbaar tegen influenza. Het gebruik van dit vaccin wordt momenteel in Nederland alleen geadviseerd voor kinderen in bepaalde risicogroepen en het universele gebruik van dit vaccin voor alle kinderen is nog onderwerp van discussie (256–258). Daarnaast is er passieve immunisatie tegen RSV beschikbaar voor zuigelingen in bepaalde risicogroepen. De ontwikkeling van andere respiratoire virusvaccins en antivirale middelen staat helaas nog in de kinderschoenen (59,80,259). Epidemiologische studies, zoals beschreven in **hoofdstuk 2 en 3**, naar het voorkomen van de verschillende respiratoire virussen in patiëntengroepen met ernstige respiratoire klachten zijn daarom van groot belang om wanneer deze vaccins of antivirale middelen beschikbaar komen, de te vaccineren risicogroepen te identificeren.

Opkomende virussen

Het proefschrift besteedt verder aandacht aan opkomende virussen zoals denguevirus (DENV) en aviaire influenza virus (H5N1). DENV infecties komen wereldwijd voor in tropische en subtropische gebieden en DENV wordt overgedragen door *Aedes* muggen. Er zijn vier serotypes van het DENV (DENV 1-4). Jaarlijks lopen ongeveer 390 miljoen mensen het risico om geïnfecteerd te worden met het DENV, een groot aantal hiervan zijn kinderen. De meeste mensen die geïnfecteerd worden met het DENV ontwikkelen geen of nauwelijks klachten. Een kwart van hen ontwikkelt wel klinische symptomen en daar weer een klein deel van ernstige symptomen. Welke patiënten uiteindelijk ernstige ziekte ontwikkelen kan gerelateerd zijn aan verschillende oorzaken. Bekende risicofactoren voor een ernstige DENV infectie zijn o.a. een eerdere infectie met een ander DENV serotype of onderliggend lijden. Tijdens een dengue-uitbraak in 2010-2011 op Curaçao hebben wij de impact van DENV bestudeerd in patiënten met onderliggend lijden. Tijdens deze uitbraak werden 1822 serologisch bevestigde dengue patiënten en vier sterfgevallen gerapporteerd. In **hoofdstuk 4** beschrijven wij twee patiënten met sikkelcelziekte en een ernstige DENV infectie. Ondanks intensieve behandeling

overleden beide patiënten. Het voorkomen van gevallen van sikkelcelziekte en DENV infectie was nog niet eerder beschreven op Curaçao. Recente studies met een kandidaat dengue vaccin hebben laten zien dat het vaccin Dengvaxia (CYD-TDV) van Sanofi Pasteur effectief en veilig is (106,109). Dit vaccin is momenteel in een aantal landen waar DENV endemisch voorkomt geregistreerd voor gebruik bij kinderen en volwassenen tussen de 9-45 jaar (11). Verdere aanbevelingen van de WHO over het gebruik van dit vaccin voor bepaalde risicogroepen zullen snel volgen. Zoals beschreven in onze studie vormen patiënten met sikkelcelziekte zeker een risicogroep waarvoor mogelijk dit vaccin of andere vaccins beschikbaar zouden moeten komen.

Virusdiagnostiek

In de laatste jaren zijn veel nieuwe detectiemethoden ontwikkeld om virusinfecties aan te tonen dan wel uit te sluiten. Het belang van het juist diagnosticeren van een virale infectie heeft invloed op het beleid voor de patiënt en het ziekenhuis. Zo kan er worden besloten om een patiënt met een positieve uitslag voor RSV of influenza geen antibiotica te geven en eventueel te starten met oseltamivir indien influenza virus is aangetoond. Daarnaast is het belangrijk om virusverspreiding te voorkomen door patiënten te isoleren en strikte hygiënische maatregelen te treffen voor het verzorgend personeel en andere patiënten.

Er zijn momenteel verschillende diagnostische methoden voor virusdetectie beschikbaar zoals de reeds eerder beschreven RT-PCR, maar ook virusisolatie *in vitro*, en methoden om snel een virus-antigeen aan te tonen zoals met directe immunofuorescentie (D-IF) en antigeendetectie doormiddel van de zogenoemde sneltesten. RT-PCR (de goudenstandaard voor virusdiagnostiek) wordt niet in elk ziekenhuis uitgevoerd omdat met de huidig tests een moleculair diagnostisch laboratorium met gespecialiseerd personeel beschikbaar moeten zijn. In plaats hiervan worden veelal sneltesten gebruikt, deze hebben als voordeel dat ze makkelijk te gebruiken zijn (geen moleculair diagnostisch laboratorium nodig) en veel sneller een uitslag geven dan bijvoorbeeld RT-PCR (15-30 minuten vs 6-24 uur) (43–45).

In dit proefschrift hebben we de diagnostische waarde en beperkingen van zulke sneltesten die in ons ziekenhuis (een tertiar centrum) werden gebruikt voor de detectie van RSV en influenza virus geanalyseerd (**hoofdstuk 5**). We hebben de uitkomsten van vier virus-diagnostische methodes namelijk: RT-PCR, virus kweek, D-IF en de sneltesten van BinaxNOW® RSV en influenza A&B, over een periode van bijna acht jaar met elkaar vergeleken. Hierbij hebben we alleen de uitslagen geanalyseerd van testen gedaan op verse neusspoelsels van kinderen jonger dan vijf jaar. Uit deze analyses kwam duidelijk naar voren dat de sensitiviteit en positief voorspellende waarde van BinaxNOW® influenza

beduidend lager was dan die van de gouden standaard RT-PCR (respectievelijk 69% en 55%). Hoewel de BinaxNOW® RSV beter presteerde dan de BinaxNOW® influenza, waren ook deze uitkomsten minder betrouwbaar dan die van de gouden standaard RT-PCR (respectievelijk 79% en 97%). Deze resultaten hebben ertoe geleid dat beide sneltesten niet meer routinematig worden gebruikt voor diagnostiek bij kinderen in het Erasmus MC-Sophia. Er worden momenteel nieuwe methodes ontwikkeld en gebruikt zoals snellere RT-PCR voor het aantonen van RSV en influenza virusantigenen.

Preventie

Het proefschrift eindigt met een hoofdstuk gericht op de preventie van een nieuw opkomende virusinfectie middels vaccinatie. In **hoofdstuk 6** worden veiligheid en effectiviteit van een kandidaat-vaccin tegen het opkomende aviaire influenza virus (H5N1) beschreven. In deze studie werd gebruik gemaakt van een gemodificeerd pokkenvirus (MVA) als vector, dat het heamaglutinine (HA) van het aviaire influenza virus (H5N1) tot expressie brengt. In totaal werden 79 vrijwilligers geïncludeerd waarna een gerandomiseerde studie met de relevante controles werd uitgevoerd gebruik makend van een kandidaat-vaccin gebaseerd op MVA-H5 dat in een escalerende dosis werd toegediend. Na een jaar werden 27 vrijwilligers die eerder gevaccineerd waren met het MVA-H5 vaccin nogmaals gevaccineerd (booster). Geen van de vrijwilligers bleek ernstige bijwerkingen te vertonen en vrijwilligers die met het MVA-H5 vaccin waren gevaccineerd ontwikkelden niet alleen beschermend geachte antistoffen tegen de homologe H5 virusstam uit Vietnam (VN/1194/04), maar na booster-vaccinatie een jaar later ook tegen de minder verwante H5 virusstam uit Indonesië (IND/5/05). Het veilig kunnen gebruiken van de MVA-vector en het boosten van een heterologe antistofrespons biedt nieuwe perspectieven voor de ontwikkeling van vaccins tegen andere potentieel pandemische influenza-virussen.

Het laatste hoofdstuk van dit proefschrift (**hoofdstuk 7**) geeft een overzicht van alle bevindingen die in het proefschrift zijn beschreven. Deze bevindingen worden samengevat en geïnterpreteerd in het licht van de huidige kennis over virusinfecties. Dit hoofdstuk eindigt met een overzicht van de laatste ontwikkelingen en aanbevelingen voor de toekomst.

Concluderend kan gesteld worden dat dit proefschrift niet alleen aandacht heeft besteed aan de rol die recent ontdekte respiratoire virussen spelen bij kinderen, maar ook aan bepaalde aspecten van opkomende virussen zoals DENV en aviaire influenza virus (H5N1). Tevens is de waarde onderzocht van diagnostische methoden die thans gebruikt worden voor het aantonen van respiratoire virussen. Daarnaast is de veiligheid en de beschermende waarde van een nieuwe methode van vaccinatie tegen aviaire influenza

virus (H5N1) bestudeerd. Het onderzoek beschreven in dit proefschrift heeft derhalve bijgedragen aan de kennis over recent ontdekte en opkomende virusinfecties, hun bijdrage aan het doen ontstaan van ernstige ziekte bij kinderen en het ontwikkelen van nieuwe interventiestrategieën tegen deze infecties. Deze inzichten kunnen een bijdrage leveren aan toekomstig onderzoek naar en het doelgericht inzetten van vaccins en antivirale middelen gericht op het voorkomen en genezen van deze virusinfecties.

Chapter 9

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Chapter 10

About the Author

ABOUT THE AUTHOR

The author of this thesis, Fleur Madeline Moesker, was born on the 29th of May in 1984 in Voorschoten, The Netherlands. She attended the Marecollege in Leiden after which she graduated from Athenaeum of the ROC Leiden in 2003. After this she studied Medicine at the Academic Medical Centre of the University of Amsterdam. Paediatrics became her interest during this study and she did here internship Children and Adolescent psychiatry in London at King's College University and her paediatric internship at the OLVG in Amsterdam. After graduating from medical school in 2010 she started working as a medical-researcher in Curaçao to study a dengue outbreak and the viral epidemiology of respiratory tract infections and gastro-intestinal diseases in paediatric and adult patients for ten months. In August 2011 she started the Research Master 'Infection and Immunity' at the postgraduate school of Molecular Medicine at Erasmus MC from which she graduated in 2013. In October 2011 she started as a PhD student at the department of Viroscience under supervision of Prof.dr. A.D.M.E. Osterhaus and dr. P.L.A. Fraaij, with a focus on acute virus infections in paediatric patients. In March 2012 she also started to work at the out-patient-clinic for Travel Medicine at Erasmus MC. In May 2016 she started working at the department of Paediatrics at the Diakonnessenhuis in Utrecht.

PHD PORTFOLIO

Name:	Fleur Madeline Moesker
Erasmus MC Department:	Department of Viroscience
Research School:	Post-graduate Molecular Medicine (MolMed)
PhD period:	2011-2015
Promotor:	Prof.dr. Albert. D.M.E. Osterhaus
Co-promotor:	Dr. P.L.A. Fraaij

Education

2003-2010	Medical Degree at University of Amsterdam, Amsterdam, The Netherlands
2011-2013	Master of Science Infection & Immunity. Postgraduate school Molecular Medicine at the Erasmus Medical Center, Rotterdam, The Netherlands

In-depth courses

2011	Study Design (NIHES)
	Biomedical Research Techniques (MolMed)
	Scientific English writing (MolMed)
	Statistics and Survival Analysis for PhD-students (MolMed)
	Summercourse 1 (part of Master Infection & Immunity)
2012	The basic introduction course on SPSS (MolMed)
	Wintercourse 1 & Summercourse 2 (part of Master Infection & Immunity)
	Course in Virology (MolMed)
	Epidemiology and Infectious Diseases (NIHES)
	Photoshop, Illustrator CS5 and InDesign course for PhD-students (MolMed)
	Basiscursus Regelgeving en Organisatie voor Klinisch Onderzoekers (Erasmus MC)
2013	Wintercourse 2 (part of Master Infection & Immunity)
2014	Biostatistics (NIHES)
	Research management for PhD-students (MolMed)
2015	English Biomedical Writing and Communication (Erasmus MC)

Presentations

Oral

European Society for Pediatric Infectious Diseases (ESPID), Milan, Italy	2013
Nederlandse Werkgroep Klinische Virologie (NWKV), Veldhoven, The Netherlands	2011

Poster

European Society for Pediatric Infectious Diseases (ESPID)	2015
3rd International One Health Congress (IOHC)	2015
Sophia research days	2014
Sophia research days	2013
European Society for Clinical Virology (ESCV)	2011

Attended conferences and symposia

European Society for Pediatric Infectious Diseases (ESPID)	2011, 2013-2015
3rd International One Health Congress (IOHC)	2015
Farewell symposium prof.dr. A.D.M.E Osterhaus	2014
Sophia Research Days	2013-2014
Training upcoming leaders in pediatric science (TULIPS): Jonge Onderzoekersdagen	2013-2014
Infectious Disease week, San Franscisco, USA	2013
Molecular Medicine days	2012-2013
Farewell symposium prof.dr. R. de Groot	2013
Oration symposium prof.dr. E.C.M. van Gorp	2013
Farewell symposium dr. N.G. Hartwig	2012
Klinische avond Tropische Geneeskunde	2012
PAOS Nascholingsavond Kindergeneeskunde	2012
European Society for Clinical Virology (ESCV), Madeira, Portugal	2011
Nederlandse Werkgroep Klinische Virologie (NWKV)	2011
1st postgraduate course on “Congenital Heart Disease” (NASHKO)	2011
HIV-monitoring (NASKHO)	2011

Teaching activities

Basisopleiding en nascholingscursussen (huis)artsen Reizigersgeneeskunde	2012-2014
Viruskenner Coach	2011-2013
Co-supervision of MSc. Thesis (I. Willems)	2011

Miscellaneous

Ambassador Research Master Infection & Immunity	2013-2014

LIST OF PUBLICATIONS

Book Chapters:

Overview of human upper and lower respiratory tract viral infections
Fleur M. Moesker, Pieter L.A. Fraaij, Albert D.M.E. Osterhaus
Book chapter in book entitled: Human respiratory viral infections, publication date June 23rd 2014

New, emerging and re-emerging respiratory viral infections
Fleur M. Moesker, Pieter L.A. Fraaij, Albert D.M.E. Osterhaus
Book chapter in book entitled: Viral Infections and Global changes, publication date October 18th 2013

Papers:

Human bocavirus infection as a cause of severe acute respiratory tract infection in children
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Chapter

11

Dankwoord

DANKWOORD

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