Clinical and epidemiological aspects of viral infections: a molecular approach

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ISBN 978-90-367-7107-8 (printed version)
ISBN 978-90-367-7106-1 (electronic version)
Financial support for the publication of this thesis was kindly provided by
Groningen Graduate School of Medical Sciences
Page layout: DTP at home
Cover Layout: DTP at home
Printed by: Gildeprint

rijksuniversiteit
groningen

Clinical and epidemiological aspects of viral infections

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Proefschrift

ter verkrijging van de graad van doctor aan de
Rijksuniversiteit Groningen
op gezag van de
rector magnificus prof. dr. E. Sterken
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

woensdag 22 oktober 2014 om 16.15 uur

door

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geboren op 26 januari 1970

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1.1 A short history of the science of viral diagnostics

Long before the actual detection of filterable viruses at the end of the 19th century, the role of transmissible agents in diseases like yellow fever and measles was recognized.[1,2] In search for therapies against frequently encountered and deadly diseases like rabies and smallpox, early vaccination strategies were developed: it was shown that inoculation with attenuated virus provided protection against these fatal diseases.[3] Because viruses are unable to grow on artificial media, it was not until the 1890s that viral diseases were first acknowledged in the laboratory, when Dmitri Ivanowsky (1892) and Martinus Beijerinck (1898) independently showed that the filtered extract of plants with mosaic disease caused disease when introduced to healthy plants (contagium vivum fluidum).[4,5] The recognition that viruses need living cells to propagate, led to the use of nonhuman hosts to isolate and characterize viral agents. However, the science of diagnostic virology was not further advanced until the development of tissue culture techniques with cytopathic effect as read outs to detect and identify a virus from a clinical specimen (Table 1.1.).[3]

Table 1.1. Timeline of highlights in diagnostic virology

1892	Discovery of filterable viruses (Dmitri Ivanowsky)
1898	Discovery of filterable viruses independently from Ivanowsky (Martinus Beijerinck)
1928	Cultivation of vaccinia virus
1937	Development of yellow fever vaccine by passaging through animals
1949	Poliomyelitis virus able to be cultivated in cell cultures of non-neural origin
1950	Demonstration of cytopathic effect
1950s	Demonstration of usefulness of fluorescent antibodies (FA) in studies of infectious diseases
1955	Diagnosis of influenza using FA
1950-1970	Development of molecular biology techniques
1985	PCR
1996	Real time PCR

The application of culture-based methods led to the recognition and characterization of a large variety of virus species, notably viruses involved in respiratory tract infections. Apart from the inability of certain viruses, like those associated with acute gastrointestinal disease, to grow on cell lines, a major disadvantage of diagnostic application of cell culture techniques is the time needed to confirmation. It may take several weeks before a diagnosis is reached, if a virus is detected at all. The development of diagnostic tests using specific antibodies for antigen detection has enabled more rapid diagnoses compared to virus culture, albeit at the cost of reduced sensitivity (the proportion of true viral diseases detected with a positive test).[6,7] These latter developments in diagnostic virology greatly enhanced the ability to associate specific viruses with clinical disease. Furthermore, those techniques enabled to fight against endemic viral infections like polio and smallpox with the development of targeted vaccination, which have proven successful for eradication. The poxvirus (variola) eradication in 1977 is the first example of the successful vaccination strategies, while poliovirus is the next target for the WHO, possibly followed by measles. Their eradication is helped by the fact that these viruses have humans as the only natural host and spread among other vectors such as animals is absent. In the early 1970s several scientists believed that virtually all viral illnesses could be fought back through effective immunization strategies. The HIV-1 epidemic in the beginning of the 1980s marked a turning point in the optimistic believes

on victory of mankind over viral diseases. The occurrence of severe opportunistic infections in young homosexual men, who were at highest risk for attracting this syndrome, led to worldwide studies into the cause of Acquired Immune Deficiency Syndrome (AIDS) and the use of molecular nucleic acid methods in clinical diagnostic virology was first explored.[8-10] The discovery of the polymerase chain reaction (PCR) in the beginning of 1990s further revolutionized clinical diagnostic virology, allowing the identification of very small amounts of genetic material by exponential expansion of a viral target sequence.[11,12] This technology also enabled scientists to detect viral nucleic acids from a variety of viruses that could not be cultured such as hepatitis B virus (HBV) and hepatitis C virus (HCV). After the discovery of HIV as the causal virus for the clinical syndrome AIDS, molecular methods played a pivotal role in the design of therapeutic drugs. The unraveling of genomic organization and viral replication was enhanced by molecular techniques, paving the way for the development of antiviral compounds targeting different parts of the virus or its lifecycle. For some viruses, like HIV-1 and HCV, antiviral therapy has completely changed the fatal prognosis of infected patients.[13,14] Despite these advances, for recently discovered and researched viruses like rhinovirus or enterovirus, no effective drugs are on the market as yet.[15] When it was shown that the risk of progression to AIDS and death was directly related to the viral load (and CD4 counts) in blood, monitoring these key test results for prognosis became an essential component of antiretroviral treatment strategies.[16] From this time onwards, clinical diagnostic virology was not only the detection of a viral infection, but has become an essential diagnostic tool in patient management.

1.2 Molecular diagnostic virology

Molecular diagnostic techniques have evolved rapidly and gained a solid position in the daily routine of microbiological laboratories, especially in the field of clinical virology.[17-19] One of the earliest applications of molecular techniques in pathogen identification was the use of PCR in patients with herpes virus (HSV) encephalitis. Detection of HSV in cerebrospinal fluid by PCR was a more sensitive and less invasive method than brain biopsy and tissue culture.[20] Similar advancements were introduced for the clinical diagnosis of enteroviral meningitis.[21] The development of multiplex PCR, where several viral targets are incorporated into one single reaction, made it possible to diagnose a causal pathogen in clinical syndromes arising from any specific viral source, for example respiratory and gastrointestinal infections. Importantly, molecular tests are useful in the detection of a causative virus during the acute phase of a viral disease when the viral load is relatively high. During this first phase, conventional methods like serology and antigen detection have disadvantages compared to molecular tests: antibody response, even the IgM response, takes several days to develop and antigen tests have reduced sensitivity compared to molecular methods. Furthermore, the development and detection of an antibody response can be severely hampered in immunocompromised patients.

Driven by these developments in molecular techniques, continuously growing lists of previously unknown or uncultivable viruses are detected (Table 1.2.). In this respect, much attention has been given to the non-human origin of human (pathogenic) viruses for example by recent studies into bats as reservoirs for viruses that are capable of infecting humans. Such studies contribute to a further understanding of viral diversity and help to determine zoonotic risks.[22-24]

Table 1.2. Newly recognized viruses over the last 20 years

1980 HTLV[66]	**2004** H7N7 [67]
1983 HIV-1[44,68-70]	**2005** Human bocavirus[71]
1989 HCV[72]	**2005** Coronavirus HKU1 [73]
1990 HHV-7 [74]	**2007** Human Rhinovirus species C [75]
1994 HHV-8 [68]	**2007** Wu [76]
1995 Sin Nombre virus [77]	**2007** Ki [78]
1996 Australian Bat lyssavirus [79]	**2009** Influenza A H1N1pdm09 [80]
1998 Influenza A H5N1 [81]	**2010** Severe fever with thrombocytopenia syndrome virus [82]
1999 Nipah virus [83]	**2010** Trichodysplasia spinulosa polyomavirus [84]
2001 Human metapneumovirus[85]	**2012** MERS-CoV [62]
2002 SARS-CoV [60]	**2013** Influenza A H7N9 [86]
2004 Coronavirus NL63 [87]	

Nowadays, the ability to detect minute amounts of genetic material of viruses challenges the clinical interpretation of a positive test result because there may be a bystander effect. Apart from pathogen detection, quantitative molecular techniques have an important role in patient management. Quantification of viral load in order to assess progress and prognosis of disease has gained solid position not only in the management of HIV infections, but also for example in reactivations of cytomegalovirus (CMV) or Epstein Barr Virus (EBV) in immunosuppressed patients.[25-27] Genotyping by molecular methods guides treatment protocols.[28] In addition, antiviral susceptibility testing by identifying known resistance mutations before and during treatment has been incorporated in routine clinical care in case of HIV-1 infection and infections with CMV, HSV, HCV or HBV when standard therapy fails.

The more recent applications of molecular diagnostics have moved towards rapid tests which are easy to perform, so called point-of-care tests.[29,30] These point-of-care tests can be performed in a satellite laboratory facility, situated for example near the emergency room thereby enhancing time efficiency.[31] Results are generated within 90 minutes after receiving the sample, providing timely input for clinical decision making.

As new developments in molecular methods are rapidly moving on, many questions about the clinical value and application of such tests in routine diagnostics are still not fully answered and molecular viral diagnostics have not been optimally incorporated into routine clinical care. To optimize patient care, such issues need to be urgently addressed.

1.3 Benefits of molecular diagnostic tests

Molecular diagnostic methods are characterized by a high sensitivity and specificity with few false positives and negatives, and have the potential of providing a rapid result. Based on these characteristics, the benefits of molecular tests can be broadly categorized into three groups.

(1) The use of molecular diagnostics benefits clinical management of a patient with signs and symptoms of a viral infection. The option of a timely diagnosis of a viral infection may lead to a reduction of unnecessary additional diagnostic laboratory tests and costly diagnostic imaging (e.g. X-ray or MRI), reduction of antibiotic use and perhaps start of proper antiviral treatment, and a reduction in hospitalization and length of stay.

(2) Molecular diagnostic tests have the advantage of a timely detection of a viral pathogen for in-hospital infection control: appropriate infection control measures can be installed to prevent further transmission within the hospital.

(3) Information generated by molecular diagnostic tests may serve as input data for public health surveillance in order to monitor trends in viral diseases. Moreover, molecular tests also play an important role in identifying causative agents in emerging infections of unknown origin (e.g. SARS CoV).

1.3.1. Benefits of molecular viral diagnostics for patient management

A timely and accurate diagnosis of a viral infection can be of help in explaining illness, thereby reducing the need for additional testing as well as guide empirical treatment. The developments in molecular diagnostic methods has resulted in the detection of a variety of new (or newly recognized) viruses in respiratory and fecal material, for instance human metapneumovirus, the polyomaviruses Wu and Ki, human bocavirus, human rhinovirus C, coronavirus NL63 and HKU1 (see also Table 1.2). For some of these new viruses, the causative relation between the detection of these viruses in the laboratory and clinical illness has become acknowledged (i.e. human metapneumovirus).[32,33] For others, like Wu and Ki virus or bocavirus, the association with clinical symptoms is still under debate, because of conflicting results in clinical studies. The discrepancies may in part be due to differences in patient populations that were studied (e.g. children, immunocompromised, patients with chronic pulmonary diseases).[34-38]

So far, only few studies have focused on the impact of viral molecular diagnostic tests on the clinical management of the patients, i.e. the effect of a positive PCR on the use of antibiotics, on the length of stay in the hospital and on the number of additional investigations. Several studies among children showed a beneficial effect of the detection of a virus as the cause of respiratory disease towards discontinuation of antibiotics and reduced length of stay in the hospital. [39-41] However, a recent Cochrane review on the impact of rapid viral diagnostics for acute febrile illness in otherwise healthy children referred to the emergency department showed that rapid diagnostics did not significantly reduce antibiotic use, although there was a trend towards less antibiotic use in the rapid diagnostic group. [42] Importantly, the studies included in this review observed lower rates of chest X-rays and blood investigations in the rapid diagnostic group compared with the control group. The question remains whether these results can be extrapolated to unhealthy children with chronic underlying conditions such as asthma or cancer. In addition, it is unclear what the reasons are for continuation of antibiotic therapy despite the presence of an alternative viral diagnosis. Undoubtedly, not being able to rapidly exclude a bacterial (super)infection is probably an important factor, together with a lack of parameters to predict, with a high probability, that the disease is indeed of viral origin.
One recent study evaluated the impact of under-diagnosis of a norovirus infection in patients with gastrointestinal disease in whom fecal material was referred only for bacterial examination and the consequences of not knowing the viral cause of disease for patient management. [43] In these patients, the presence of norovirus was retrospectively determined. During the 8-month study period, the number of detected norovirus infected patients almost doubled by this approach (an additional 45 patients with norovirus infection were retrospectively detected to 50 routinely diagnosed patients). Patients who were norovirus positive in retrospect underwent more diagnostic imaging (including colonoscopy) and had a longer stay in the hospital as compared to patients who had norovirus detected at the moment they were in the hospital. Besides, missed norovirus positive patients were more often involved in a cluster of nosocomial transmission of norovirus.

It may be that the clinical impact of a negative test is more important than the value of a positive test result. The ability to rule out an infection within a relevant short time-frame with high certainty helps

in focusing on additional testing and empiric therapy towards another likely cause of disease. There is a need for rapid test results with a high positive as well as negative prognostic value that allow prediction of those patients that need hospital admission because of increased risk towards serious illness, and those that can be discharged safely. Point-of-care tests that can reliably detect viral pathogens with high sensitivity and specificity could meet these criteria, but are currently not incorporated in routine clinical care.

Overall, the majority of studies using PCR as a rapid viral respiratory diagnostic test showed no significant differences in clinical management between patients with and without a positive viral test result, despite mounting data on clinical illness related to viral detection. [44-49] Besides the need for additional studies into clinically relevant outcome data (complications, mortality), education on the meaning and interpretation of a positive test result is the key requirement for the successful implementation of molecular methods into routine clinical care.

1.3.2. Benefits of molecular viral diagnostics for in-hospital infection control

A timely detection of a viral pathogen benefits infection control. Appropriate infection control measures can be installed after diagnosis of a viral pathogen, for example through "cohorting" patients with similar infections in order to reduce further nosocomial spread. Again, the exclusion of a viral infection based on a negative test results is at least as important, especially during the respiratory season when resources are under pressure because of the high admission rates of patients with viral infections. The benefits of a timely and accurate viral diagnosis in reducing nosocomial transmission have been well described for certain viral pathogens, most notably RSV and norovirus. The combination of early diagnosis, strict cohorting of patients and the implementation of appropriate infection control measures have been shown to reduce nosocomial transmission. [50-53] In recent years, the use of molecular epidemiology in tracing nosocomial transmission of viral pathogens has gained much interest, because of the increasing possibilities in clinical laboratories to characterize pathogens by sequence analysis. [54-56] Combining classical epidemiological tools, i.e. the clustering in time, place and person, with molecular data contributes to the understanding of how pathogens are being transmitted from one patient to the other with the ultimate goal of providing evidence based data to guide the implementation of appropriate infection control measures. To this date, the use of sequence based data in the surveillance of (hospital acquired) viral infections and in infection control is not common practice and mainly confined to characterization of pathogens when the outbreak is already over in order to understand the possible transmission routes in retrospect.

1.3.3. Benefits of molecular viral diagnostics for public health

A third benefit of molecular diagnostic tests is the opportunity to provide accurate information to public health authorities for regional, national and international surveillance of viral diseases. These data are needed in order to know and understand what (sub) types of viruses are circulating and to detect changes in distribution patterns. This should ideally be combined with relevant clinical information on associated clinical illness.[57] An example of a viral diagnostic network is Noronet, an informal network of scientists working in public health institutes and/or universities sharing virological, epidemiological and molecular data on the occurrence of the norovirus dynamics (www.noronet.nl). Molecular data shared through this network enables the linkage of an increase in norovirus activity to the emergence of new norovirus variants in a timely manner. A more severe norovirus season can be thus predicted and may serve as a warning for health care institutions to install outbreak management measures.[58] Another example of a data-sharing network is the European Influenza Surveillance Network (EISN) based at the European Centre for Disease Prevention and Control (ECDC) in which epidemiological and virological data on influenza are collected and reported on a weekly basis. The development of a jointly owned

database within the Netherlands in which epidemiological, clinical and sequence data of enteroviruses are shared between the national public health institute and clinical diagnostic laboratories (VIRO-Typened) is an example of the possibilities on a national level to add sequence based information to regular surveillance systems, in this case primarily to exclude poliovirus circulation.[59]

Molecular tests play also an important role in identifying causative agents of epidemics and pandemics and in understanding their epidemiology, as illustrated by recent global outbreaks of infections caused by newly recognized viruses, like SARS CoV, MERS-CoV and avian influenza H5N1 and H7N9. [60-62]

1.3.4. Questions associated with benefits from molecular viral diagnostics

Several questions arise when examining the role of molecular diagnostic tests. The actual advantages of molecular techniques may potentially counteract the beneficial role of these tests in clinical management of a patient with an infectious disease: because of its high sensitivity possibly insignificant amounts of nucleic acids of virus can be detected. The question then arises if these are from a pathogenic active virus or from degradation products. Furthermore a positive PCR test may reflect carriage of the virus after symptomatic infection or may indicate the period just before development of disease. Also, certain viruses are commonly found in healthy, asymptomatic persons, questioning their role in a particular symptomatic disease. [63-65] Using a multiplex format provides the opportunity to examine the presence of more viruses simultaneously. However, whenever co-infections are detected, do they correlate or contribute to more serious illness? And which virus may be the causal component? Apart from the explanation for illness, the detection of a causative viral pathogen should lead to a reduction in unnecessary follow-up investigations and to a discontinuation of unnecessary treatment, especially antibiotics. But is this really the case in daily clinical practice? And if not, what are the reasons for not doing so?

Molecular detection of a viral pathogen could benefit infection control because actions to reduce nosocomial transmission can be taken in a timely matter. However, to know which infection control measures are the most appropriate in reducing nosocomial transmission, one has to have knowledge on the transmission dynamics of a pathogen: what are the main transmission routes, what is the role of visitors and health care workers in introducing pathogens from outside into the hospital and which percentage of nosocomial infections is actually due to patient-to-patient contact? Knowledge on the relative contribution of these factors to nosocomial infection rates is necessary to guide infection control policy and to focus the control efforts on key intervention points.

In order to fully exploit the potential benefits of molecular diagnostic tests answers to the above raised questions need to be found. Clinical and epidemiological data are essential to relate the outcome of the molecular test results to the clinical illness of the patient. Information on patient characteristics like the presence of specific chronic underlying illnesses is necessary to gain more insight into the diagnostic value of molecular tests. Such knowledge may consequently be incorporated into the clinical management of the patient in order to avoid further investigations or unwarranted treatment. It is important that clinicians acquire knowledge on the clinical value of viral molecular tests.

Epidemiological and clinical data are also needed to fully exploit the benefits of molecular diagnostic tests for infection control. Understanding the transmission dynamics of viral pathogens by combining mathematical modeling techniques with epidemiological methods further contribute to the implementation of appropriate infection control measures.

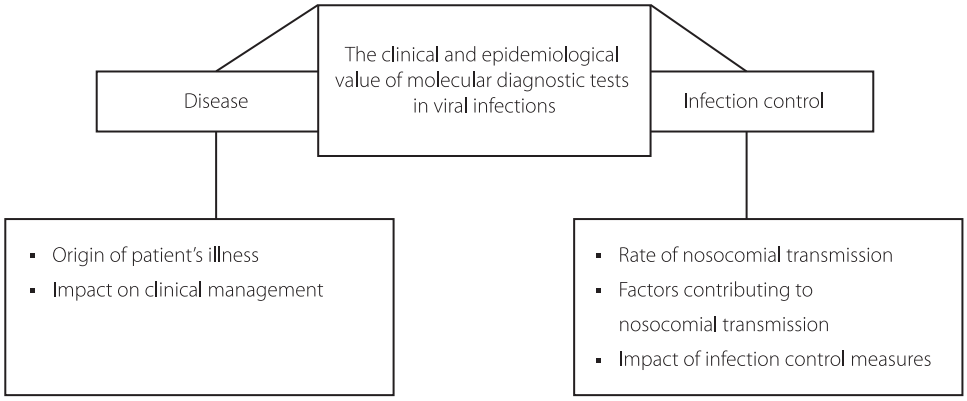
1.4. Scope of this thesis

The main focus of the scientific studies as part of this thesis is the clinical and epidemiological value of molecular diagnostic tests in viral infections. Two main issues and associated central research questions are studied (see also Figure 1.1.):

1. The value of molecular diagnostic tests in clinical management of the patient.
   - What is the association between the molecular detection of a virus, clinical syndromes and patient characteristics?
   - What is the influence of molecular detection of a virus on patient management?
2. The value of molecular diagnostic tests in infection control.
   - What is the benefit of molecular detection and characterization of a virus for in-hospital infection control practices?

The value of molecular diagnostic tests for respiratory and gastrointestinal illness caused by viruses is the object of this thesis. The usefulness of molecular techniques in clinical management and infection control is illustrated by studies into human rhinovirus, influenzavirus, enterovirus 68 and norovirus. Clinical as well as epidemiological data were systematically collected and related to the outcome of viral diagnostic tests and were used to gain more insight in the transmission of viral pathogens within the hospital.

Figure 1.1. Framework of this thesis



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Abstract

Background

Enterovirus 68 (EV68) belongs to species Human enterovirus D. It is unique among enteroviruses because it shares properties with human rhinoviruses. After the first isolation in 1962 from four children with respiratory illness, reports of (clusters of) EV68 infections have been rare. During the autumn of 2010, we noticed an upsurge of EV68 infections in the Northern part of the Netherlands in patients with severe respiratory illness.

Objectives

To give a detailed description of the clinical and virological data of patients with EV68 infection identified in 2010, and compare these with data collected in 2009.

Study design

We systematically collected clinical data from patients with an EV68 infection detected in 2010. We added four patients with an EV68 infection from 2009. Further characterization of EV68 was performed by partial sequence analysis of the VP1 genomic region.

Results

In 2010, EV68 was identified as the only cause of respiratory illness in 24 patients, of which 5 had to be admitted to the intensive care unit. Sequence analysis revealed different lineages in the majority of EV68 detected in 2010 as compared to the 2009 isolates.

Conclusions

We noticed an increase of EV68 infections and present clinical as well as sequence data, in which two distinct phylogenetic clusters could be identified.

Background

Enteroviruses (HEV, family of Picornaviridae) are amongst the most common pathogens in humans. Based on molecular characteristics, enterovirus can be divided into four species, HEV-A to HEV-D. Currently, the serotypes EV68, EV70 and EV94 are the only identified members of HEV-D. EV68 is unique amongst enteroviruses because it has characteristic properties of both enteroviruses and rhinoviruses (HRV): it is acid sensitive in which it resembles HRV; however, it has close genetic similarities with EV70. Strains of EV68 have been independently identified as HRV serotype 87. However, molecular and antigenic characterization has shown that HRV87 is genetically identical to EV68.[1-3] EV68 is almost exclusively associated with respiratory disease, in contrast to other enteroviruses which typically, but not exclusively, replicates in the intestinal tract. [4]

Objectives

In August 2010, we noticed an upsurge of patients with serious respiratory illness in both our university hospital and adjacent regional hospitals. In several of these patients, EV68 was the only viral cause detected. We systematically collected clinical data from these patients and added four patients with an identified EV68 infection from 2009. We characterized the detected EV68 to determine whether different lineages were present in both years.

Study Design

Patients and sample collection

As part of a prospective study into respiratory infections in hospitalized children from 2009 onward, we systematically collect clinical data of all hospitalized children in whom samples are taken for the detection of a panel of 15 respiratory viruses. Besides, from August till October 2010, data of patients were added who were notified to us by clinicians because of a remarkable pattern of clinical illness (respiratory disease and wheezing during a "non-respiratory season"). Samples positive for HRV were further characterized and typed by sequence analysis of the VP4/VP2 region.
Also, from July 2010 till January 2011, we systematically tested HRV positive samples from adult patients for the presence of enterovirus, as part of the validation of a re-optimized RT-PCR for the detection of HRV. All EV68 positive samples were characterized by sequence analysis of the VP1 region.

RT-PCR and sequencing

In the majority of patients nasopharyngeal swabs or nasopharyngeal aspirates were taken for the detections of respiratory viruses by a laboratory developed real-time PCR as has been described elsewhere.[5] RNA was extracted using the NucliSense EasyMag (bioMérieux, Lyon, France). RT-PCR for the detection of HRV (also detecting EV68 due to the close sequence relationship between HRV and EV68) was performed on all samples.(Rahamat-Langendoen et al., manuscript in preparation) Identification of HRV was done by amplification and sequencing of a 549 nucleotide fragment spanning the hypervariable part of the 5'NTR, the entire VP4 gene and the 5' terminus of the VP2 gene as has been described by Savolainen et al (Table 2.1).[6] For EV68, these primers generated a 660 bp nucleotide fragment (nucleotide 534-1193) based on the Fermon strain (AY426531). cDNA synthesis was carried out in a reaction volume of 50 µl, which contained 20 µl RNA, 5.5 mM $MgCl_2$, 2 mM dNTPs, 1x RT buffer, 2.5 µM random hexamers, 20 U RNAse inhibitor (RNasin) and 62.5 U MultiScript reverse transcriptase (Applied Biosystems, Foster City, California, USA).

The following cycling conditions were used for cDNA synthesis: 10 min at 25 °C, followed by 60 min at 48 °C, and 5 min at 95 °C. For amplification, 10 µl cDNA was added to a total of 50 µl PCR mix, consisting of 1x PCR buffer, 1 mM $MgCl_2$, 25 pmol of both upstream and downstream primers, 2.5 U DNA polymerase (Hotstar polymerase, Qiagen), 1mM dNTP's (Roche) and DNase/RNase free water (Sigma). PCR was subsequently performed with the following cycling conditions: 15 min at 95 °C, followed by 40 cycles of 1 min at 95°C, 1 min at 60°C and 1 min at 72°C, followed by 10 min at 72°C. For the EV68 strains, part of VP1 gene (741 base pairs, nucleotide 2323-3154) was amplified by RT-PCR using the primer pairs described in Table 2.1 and the same conditions as described for VP4/VP2, using an annealing temperature of 55°C instead of 60°C. The PCR products were sequenced on an ABI 3130 automated sequencer, using fluorescent dideoxy-chain terminators (3.1 BigDye Terminator kit; Applied Biosystems). For phylogenetic analysis a fragment of 731 bp (2399-3129bp Fermon AY426531) were aligned with Clustal W 2.0 and phylogenetic trees were constructed by the neighbor-joining method using MEGA 4.0 with the maximum likelihood model and complete deletion for missing data. The EV68 sequences derived from this work are submitted to GenBank (accession numbers JF896287-JF896312).

Table 2.1. Primers for amplification and sequencing of EV68 isolates

Primers	Sequence (5'→3')	Gene	Orientation	Position *	Reference
Rhinoseq-FW	GGG-ACC-AAC-TAC-TTT-GGG-TGT-CCG-TGT	VP4/VP2	Sense	534-560	Savolainen et al 2002[5]
Rhinoseq-RV	GCA-TCI-GGY-ARY-TTC-CAC-CAC-CAN-CC	VP4/VP2	Antisense	1168-1193	Savolainen et al 2002[5]
EV68-VP1-2325-fwn	GGR-TTC-ATA-GCA-GCA-AAA-GAT-GA	VP1	Sense	2323-2345	This study
EV68-VP1-2547-fwni	AAG-CCA-TAC-AAA-CTC-GCA-CRG-T	VP1	Sense	2546-2567	This study
EV68-VP1-2772-rvni2	AGT-TGT-GAG-TAT-GGT-RAY-TTC-AGC-A	VP1	Antisense	2796-2772	This study
EV68-VP1-3121-rvni	TAG-GYT-TCA-TGT-AAA-CCC-TRA-CRG-T	VP1	Antisense	3154-3130	This study

I=inosine; Y=T, C; R=G, A; N=A, G, C, T

*Primer positions are given according to the orientation of the primer, either sense or antisense; numbers are given according to the EV68 Fermon strain (AY426531)[3]

Results

Patients and clinical data

During 2010, we did not detect EV68 within our ongoing study until August. Between August and November 2010, we tested 272 samples from 231 patients for the presence of respiratory viruses (134 patients < 18 years, 97 patients ≥18 years). In 112 (41%) respiratory samples HRV was detected (as mono-infection or in co-detection with other viruses): 30 out of 97 adult patients tested positive for HRV, of which 12 were identified as EV68 (12.4%). In children, HRV was detected in 82 out of 134 patients; EV68 was found in 5 patients (3.7%). Samples from 21 children admitted to adjacent hospitals with respiratory infection and wheezing were sent to our laboratory for the detection of respiratory viruses; 14 patients tested positive for rhinovirus of which 7 were identified as EV68 (33.3%). All together we detected 24

patients with EV68 in a three months time. The remaining HRV positive samples were mainly typed as HRV species A and C, with a predominance of these species over HRV species B.
The EV68 positive patients ranged in age from 1 month to 72 years, with a median age of 14 years. Ten patients (41.7%) were under two years of age. Male and female were equally divided. Twenty patients (83.3%) were hospitalized because of a respiratory illness, with a median duration of illness before admission of 3.0 days (0-10 days). Three patients (12.5%) were already hospitalized for other reasons before they developed respiratory symptoms (time from admission to first day of respiratory illness between 37 and 68 days). Five patients (20.8 %) had no relevant medical history; the remaining 19 patients with EV68 had an underlying chronic illness, of which 14 related to the pulmonary tract (hyperreactivity of the lungs (6 EV68 positive patients), lung transplantation (3 EV68 positive patients), chronic need for oxygen or ventilation at home (4 EV68 positive patients)).

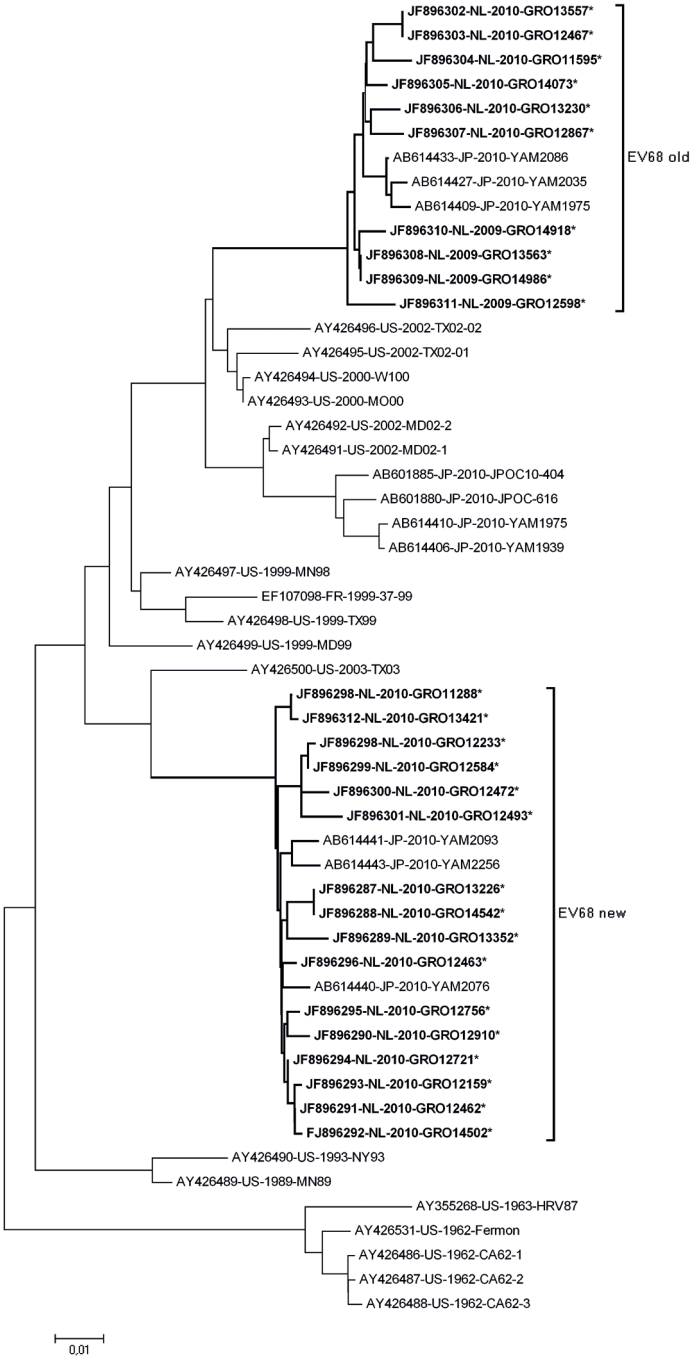
Five patients (20.8%) had to be admitted to the Intensive Care Unit (ICU) and required mechanical ventilation; one of them had no previous underlying illness. Dyspnoea and cough were predominant symptoms among all patients with EV68. Clinical diagnosis consisted mainly of exacerbation asthma/ wheezing (10 patients), pneumonia (6 patients) and upper respiratory tract infection (8 patients). In none of the patients other respiratory viruses were detected besides EV68. One patient (< 18 years) had a culture proven respiratory tract infection with *Haemophilus influenzae*, interpreted as a bacterial super infection in the presence of EV68. The median duration of hospitalization in patients with community acquired infection was five days (range 1-25 days). All patients survived the infection with EV68.
In our ongoing prospective study, we identified four patients, all children, with EV68 infection in the same period in 2009. The median age was 5.5 years; all four had an underlying chronic illness related to the pulmonary tract. None of these patients had to be admitted to the ICU. One also had bocavirus detected in the respiratory sample.

Sequence analysis and phylogeny of clinical isolates

Based on our VP4/VP2 sequence analysis, EV68 clinical isolates were divided into two distinct phylogenetic clusters, one containing all EV68 detected in 2009 and part of EV68 detected in 2010, the other containing only EV68 detected in 2010 (data not shown). Further characterization of EV68 by partial sequencing of the VP1 genome showed a 3 nucleotide deletion in EV68 in the 2010 cluster as compared to the 2009 cluster, further confirming that the 2010 cluster was genetically distinct from the cluster which included the 2009 isolates (Figure 2.1 and 2.2). The VP1 sequence identity between the two clusters was 88.6-90.7%. The phylogenetic clustering did not coincide with epidemiological clustering of patients in time or place.

Figure 2.1. Clustal W alignment of a 242- amino-acid region of VP1 of a subset of EV68 detected in this study and representatives of major lineages available in GenBank. The deletion is marked with an arrow.

Figure 2.2. Neighbor-joining phylogenetic tree based on nucleotide sequences of a 731 bp fragment of the VP1 genomic region. The EV68 detected in this study are in bold and marked with an asterix. EV68-Fermon is used as reference strain (2399-3129bp GenBank accession no. AY426531).



Discussion

We report 24 patients identified in the autumn of 2010 with serious respiratory disease in whom only EV68 could be detected as the possible cause of illness. To our knowledge, this is the first detailed description of such a large group of patients infected with EV68. After the first isolation in 1962 from four children with respiratory illness, EV68 has been isolated rarely.[3, 4, 7-12] Detailed clinical and epidemiological data are mostly limited or even lacking.

Recently, two studies reported the detection of EV68 in patients with respiratory illness: one in military recruits in the USA (7 recruits with febrile respiratory illness not further specified tested positive for EV68), and one in France, where 19 patients with lower respiratory tract infection had EV68 identified as most likely cause of illness.[12, 13] The French study also noticed the upsurge of infections during autumn as we did; however, patient characteristics like age and underlying illness were not described. No comparison with EV68 detected in previous years was made.

EV68 is reported to be most frequently associated with lower respiratory tract infections, notably bronchiolitis, pneumonia and exacerbation of asthma.[3] This pattern of clinical illness was also present in our patients; indeed, the seriousness of illness ("RSV-like") during a non-respiratory season in the absence of evidence for other causes of disease alerted the clinicians. Most of our patients had a chronic underlying illness, with the majority related to the pulmonary tract. This could explain the severity of clinical symptoms, as these patients are probably more vulnerable for severe respiratory infections. However, five patients had no relevant medical history, of which one even had to be admitted to the ICU. The fact that our institution is a tertiary referral hospital could have biased our findings towards more severely ill patients. In adjacent non-referral hospitals though the same observations were made, suggesting that referral bias probably was not of main importance.

Detection and identification of EV68 in respiratory samples are not incorporated into routine clinical use and could lead to underestimation of the prevalence of EV68. Also, in contrast to the rare detection of EV68 in clinical and environmental samples, seroprevalence studies have shown a high percentage of neutralizing antibodies against EV68, suggesting that EV68 may be underrepresented in clinical samples because of mild or subclinical symptoms.[1,14]

We systematically typed all HRVs detected in children hospitalized in our tertiary hospital from 1 September 2009 until 1 January 2011. Additionally, we tested HRV positive samples from adult patients for the presence of HEV from July 2010 till January 2011. We only detected EV68 during August till November 2010, suggesting that the observed rise in infections caused by EV68 was indeed a real upsurge. No other enteroviruses were detected.

The outcome of VP1 sequence analysis showed two distinct phylogenetic clusters, which were confirmed in the VP4/VP2 sequence analysis. This strengthened our hypothesis of a change in the epidemiological and clinical spectrum of EV68 infections. The majority of EV68 detected and identified in 2010 were phylogenetically distinct from the ones detected in 2009. These findings could suggest that different genetic lineages of circulating EV68 strains might have contributed to a rise in (serious) infections.

The upsurge of serious infections caused by EV68 in our patients coincided with a peak in EV68 detections in the Dutch sentinel surveillance system into respiratory illness among patients seen by general practitioners. Preliminary sequence data showed genetic changes in EV68 comparable to our findings (personal communication Dr. A. Meijer, National Institute for Public Health and the Environment, The

Netherlands). This provides further evidence for the hypothesis of a potential widespread distribution of a phylogenetically distinct virus, which could have accounted for more infections in the population, with serious illness in hospitalized patients. However, although the majority of EV68 identified in 2010 were phylogenetically distinct from those detected in 2009, some EV68 detected in 2010 clustered with the ones identified in 2009, which could not be explained by the available epidemiological data. Probably, the changes in the virus do not exclusively explain the observed rise in patients with serious respiratory illness. Virological and epidemiological data over a longer period are needed to give further evidence to our hypothesis.

Although antiviral therapy is not yet available for patients infected with HRV in general and EV68 in particular, the detection and identification of these viruses could help in explaining serious respiratory illness, giving guidance to medical care and preventing unnecessary treatment with antibiotics. Also, we detected three hospital acquired infections, emphasizing the need of awareness of the possibility of EV68 infection in already hospitalized patients.

In conclusion, during the autumn of 2010 we noticed an upsurge of severe respiratory illness caused by infection with EV68. These epidemiological and clinical observations coincided with distinct phylogenetic clustering of the virus, suggesting sequence variation as possible contributing factor to more and severe infections caused by EV68.

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Introduction

Enterovirus 68, a member of the genus *Enterovirus* species D, has first been isolated in 1962, in four children with respiratory disease. Until 2010, it was only sporadically reported. However, it gained much more interest in recent years because its association with outbreaks of respiratory illness with varying seriousness. Currently, the virus is regarded as an emerging pathogen. Although much about the virological, epidemiological and clinical features of Enterovirus 68 still remains to be elucidated, information is rapidly evolving. This chapter gives an overview of the current knowledge of Enterovirus 68, from virology to clinical disease patterns and prevention and control.

Virology of Enterovirus 68, classification and replication

Enterovirus 68 (EV68) belongs to the family of *Picornaviridae* ("pico" meaning small, "rna" for RNA genome), genus *Enterovirus*, which gained its name because members were known to replicate in the human gastrointestinal tract. Other well known members of the *Picornaviridae* family capable of infecting humans are the genus *Rhinovirus* (with over a hundred different serotypes), *Kobuvirus* (with Aichivirus A, B and C), *Hepatovirus* (with Hepatitis A virus as single species) and *Parechovirus*. Enteroviruses are common viruses associated with a variety of clinical syndromes, ranging from mild febrile illness to aseptic meningitis, myocarditis and neonatal sepsis.[1] The initial taxonomic classification within the genus *Enterovirus* was based on the ability of different serotypes to grow in various cell cultures, followed by further characterization using virus neutralization by type-specific reference antisera.[2] However, the advent of molecular techniques in clinical virology has led to a further refinement of the classification of the genus *Enterovirus* and its species. The current classification is now based more upon genome organization and sequence similarity combined with their biological properties. This divides the genus *Enterovirus* into four species (A-D) known to cause human disease (Table 3.1, www.picornaviridae.com).

Table 3.1. Classification of *Enteroviruses*

Enterovirus A	Coxsackievirus* A2-A8, A10, A12, A14, A16 Enterovirus 71, 76, 89-91, 114, 119
Enterovirus B	Coxsackievirus A9, Coxsackievirus B1-B6 Echovirus E1-E7, E9, E11-E21, E24-E27, E29-E33 Enterovirus 69, 73-75, 77-88, 93, 97, 98, 100, 101, 106, 107
Enterovirus C	Poliovirus 1-3 Coxsackievirus A1, A11, A13, A17, A19-A22, A24 Enterovirus 95, 96, 99, 102, 104, 105, 109, 113, 116-118
Enterovirus D	Enterovirus 68, 70, 94, 111

*) named after a small town in New York State where the virus was first isolated.[71]

Enteroviruses are small (30 nm in diameter), non-enveloped viruses with an iscosahedral symmetry. Because of their lack of a lipid envelope, enteroviruses are able to resist organic solvents and nonionic detergents. Much of the information about the biophysical characteristics of enteroviruses are based on studies with poliovirus.[2] Formaldehyde, gluteraldehyde, sodium hypochlorite and chlorine are able to inactivate enteroviruses. Most enteroviruses are already inactivated at 42°C, although some chemical agents as sulfhydral reducing agents and magnesium cations can stabilize the viruses at higher temperatures up to 50°C.[3,4] Comparable to other infectious agents, ultraviolet light also can be used to inactivate enteroviruses, particular on surfaces, because it covalently links RNA molecules. Enteroviruses

are even stable at a pH lower than 3, which allows them to pass the stomach and gain access to the mucosa of the gastrointestinal tract where they replicate. These features permit enteroviruses to survive in this hostile environment for days to weeks.

Picornaviridae with the same physical characteristics as enteroviruses have been found in many animals, however, these animal *Picornaviridae* are not able to infect humans.[2] One of the most well known animal *Picornaviridae* is Foot-and-mouth disease virus, member of the genus *Aphthovirus*, with a high economic impact.

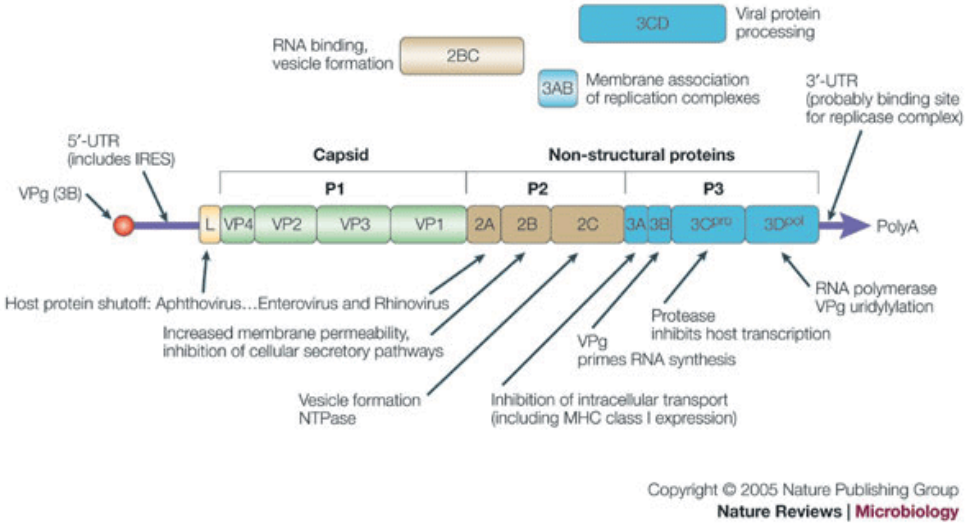
This chapter deals specifically with Enterovirus type 68 (EV68). EV68 is a member of *Enterovirus* species D, together with EV70, EV94 and EV111 and is almost exclusively related to respiratory tract illnesses. In contrast to other enteroviruses, its biological properties resemble those of Human rhinoviruses: EV68 is acid labile and grows efficiently at an optimum temperature of approximately 33°C, the temperature of the upper respiratory tract.[5,6] In fact, Human rhinovirus type 87 (HRV87) was demonstrated to be genetically and antigenically identical to EV68 and both viruses are now regarded as strains of the same *Enterovirus* serotype.

The cytopathic effect induced by EV68 was shown to be inhibited by monoclonal antibodies to the decay-accelerating factor (DAF, a membrane protein involved in the regulation of complement), which is also known to be the receptor of EV70.[5,7] Besides, phylogenetic analysis of the EV68 prototype strain (Fermon strain, GenBank ID: AY426531) showed clustering of EV68 with EV70 in four genomic regions, thus providing convincing evidence of EV68 being a member of *Enterovirus* D. The other members of *Enterovirus* D are, as far as now known, not associated with respiratory disease: EV70 is regarded as an important cause of acute hemorrhagic conjunctivitis and EV94 is associated with acute flaccid paralysis.[8,9] Recently, EV111 is identified as a new member of species D in fecal samples collected from chimpanzees in Cameroon. Phylogenetic analysis revealed that this isolate clustered with a human isolate from a patient with acute flaccid paralysis in Congo, which was at first identified as EV70.[10,11]

The length of the enteroviral single stranded RNA genome is approximately 7.500 bases with a positive polarity. It has one single open reading frame (ORF) which is preceded at the 5'end by a long noncoding region (NCR) of approximately one tenth of the total length of the genome, and followed by a short 3'NCR. This 5'NCR is a very complex structural part of the genome with a lot of structured series of branched and unbranched stem structures and pseudoknots.[12] The largest and most dominant structure within this part of the genome is the socalled IRES, or Internal Ribosomal Entry Site, which is immediately followed by the AUG startcodon for the polyprotein. Attached at the 5' end is a covalently linked protein called VPg, coded by gene segment 3B, allowing a CAP-independent translation process. Since this virus replicates in an eukaryotic cell, only one polyprotein can be synthesized which is subsequently processed through a series of primary, secondary and maturation cleavages.[12]

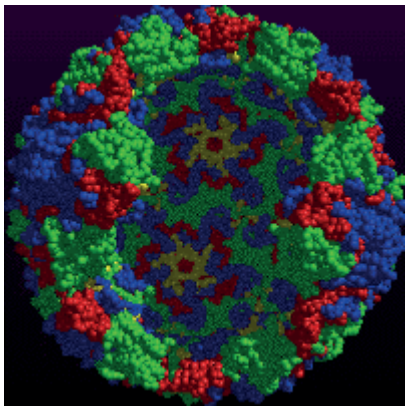
The 5'NCR is a highly conserved, but species specific region and can therefore be the chosen target for the design of primers and probes for the detection of *Enteroviruses* by molecular techniques.
The enterovirus ORF can be divided into three regions, P1-P3 (Figure 3.1). The P1 region encodes for the viral structural proteins VP1-VP4, arranged 5' to 3' as VP4, VP2, VP3 and VP1. VP1 contains type-specific epitopes and has the highest density of neutralization sites. The VP1 sequence, or part of it, is used as the target for molecular typing of enteroviruses.[13] The P2 and P3 region encode for non-structural proteins, necessary for the viral life cycle. The viral genome gives rise to a viral polyprotein which is further processed by viral encoded proteases encoded by segment 2A and 3C, some of which can act in a *cis* or in a *trans* form.[12] The RNA dependent RNA polymerase is encoded by $3D^{pol}$.

Figure 3.1. Picornavirus genome organization.[70]



The viral capsid consists of four proteins, VP1-VP4. These four proteins form a protomer with VP1, VP2 and VP3 located on the surface of the virion (Figure 3.2). VP4 is beneath them and lacks surface exposure. Five protomers form a pentamer, and one virion consists of twelve pentamers. Thus a total of 60 copies of each of the four proteins surround the viral RNA. This gives the virus the icosahedral structure. VP1, VP2 and VP3 together form a so-called canyon structure into which the cellular receptor for enteroviruses fits. When the virus binds to its receptor, destabilization of VP4 leads to uncoating of the virus.

Figure 3.2. Rhinovirus 16 with view of the interior, by X-ray crystallography, as example of the structure of picornaviruses



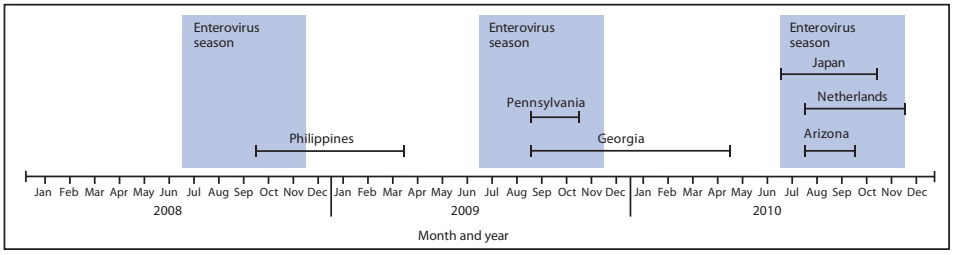
(source: http://www.virology.net/big_virology/BVRNApicorna.html)

Epidemiology

EV68 was first isolated in 1962 in respiratory samples received from four children with pneumonia and bronchiolitis, using primary monkey-kidney cell cultures.[14] Until 2008, reports of respiratory disease caused by EV68 were rare. Between 1970 and 2005, EV68 was reported only 26 times within the framework of the enterovirus surveillance system in the United States.[15] Patients with a respiratory disease are generally not systematically tested for the presence of enterovirus. Besides, most surveillance systems focus on the detection of enteroviruses in stool specimen, in which EV68 cannot be detected. Therefore, the absence of reports of EV68 does not necessarily indicates the absence of EV68 itself. A seroprevalence study in Finland among pregnant women in the first trimester of pregnancy showed that the prevalence of antibodies against EV68 was 100%, with declining antibody titers over the years.[16] This suggest that EV68 might have been around for a long time, despite the absence of reports about clinical illness caused by EV68.

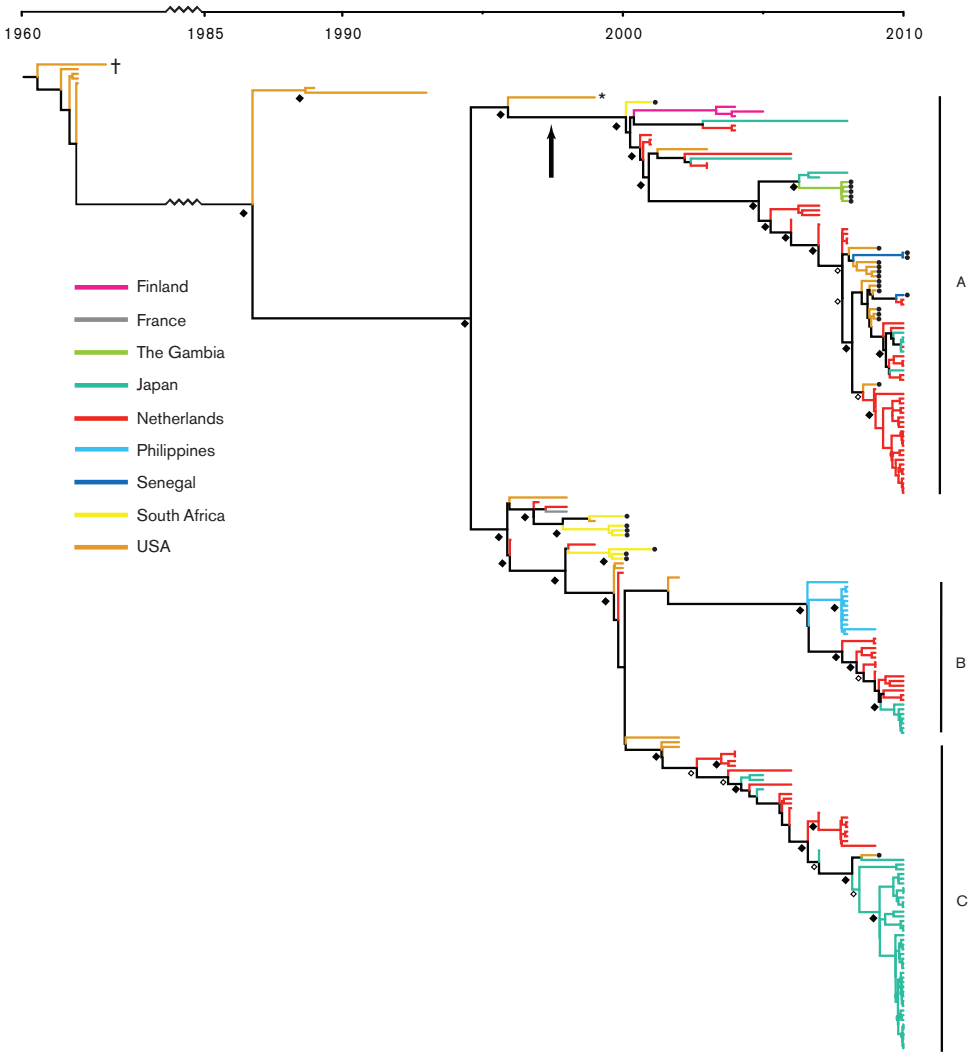
From 2009 onwards, a growing number of reports are published describing outbreaks or clusters of EV68 infection, indicating that EV68 is an emerging respiratory pathogen.[17-20] Although the observed increase in cases might be caused by enhanced surveillance for example in patients with asthma, the fact that the increase in EV68 infections is independently reported from different countries in different continents during the same time period favors a real re-emergence (Figure 3.3).

Figure 3.3. Occurrence of human enterovirus 68, by month, duration, and geographic location – Asia, Europe, and United States, 2008-2010.[20]



Several retrospective studies looking for EV68 in respiratory samples from 1996 onwards, confirmed the upsurge of EV68 infections in recent years. In several clusters, new genetic variants were described based on VP1 sequence analysis. Phylogenetic analysis of all available EV68 sequences in 2012 revealed the presence of three primary clades of EV68 (clade A, B and C, Figure 3.4).[21] Based on the evolutionary relationship of the viral sequences it was determined that between 1960 and mid-1990s, the EV68 genome underwent a rearrangement in the 5'NCR, which resulted in a 24 nucleotide deletion. In the mid-1990s, the virus underwent a large diversification, resulting in two clades (A and C). The further deviation of clade B from clade C occurred around 2007. All three clades were distributed globally.

Figure 3.4. Evolutionary history of EV-D68 based on complete VP1 sequences (adapted from Tokarz R et al. Worldwide emergence of multiple clades of enterovirus 68[21])



Clade C is characterized by an additional 11 nucleotide deletion in the 5'NCR region. Little is known about the effect of these deletions in the 5'NCR on the virulence or fitness of the virus. However, these deletions might alter the virulence of the virus by enhancing the translational efficiency and thus could be associated with the recent increase in EV68 cases. Clade A lacks the 5'NCR deletion, but instead has a 3 nucleotide (or 1 amino acid) deletion in the VP1 gene. Besides this deletion in the VP1 gene, it has been shown that in the exposed immunogenic parts of the VP1 gene, amino acid substitutions relative to the Fermon strain could be identified.[21,22] These changes could have influenced the immune response to the virus. The observation in the Finnish study among pregnant women that the mean titers of neutralizing antibodies against EV68 (Fermon strain) declined over the years is in line with the hypothesis that the amino acid substitutions in VP1 could have had an immune modulatory effect.[16,22]

Thus, the antigenic drift in the VP1 gene might explain the recent increase in incidence of EV68 infections. In temperate regions, EV68 infections display a clear seasonality, with most cases reported through autumn and into the winter period.[19,23,24] This is in contrast to other enteroviruses, which typically circulate during the summer and early in autumn. Until now, little information is available on seasonality in tropical regions.

Transmission of EV68 from one person to the other most likely occurs via the respiratory route. Similar to other viruses causing respiratory tract infections, hand contact with respiratory secretions and autoinoculation to the mouth, nose or eyes are probably the most important routes. Also, airborne droplets may contribute to transmission of respiratory viruses in general and EV68 in particular.[25] In general, children are thought to play a prominent role in the introduction and transmission of respiratory viruses within the household.[26,27] Crowding and poor hygiene may facilitate further spread, not only in households, but also in institutions and hospitals. Nosocomial transmission has been well documented for respiratory viruses and for enteroviruses.[28-32] However, EV68 specific information on the transmission dynamics in the community and in healthcare associated institutions is lacking.

The high seroprevalence of EV68 within the community and the relatively few reports of clinical illness suggests that infection with EV68 occurs very frequently, in majority associated with mild or asymptomatic disease. Which host factors contribute to the occurrence of more serious, symptomatic respiratory disease are largely unknown. Until now, most studies suggest that clinical illness caused by EV68 is most commonly found in children.[33-35] However, this could have been biased because most studies were performed in pediatric patients. In studies where patients regardless of their age were included, 20-50% of EV68 infections were detected in adults.[15,19,22] The age distribution of infections might thus have been blurred by the study designs and EV68 could probably be recognized as a pathogen for all age groups.

The close relationship of EV68 with human rhinovirus and their cross-reactivity in molecular diagnostic tests (see also section on diagnosis), together with the fact that diagnostics for enteroviruses are mainly performed on fecal samples instead of respiratory specimen, further contributes to the gaps in knowledge on EV68 as a true respiratory pathogen.[36] This diagnostic bias is illustrated by the fact that several outbreaks of EV68 are reported from various parts of the world from 2010 onwards: it is highly unlikely that EV68 limits its circulation to these regions and is not present elsewhere. These findings provide additional arguments that the reported burden of EV68 infections is also influenced by the diagnostic capacities of the clinical laboratories.

Clinical Features

Until recently, reports of respiratory illness caused by EV68 have been rare. As a consequence, very limited information about the association of EV68 with clinical symptoms and disease severity was available. During the last four years however, outbreaks and clusters of EV68 in different countries provided more data on EV68 as a respiratory pathogen. The clinical presentation of EV68 infections in these reports ranged from mild illness to complicated respiratory disease, for which hospitalization was necessary (Table 3.2). In a few cases, death as a consequence of EV68 infection was recorded.[18,37] All cases presented with respiratory disease, except in one, where EV68 was detected in the cerebrospinal fluid and was implicated in a fatal infection of the central nervous system.[37]

Table 3.2. Clinical and demographic features of patients with EV68 as reported in literature.

Period	Study population	EV68 (N)	Age	Sex	Symptoms	Diagnosis	Comorbidities	Hospitalization	ICU, death	Ref
Sept – Nov 2009	Pediatric patients	10	6 mo-10 yrs	M 60%		50% bronchitis 50% asthma	80%, n.s	100%	NK	[23]
Aug – Sept 2010	Pediatric patients	7	6 – 18 yrs	M 39%	Cough, difficulty breathing	56% pneumonia	39%, reactive airway disease	100%	N=1 ICU	[24]
2005-2010	Pediatric patients	55	5 mo-15 yrs	M:F= 1.5:1		75% URTI 15% bronchitis/ bronchiolitis	NK	NK	NK	[33]
2006-2011	Pediatric patients	25	40% 5-12 yrs	M:F= 1:1.5	Fever, cough, dyspnoea (hospitalized patients)	32% pneumonia 64% ILI	56% of hospitalized patients (40% asthma	36%	N=1 ICU	[35]
2009-2010	All ages	17	7 wks-45 yrs	M:F= 10:7		44% asthma, 38% LRTI, 19% URTI	82% (53% chronic respiratory illness)	94%	N=8 ICU	[72]
2008-2009	All ages	12	1 mo-57 yrs	M:F= 1:2		50% URTI 50% LRTI	17%, transplantation	100%	NK	[38]
1994-2010	All ages	71	26% 50-59 yrs	NK	Dyspnoea, cough	23% bronchitis, 56% ARI/ILI	NK	NK	NK	[22]
2009-2010	Adults	6	>20yr	NK	Fever, cough	NK	33% n.s.	50%	-	[20]
2009	Pediatric patients	28	54% 0-4 yrs	NK	NK	NK	NK	NK	N=15 ICU	[20]
2010	Pediatric patients	26	4 ± 2.7 yrs	M:F= 19:7		54% moderate asthma attacks, 42% severe	65% asthma	100%	NK	[17]
2010	All ages	15	3 mo-5 yrs	M:F= 2:1	Fever, wheezing	47% bronchitis, 20% pneumonia, 13% pharyngitis	NK	NK	NK	[34]
2010	All ages	24	1 mo-72 yrs	M:F= 1:1	Dyspnoea, cough	42% asthma, 25% pneumonia, 33% URTI	80% (74% chronic respiratory illness)	96%	N=5 ICU	[19]

Period	Study population	EV68 (N)	Age	Sex	Symptoms	Diagnosis	Comorbidities	Hospitalization	ICU, death	Ref
NK	Case report	1	5 yrs	M	Flaccid paralysis	pneumonia, meningoencephalitis	None	Yes	death	[37]
2008-2009	Pediatric patients	21	1 mo-9 yrs	M:F= 13:8	Cough, difficulty breathing, wheezing	100% pneumonia	NK	100%	N=2 death	[18]
1962-'63; 1989-2003	All ages	12	3 mo-43 yrs	M:F= 7:5		42% pneumonia, 42% bronchiolitis	NK	NK	NK	[6,14]
1970-2005	All ages	26	25% > 20 yrs	M 59%	NK	NK	NK	NK	NK	[15]
2005-2007	All ages	6	8 mo-71 yrs	NK	NK	NK	NK	NK	NK	[73]
2004-2005	Adults	3	NK	NK	NK	ARI	NK	NK	NK	[74]
2008	All ages	19	NK	NK	NK	68% LRTI (77% bronchiolitis/ bronchitis, 38% pneumonia), 37% asthma	NK	89%	NK	[75]

NK= not known; n.s.= not specified
mo=months, yrs=years
M=male, F=female
ICU = intensive care unit
URTI= upper respiratory tract infection
LRTI= lower respiratory tract infection
ARI= acute respiratory illness; ILI = influenza like illness

Based on observational studies, clinical presentation is dominated by signs and symptoms of acute respiratory illness like cough, fever and shortness of breath. A study of Meijer at al. in 2012 looked retrospectively in specimens from patients with respiratory symptoms, collected as part of the surveillance of respiratory illness among general practitioners and compared patients with EV68 with patients who were EV68 negative. EV68 positive patients had significantly more shortness of breath than EV68 negatives. In addition, EV68 positive patients had significantly more bronchitis. However, within this community based study, EV68 infection did not more often lead to complications compared with patients diagnosed with an acute respiratory illness because of other causes. EV68 was also sporadically found in asymptomatic patients.[22]

Reports from hospitalized patients, mainly pediatric patients, showed similar clinical patterns, with clinical diagnosis ranging from upper respiratory tract disease to bronchitis and pneumonia. However, only few studies mention the outcome of chest radiographs, and the diagnosis is mostly recorded based on clinical signs and symptoms. Whether the presence of a chronic underlying illness makes a patient more vulnerable to symptomatic EV68 infection is unclear. The majority of studies that recorded the presence of comorbidities focused on hospitalized patients (Table 3.2). Data suggest that the presence of a chronic underlying illness, more specifically chronic respiratory disease like asthma, might be associated with the presence of EV68 infection, however studies that systematically address this issue are lacking.

The pattern of illness associated with EV68 resembles that of rhinoviruses, although rhinovirus detection is much more frequently reported.[18,23,38] Few studies directly compared clinical symptoms and patient characteristics of rhinovirus positive patients with patients with EV68. In a study in pediatric patients hospitalized for acute wheezing and bronchitis, enterovirus (of which 70% was EV68) was more frequently associated with respiratory distress and a need for oxygen therapy at the time of admission compared to patients with rhinovirus.[23] Others detected comparable disease patterns in patients with EV68 and rhinovirus, particularly in patients with lower respiratory tract infections.[38,39]

Also, the association of rhinovirus with reactive airway disease like asthma, has become well established in recent years. Clinical data, although largely based on observational studies in selected study populations, suggest that this also could be true for EV68.
In summary, the hypothesis is that the spectrum of clinical disease caused by EV68 ranges from asymptomatic carriage to severe respiratory illness requiring hospitalization, with rare fatal cases. The association with reactive airway disease remains to be determined.

Pathogenesis and Immunity

The pathogenesis of EV68 in humans is largely unknown. However, as EV68 has been almost exclusively detected in respiratory material, it is likely that replication of EV68 takes place in the respiratory tract. Given the similarity between EV68 and rhinoviruses, it might be assumed that, comparable to rhinovirus, EV68 replicates in the epithelial cells of the nasopharynx and the nasal passages.[40] Besides, the optimum temperature for growth of EV68 lies at 33°C, which is the temperature of the upper respiratory tract. Whether EV68 is capable of replicating in the lower respiratory tract is unknown. For rhinovirus, there is increasing evidence that infection from rhinovirus can occur throughout the airway and is not only confined to the upper respiratory tract.[41,42]

Experimentally infected persons with rhinovirus start shedding the virus after 8 till 18 hours, coinciding with the occurrence of respiratory symptoms.[43] Virus shedding reaches a peak in 2 to 3 days after

infection and then rapidly declines. After three weeks, rhinovirus is no longer detectable in respiratory samples, however in immune-compromised patients, viral shedding can be prolonged and infection may be persistent.[44] Whether EV68 has similar replication patterns as rhinovirus remains to be elucidated.

Rhinovirus induced wheezing in infancy and early childhood is associated with the development of asthma in children.[45] Also, exacerbations of asthma, chronic bronchitis and cystic fibrosis can be precipitated by rhinovirus infections. Enhanced responsiveness of the lower airways has been described in rhinovirus infected persons.[46,47] The relationship between EV68 and reactive airway disease remains to be determined. However, clinical data suggest that EV68 is indeed associated with the occurrence of bronchitis or bronchiolitis (Table 3.2). Also, EV68 has been shown to increase the severity of asthma attacks in children with pre-existing reactive airway disease.[17] These data suggest that EV68, similar to rhinovirus, might be associated with enhanced responsiveness of the airways. Further studies are needed to explore this hypothesis.

In patients with a symptomatic rhinovirus infection, inflammatory response occurs rapidly after infection. In asymptomatic individuals, there is less viral shedding and also a decreased inflammatory response, however no differences are present in time to clearance of the virus between symptomatic and asymptomatic patients.[40] The adaptive immune response with cell mediated and humoral components follows upon the initial inflammatory response. T-cells are recruited to the lung and may contribute to clearance of the virus through the production of cytokines.[48] The termination of viral shedding and protection for subsequent infection coincides with the occurrence of neutralizing antibodies in serum as well as in nasal secretions several days after infection.[49] Thus, the humoral immune response seems to play an important role in the final clearance of rhinovirus infection and not in the initial recovery of illness.

Diagnosis

Before the advent of molecular diagnostic techniques, identification of enteroviruses relied on culture and subsequent virus antibody neutralization tests using specific antibodies for further (sero)typing. Besides being time consuming and labor intensive, identification of enteroviruses may fail because of the antigenic variability or lack of available specific clonal antibodies.[50] In the last decade, the use of polymerase chain reaction (PCR) for the detection of enteroviruses has become increasingly common in diagnostic laboratories.

PCRs usually targets the highly conserved 5'-NCR.[51-53] The detection of enteroviruses is complicated by the fact that enteroviruses and rhinoviruses have parts of their genome in common that are highly similar in nucleotide sequence. Thus, enterovirus could be misidentified as rhinovirus in routine molecular diagnostic tests. The detection of EV68 is further complicated by the fact that respiratory samples are in general not routinely tested for the presence of enteroviruses, whereas other patient material like feces or cerebrospinal fluid/liquor is much more likely to be tested for the presence of enteroviruses. However, EV68 is exclusively related to the respiratory tract and thus will not be found by regular and specific enterovirus testing. Recently, a survey was performed among ten clinical laboratories in the Netherlands to assess the capability of detecting EV68 during an EV68 epidemic in September and October 2010.[36] This study demonstrated that these ten laboratories were able to detect EV68 with their enterovirus specific real-time PCR, however, typing of enteroviruses was usually performed on stool specimens in the context of poliovirus eradication and not on respiratory samples, in which EV68 is found. The absence of reports of EV68 detection is thus most likely biased because of the specimens used for detection and typing of enteroviruses.

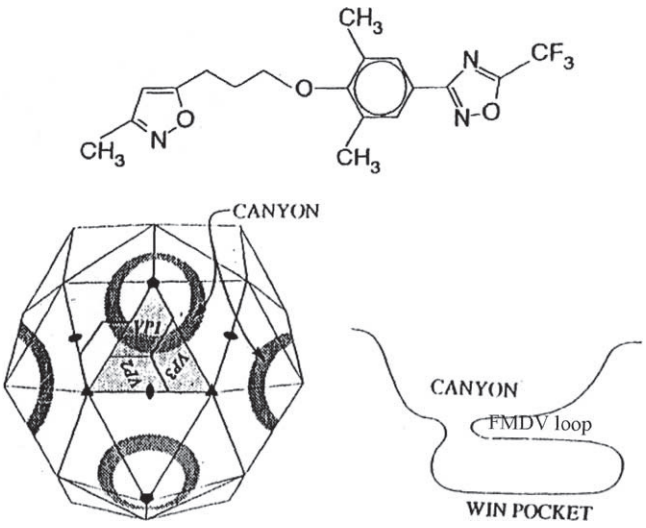
The 5'NCR used for detection of enteroviruses, is not discriminatory enough for enterovirus typing. Sequence based information of a part of the VP1 gene containing important neutralization sites has shown to have a good correlation with antigenic typing using the standard neutralization test.[13] Also, sequencing of the VP4 and/or VP2 gene has been used as method for typing enterovirus. However, these regions have also shown to be less discriminatory as VP1, particularly for the enterovirus species B and C.[54,55] Currently, the combination of PCR for detecting enterovirus and partial VP1 sequencing for further typing greatly reduces the time needed for identification and characterization of enteroviruses.

Besides molecular techniques to diagnose enteroviral infections, serologic diagnosis can be made by determining the antibody titers in acute and convalescent serum specimen. The detection of IgM antibodies using enzyme-linked immunosorbent assays (ELISA) is regarded as evidence for a recent enterovirus infection. However, these tests measure broadly reactive antibodies and are not strictly serotype specific; thus, they are less useful in determining the infecting serotype.[56] Besides, a definite serological diagnosis of enteroviral infection can be made only after the collection of two (acute and convalescent) serum samples, with at least 7-10 days in between. The use of molecular diagnostic techniques has therefore shortened the time to diagnosis considerably.

Treatment

Theoretically, every step in the lifecycle of enterovirus is a potential target in antiviral therapy (virus attachment, viral uncoating and capsid function, viral replication, viral protein synthesis). However, to date no therapy has been approved for the treatment of enteroviral or rhinoviral infection. The most well characterized targets are the viral capsid proteins and the viral proteases 3C and 2A.

Figure 3.5. Structure of Pleconaril 3-[3,5-dimethyl-4[[3-(3-methyl-5-isoxazolyl)propyl]oly]phenyl]-5(trifluoromethyl)-1,2,4-oxadiazole. Pleconaril binds to the hydrophobic sites in pockets in the base of canyons on the surface of picornaviruses.[57]

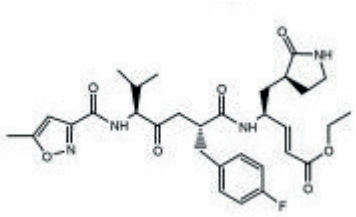


Pleconaril is a capsid function inhibitor (Figure 3.5).[57,58] Capsid inhibiting compounds block viral uncoating and attachment to the host cell by binding to the hydrophobic pocket formed between VP1 and VP3. The filling of the pocket makes the virus more stable, thus preventing the necessary uncoating at entering the cell. Also, changes in the conformation of the canyon cleft may affect the attachment of the virus to the host cell receptor. Pleconaril has demonstrated activity against entero- and rhinoviruses and has a high oral bioavailability. It was made available in 1996 for compassionate use in patients with life-threatening enterovirus infections, including meningo-encephalitis, neonatal sepsis and myocarditis. Although small series of patients treated with pleconaril supported the clinical impression that it was effective in the majority of patients, other studies were not able to show significant improvement in patients treated with pleconaril.[59,60] Because of difficulties in conducting trials in an epidemic illness and the inability to show a consistent treatment effect, further pursuit for licensing pleconaril for viral meningitis indication was stopped in 2000.

In patients with common cold, pleconaril was able to reduce the length of symptoms by one day with some minor gastrointestinal side effects of nausea and diarrhea.[61] However, in a 6-week prophylactic study for the prevention of picornaviral respiratory tract infection, it was found that pleconaril induced cytochrome P450 3A, an enzyme of major importance for drug metabolism, thereby raising the possibility of drug interactions, in particular with oral contraceptives. Within the prophylactic study, a side effect of the use of pleconaril was increased menstrual irregularities, raising concerns that pleconaril might reduce the effectiveness of oral contraceptives. The Food and Drug Administration (FDA) concluded that the risks of using pleconaril did not outweigh the potential benefits and no license was given for pleconaril as treatment for the common cold.[62] After this, the production of pleconaril was stopped and currently the drug is not available for clinical use.

Protease inhibitors are another attractive target for antiviral therapy, because proteases are important in the viral replication cycle and because they have a unique and specific viral structure that is not present in host-cell proteases.[58,63] However, until now only one drug (rupintrivir, Pfizer) entered clinical trials (Figure 3.6). Rupintrivir showed in vitro strong antiviral capacity against a broad spectrum of rhinoviruses and enteroviruses.[64] However, in phase II clinical trials, rupintrivir had only limited effect on virus reduction and disease severity in patients with experimental rhinovirus infection, thus halting further development for clinical use.[61]
Currently, treatment of respiratory infection caused by enteroviruses or rhinoviruses in general and EV68 in particular consists mainly of supportive care and symptomatic treatment (oral or inhaled steroids, nasal decongestion, supplemental oxygen, antipyretics).

Figure 3.6. Structure of AG7088/Rupintrivir.[64]

Compound	Structure
AG7088	

Prevention and Control

Currently, no vaccine is available to prevent infections with enteroviruses, with the exception of poliovirus. Prevention of an infection thus relies on adequate hygienic measures, particularly in healthcare related institutions where vulnerable people are admitted. EV68 is a respiratory pathogen closely related to human rhinovirus; transmission from one infected person to the other most likely occurs via the respiratory route. Direct contact with respiratory secretions and subsequent autoinoculation of nose, eyes and mouth contribute probably the most to transmission. Enteroviruses and rhinoviruses are known to survive for days to weeks upon environmental surfaces. Also, transmission via droplets is likely to play a role.

There is no information available about the occurrence of nosocomial infections by EV68. One study by Rahamat-Langendoen et al. mentioned three out of 23 hospitalized patients, who acquired EV68 infection while being admitted in the hospital, based on the time period between first day of illness and date of admission to the hospital. This equals a percentage of 13%.[19] However, for rhinovirus, outbreaks in long term care facilities have been recorded.[65,66] Also, an outbreak of rhinovirus species C was reported on a neonatal intensive care unit.[67] In a hospital based prospective cohort study among hospitalized children, 22% of rhinovirus positive disease episodes was acquired in the hospital.[68] Besides, several clusters of infection could be identified in which nosocomial transmission of rhinovirus on the same hospital ward probably occurred. It is likely that nosocomial EV68 infections occur in comparable percentages.

In general, the detection of a respiratory pathogen leads to the implementation of infection control measures to prevent transmission in healthcare institutions. Interventions are focused on the most likely route of transmission. For respiratory pathogens, like and including EV68, precautions should be aimed at either direct contact with secretions or transmission via droplets. However, infectiousness is probably highest in the early stages of disease before the results of diagnostic tests are available. As a consequence, the implementation of infection control measures should probably be driven by the presence of respiratory symptoms themselves. To what extent asymptomatic patients or patients with only mild disease contribute to transmission to others is largely unknown, however the general assumption is that symptomatic patients are more likely than asymptomatic ones to spread infections.[69] Within families, children are thought to be the ones that introduce and spread a respiratory virus, probably because of their level of personal hygiene and their need for parental attention.[69] The contribution of visiting children or siblings to intra-hospital transmission of respiratory viruses remains to be determined.

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Abstract

Recent developments in molecular diagnostic tools have led to the easy and rapid detection of a large number of rhinovirus (HRV) strains. However, the lack of clinical and epidemiological data hampers the interpretation of these diagnostic findings. From October 2009 to January 2011, we conducted a prospective study in hospitalized children in whom samples were taken for the detection of respiratory viruses. Clinical, epidemiological and microbiological data from 644 patients with 904 disease episodes were collected. When HRV tested positive, strains were further characterized by sequencing the VP4/VP2 region of the HRV genome. HRV was the single respiratory virus detected in 254 disease episodes (28%). Overall, 99 different serotypes were detected (47% HRV-A, 12% HRV-B, 39% HRV-C). Patients with HRV had more pulmonary underlying illness compared to patients with no virus (p=0.01), or patients with another respiratory virus besides HRV (p=0.007). Furthermore, cough, shortness of breath and a need for oxygen was significantly more present in patients with HRV infection. Particularly, patients with HRV-B required extra oxygen. No respiratory symptom, except for oxygen need, was predictive of the presence of HRV. In 22% of HRV positive disease episodes, HRV infection was hospital acquired. Phylogenetic analysis revealed several clusters of HRV; in more than 25% of these clusters epidemiological information was suggestive of transmission within specific wards.

In conclusion, the detection of HRV may help in explaining respiratory illness, particular in patients with pulmonary comorbidities. Identifying HRV provides opportunities for timely implementation of infection control measures to prevent intra hospital transmission.

Introduction

In recent years, human rhinoviruses (HRVs) have been increasingly recognized as a potential cause of acute otitis media, bronchiolitis, asthma and pneumonia in children [1-3]. The development of sensitive and rapid molecular techniques markedly improved the detection rate of HRV and revealed the high genetic diversity. Over 150 serotypes of HRV have been described, classified into three main species: HRV-A, HRV-B and HRV-C [4]. HRV-C is the most recently discovered species and is thought to contribute more to recurrent wheezing and exacerbations of asthma compared to HRV-A and HRV-B [1,5]. Also, recently published data suggest that HRV-A and HRV-C cause more severe illness than HRV-B, with greatest virulence during the winter [6]. Currently, molecular diagnostics are increasingly integrated into routine practice allowing detection and quantification of HRV, thereby also raising questions about the value in direct patient care and infection control. We performed a hospital based prospective study to determine the clinical, epidemiological and viral characteristics associated with HRV infection in children.

Material and methods

Patients and sample collection

In October 2009 a prospective study into respiratory infections in hospitalized children was initiated at the University Medical Center Groningen (UMCG), The Netherlands. The UMCG is a tertiary referral hospital with more than 1300 beds in the northern region of the country. Demographical, clinical and microbiological data were systematically collected from all children under 18 years, of whom respiratory samples have been taken for the detection of 15 respiratory viruses (influenza A/B, respiratory syncytial virus A/B, coronavirus 229E/NL63/OC43, para-influenzavirus type 1-4, metapneumovirus, adenovirus, bocavirus and rhinovirus). Samples positive for HRV were further characterized by sequence analysis of the VP4/VP2 region. Information on the presence of bacterial respiratory pathogens was included when bacteriological culture was performed at the same day, one day before or one day after the virological sample was taken.
Clinical data were collected using a standardized case record form with items regarding the presence of an underlying chronic illness (pulmonary, cardiovascular, gastrointestinal, neurological) and/or immune suppression (transplantation, malignancy, immune suppressive therapy), clinical symptoms (fever, cough, shortness of breath, otitis media, wheezing, vomit, diarrhea, oxygen need, mechanical ventilation), treatment (antibiotics, antivirals, inhalation therapy), clinical diagnosis (upper respiratory tract infection: pharyngitis, coryza, otitis media; lower respiratory tract infection: pneumonia, bronchiolitis, exacerbation of asthma, croup) and outcome. Pulmonary underlying illness included asthma, congenital pulmonary illness or anatomic malformations, cystic fibrosis and broncho-pulmonary disease. Cardiovascular disease was divided into inborn or acquired heart disease. Patients with partial resection of bowel, failure to thrive, and those who were waiting for a liver transplantation, were categorized as having gastrointestinal disease as underlying illness. Neurological disease was not further specified.

Epidemiological data were gathered to determine whether the respiratory infection was community or hospital acquired, including measures that were taken in the hospital to prevent further transmission of respiratory viruses. Hospital acquired HRV infection was defined as a first day of illness two or more days after admission to the hospital. Infection control measures consisted of a combination of droplet and contact precautions (gown, gloves and mask for health care workers during patient care, patient in one person room) and were installed when HRV was detected.
The study was approved by the local Medical Ethical Committee of the UMCG. Informed consent was obtained from parent or guardian.

Real-time PCR and sequencing

The majority of samples (91%) arrived at the laboratory within one day after collection. Samples were divided in aliquots and stored at 4° Celsius if PCR testing was performed the same or the next day. Longer storage was carried out at -80° Celsius. In general, PCR testing was performed on a daily basis, providing results within 48 hours after arrival of the sample at the laboratory.
All respiratory samples, either nasopharyngeal swabs, aspirates or sputum, were tested by a laboratory developed (LDT) real-time PCR as has been described elsewhere [7]. For rhinovirus detection, a real-time LDT-PCR was introduced using the SuperScript® III Platinum® One-Step qRT-PCR Kit. All reactions were performed with Phocine Distemper Virusas an internal control in a total volume of 25 µl containing 12.5 µl of 2x reactionmix, 0,5 µl SuperScript® III RT/ Platinum® Taq mix, 0,5 µl of 1:10 Rox reference dye, 300 nM of each forward primers, 600 nM reverse primer and 100 nM of each probe, and 5 µl of genomic RNA template. Primers and probes used are listed in Table 4.1. The inclusion of this large set of primers and probes is performed in a specific reaction to avoid bias and favoring against other respiratory viruses. Our rhinovirus PCR has been optimized during the last years based on the available genetic information, in particular regarding species C. By re-optimizing the assay with new primers and probes, together with the use of Invitrogen SuperScript enzymes, we detected retrospectively more HRV positive samples at a lower Ct value. To ensure that these HRVs were not enteroviruses, all samples were sequenced. We only detected HRV, eventually 99 serotypes, as well as enterovirus 68, which is genetically identical to HRV.

The cycle threshold (Ct) value (the number of amplification cycles needed for a PCR to become positive) was used as relative estimate for the amount of HRV present in the samples.

Characterization of HRV was done by amplification and sequencing of a 549 nucleotide fragment spanning the hypervariable part of the 5'NTR, the entire VP4 gene and the 5' terminus of the VP2 gene as described before [8,9]. For phylogenetic analysis a fragment of 395-401 bp were aligned with Clustal W 2.0 and phylogenetic trees were constructed by the neighbor-joining method using MEGA 4.0 with the maximum likelihood model and complete deletion for missing data. The HRV sequences derived from this work are submitted to GenBank (accession numbers JQ042307-JQ042680).

Table 4.1. Primers and probes for HRV detection used in this study.

Primers/probes	Sequence (5'→ 3')	Position*	Tm**
Rhino-fwdB-mod-TM	GGTGTGAAGACTCGCATGTGC	408-427	60.1
Rhino-fwdA-mod-TM	GGTGTGAAGAGCCCCGTGTG	408-426	62.4
Rhino-fwd-C-TM	GGTGTGAAGAGCCNANTGYGCTC	408-429	58.9
Rhino-fwd-D-TM	GGTGYGAAGANCCNANTGTGC	408-427	58.9
Rhino-fwd-E-TM	GGTGTGAAGACYTGCATGTGC	408-427	57.9
Rhino-fwd-F-TM	GGTGTGAAGAGYCNCGTGTGCT	408-428	58.1
Rhino-rev3	CCAAAGTAGTYGGTYCCRTCCC	523-544	58.4
Rhino-Probe-TM	TCCTCCGGCCCCTGAATGCG	438-457	70.2
Rhino-Probe3	TCCTCCGGCCCCTGAATGTGG	438-458	69.1

*Primer positions are given according to the orientation of the primer; numbers are given according to a HRV-A16 reference strain (GenBank no. L24917)

**) Tm = melting temperature

Data analysis

For data analysis, the first respiratory sample of each episode of infection was included. An episode of infection, or disease episode, was defined as a time period starting with a first day of illness and ending with discharge from the hospital or resolvement of clinical symptoms, during which a respiratory sample was taken for viral diagnostics. Statistical analyses were performed using SPSS software version 20.0. For normally distributed continuous variables, parametric tests were used (Student t-test). The distribution of categorical variables in comparison groups were analyzed using the Chi-square test. The association of HRV with chronic underlying illness and symptoms was determined using binary and multinomial logistic regression (with no infection, HRV mono-infection, HRV mixed infection, other respiratory infection as the dependent variable). A two sided p-value < 0.05 was considered statistically significant.

Results

Patients characteristics

From October 2009 till January 2011, 644 unique patients were included with 904 disease episodes, 366 (57%) were male and 278 (43%) female. Characteristics of the patients with no virus detected (n=242, with 341 disease episodes), those with only HRV (n=162, 254 disease episodes) and those with another respiratory virus than HRV (n=157, 195 disease episodes) are summarized in Table 4.2. In 83 patients (111 disease episodes), mixed infection of HRV with one or more other respiratory viruses was found, most frequently adenovirus (37 episodes), RSV-A/B (35 episodes), bocavirus (20 episodes) and influenza A virus (11 episodes). These patients did not differ significantly from the ones with HRV mono-infection (Table 4.2).

Table 4.2. Characteristics of patients with no respiratory virus detected (PCR negative), with HRV mono- or mixed infection and with another respiratory virus than HRV.

	PCR negative	HRV mono-infection	HRV mixed infection	Other respiratory virus
	N=242 (%)	N=162 (%)	N=83 (%)	N=157 (%)
Sexe	M 135 (55.8)	M 92 (56.8)	M 51 (61.4)	M 88 (56.1)
	F 107 (44.2)	F 70 (43.2)	F 32 (38.6)	F 69 (43.9)
Age (months)	Mean 31.6	Mean 32.7	Mean 24.2	Mean 36.6
	Median 1.0*[a]	Median 11.0	Median 13.0	Median 13.0
Underlying illness	142 (58.7)	108 (66.7)*[b]	45 (54.2)	69 (43.9)
Pulmonary	52 (21.5)	54 (33.3)*[c]	19 (22.9)	31 (19.7)
Cardiovascular	50 (20.7)	37 (22.8)	12 (14.4)	15 (9.6) *[d]
Gastro-intestinal	33 (13.6)	14 (8.6)	8 (9.6)	8 (5.1)
Neurology	21 (8.7)	15 (9.3)	7 (8.4)	13 (8.2)
Immune suppression†	36 (14.9)	36 (22.2)	11 (13.3)	14 (8.9)

*[a] p<0.0001

*[b]HRV mono-infection versus other virus: p<0.001

*[c]HRV mono-infection versus PCR negative p=0.01, versus other virus p=0.007

*[d]other virus versus PCR negative p=0.003, other virus versus HRV mono-infection p=0.001

†malignancy, transplantation, use of immune-suppressive therapy

Patients with HRV infection had more pulmonary underlying illness, mainly caused by asthma (39%) or congenital pulmonary illness/anatomic malformations (44%), compared to PCR negative patients (p=0.01) or patients with another respiratory virus than HRV (p=0.007).

Although the majority of patients were under the age of 5 years (80%), the prevalence of underlying illness was significantly higher in patients older than 5 years: in patients with HRV mono-infection, 86% had comorbidities compared to 64% in those under 5 (p=0.03), for PCR negatives this was 83% versus 56% (p=0.001) and in patients with another respiratory virus 70% had a chronic underlying disease compared to 38% in those under 5 (p=0.002). These differences in prevalence of chronic underlying disease was mainly caused by a significantly higher prevalence of chronic respiratory disease in the older age category (data not shown).

Clinical symptoms

To determine the relationship between HRV and clinical symptoms, PCR negative disease episodes were compared with those with a HRV mono-infection. Because patients with HRV had significantly more chronic respiratory disease, we stratified disease episodes for this underlying condition. HRV positive patients who also had a chronic respiratory disease, experienced significantly more cough, shortness of breath and a need for oxygen compared to PCR negatives. However, in HRV positive patients without a pulmonary underlying illness, only cough and fever were significantly more present (Table 4.3).

Table 4.3. Clinical symptoms in disease episodes of patients with HRV mono-infection compared to PCR negative patients

Symptoms	Pulmonary underlying illness		No pulmonary underlying illness	
	PCR negative	HRV mono-infection	PCR negative	HRV mono-infection
	N=83 episodes (%)	N=98 episodes (%)	N=238 episodes (%)	N= 139 episodes (%)
fever	29 (34.9)	48 (49.0)	111 (46.6)	82 (59.0)§[a]
cough	13 (15.7)	38 (38.8)*[a]	28 (11.8)	35 (25.2) §[b]
dyspnoea	18 (21.7)	54 (55.1)*[b]	53 (22.3)	34 (24.5)
diarrhoea	4 (4.8)	12 (12.2)	27 (11.3)	24 (17.3)
vomit	6 (7.2)	12 (12.2)	27 (11.3)	19 (13.7)
oxygen need	31 (37.3)	62 (63.3)*[c]	57 (23.9)	29 (20.9)
mechanical ventilation	34 (41.0)	30 (30.6)	91 (38.2)	40 (28.8)

*[a]p=0.001 (HRV positives versus PCR negatives in patients with pulmonary underlying illness)

*[b]p < 0.001 (HRV positives versus PCR negatives in patients with pulmonary underlying illness)

*[c]p=0.001 (HRV positives versus PCR negatives in patients with pulmonary underlying illness)

§[a]p=0.02 (HRV positives versus PCR negatives in patients with no pulmonary underlying illness)

§[b]p=0.001(HRV positives versus PCR negatives in patients with no pulmonary underlying illness)

In multivariate analysis, the presence of a chronic respiratory disease remained positively associated with HRV mono-infection (OR 2.06, CI 1.43-2.97). This association was unique for HRV mono-infections and was not found for HRV mixed infections or other respiratory viral infections. Besides, in patients with a chronic respiratory illness a need for oxygen was exclusively related to HRV detection (odds ratio 3 for HRV mono-infection, and 3.3 for mixed infection). Cough and shortness of breath were equally strongly associated with the detection of other respiratory viruses as with HRV (Table 4.4).

Table 4.4. Association of clinical symptoms with the detection of HRV and other respiratory viruses in patients with chronic respiratory disease.

Symptoms	**HRV mono-infection**	**HRV co-detection**	**Other respiratory virus**
	(OR[a], 95% CI[b])	**(OR, 95% CI)**	**(OR, 95% CI)**
Fever	1.37 (0.69 – 2.71)	2.62 (1.02 – 6.70)	4.19 (1.68 – 10.42)
Cough	2.52 (1.17 - 5.4)	2.7 (1.03 – 7.16)	3.77 (1.53 – 9.28)
Dyspnoea	4.12 (2.10 – 8.09)	6.74 (2.68 – 16.94)	6.13 (2.60 – 14.44)
Diarrhea	2.04 (0.55 -7.57)	1.43 (0.28 – 7.25)	1.36 (0.31 – 5.94)
Vomit	1.33 (0.43 – 4.14)	1.60 (0.39 – 6.58)	2.50 (0.72 – 8.72)
Need for oxygen	3.02 (1.63 – 5.58)	3.29 (1.38 – 7.84)	1.39 (0.66 – 2.92)
Mechanical ventilation	0.63 (0.33 – 1.19)	0.19 (0.06 – 0.61)	0.46 (0.20 – 1.05)

[a]) OR, odds ratio

[b]) CI, 95% confidence interval

Patients with a HRV infection were more often diagnosed with an exacerbation of asthma compared to PCR negatives and to patients with another respiratory virus, although the difference with the latter group was not statistically significant (Table 4.5). In contrast, pneumonia and bronchiolitis was more often associated with the detection of other respiratory viruses than HRV.

Table 4.5. Clinical diagnosis in patients with HRV compared to patients who were PCR negative or who had another respiratory virus (% of episodes).

Clinical diagnosis	PCR negative	HRV infection	Other respiratory virus
Upper respiratory tract infection	13.5%	52.8%[a]	49.7%[a]
Otitis media	0.9%	1.2%	3.6%
Exacerbation asthma	0.6%	5.1%[b]	1.0%
Bronchiolitis	0.9%	2.0%	15.4%[c]
Pneumonia	10.9%	12.2%	18.5%[d]

[a]) p<0.001 compared to PCR negative
[b]) p=0.008 compared to PCR negative, p=0.12 compared to other respiratory virus
[c]) p<0.001 compared to PCR negative and HRV positive
[d]) p=0.02 compared to PCR negative, p=0.04 compared to HRV positive

The median length of stay in the hospital was significantly shorter for patients who had any respiratory virus compared to patients with no virus (7 days versus 25 days, p<0.001). Also, patients with HRV had to stay longer in the hospital compared to patients with another respiratory virus (median length of stay 11 days versus 6 days, p<0.001).

The median Ct value of HRV in HRV mono-infection was 24, significantly lower than the median Ct value of HRV in HRV mixed infections (28, p< 0.001). However, we found no relationship between the presence of symptoms and the relative amount of virus in the samples.

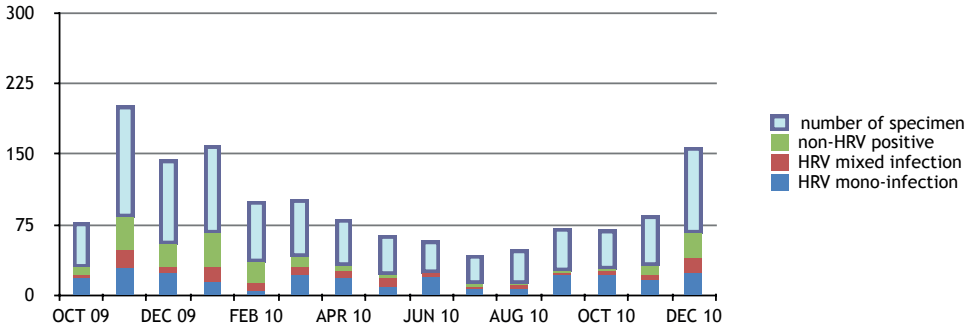
Bacteriology and antibiotic use

Antibiotic use did not differ between the three patient groups (in 52% of PCR negatives, 50% of those with HRV and 53% with another respiratory virus, antibiotics were given). Also, the duration of antibiotic therapy was comparable (median duration of 7 days). In more than 60% of the disease episodes, no bacteriological culture was recorded around the time the virological sample was taken. In case bacteriological cultures were taken, significantly more *H. influenzae* was found in patients with HRV (17.4%, p=0.04) and in patients with another respiratory virus (19.4%, p=0.02) compared to the patients who had no respiratory virus detected (3.6%). *S. pneumoniae* was more often found in patients with another respiratory virus (19.4%) than in patients with HRV (2.2%, p=0.02). The use of antibiotics was strongly associated with the clinical diagnosis pneumonia and the submission of samples for bacteriological culture: antibiotics were prescribed in 94% of the patients with pneumonia and in 75% of patients who had samples collected for bacteriological culture (both p<0.001). However, the outcome of the bacteriological culture (positive or negative) did not influence the use of antibiotics (p=0.5), nor did the presence of a chronic underlying illness (p=0.6).

HRV characterization

HRV was found throughout the study period, with the least frequent detection during February, July and August 2010 and peaks in spring (2010) and fall (2009 and 2010) (Figure 4.1). Still, during the winter period (December and January) HRV was detected in 40%-60% of the samples positive for respiratory viruses.

Figure 4.1. Monthly distribution of the number of respiratory specimen taken in our patient population, the number of HRV detections (either as mono-infection or mixed infection) and the number of detected respiratory viruses other than HRV (non-HRV positive).

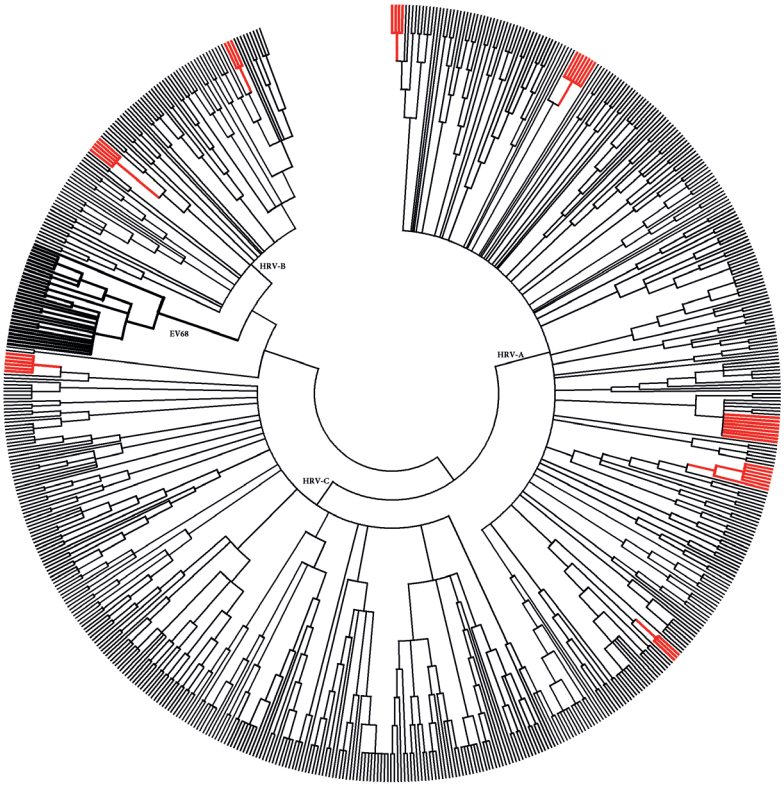


Sequence analysis of HRV was possible in 303 out of 365 HRV positive disease episodes; 221 episodes with HRV mono-infection and 82 with HRV mixed infection. The reason for unsuccessful sequence analysis was the low amount of HRV present in the samples.

Overall, 99 different serotypes were detected, most often species HRV-A (47%) and HRV-C (39%) (Figure 4.2). In 12% of the episodes, HRVs were detected belonging to species HRV-B. We did not observe a trend in circulating serotypes. Almost 50 patients were identified with serial infections with different serotypes or with different species. Three patients had detection of serotypes belonging to HRV-A, HRV-B and HRV-C consecutively.

In patients with HRV-B, more pulmonary underlying illness was present (54%) compared to those with HRV-A and HRV-C (32% and 39%), although the difference was only statistically significant for the comparison of HRV-B with HRV-A (p=0.045). No significant differences were seen in clinical symptoms related to the species of HRV, except for the need for oxygen which was more present in patients with HRV-B (p=0.012). Patients with HRV-C had a shorter duration of stay in the hospital than patients with HRV-A (median 6 days versus 10 days for HRV-A, p=0.009) and HRV-B (median 16 days, p=0.008).

Figure 4.2. Phylogenetic tree of detected HRV serotypes in this study. In red the clusters of infection based on epidemiological and virological information. In bold the clusters of EV68 as described before [8].



Nosocomial infection

Based on epidemiological data (first day of illness in relation to admission to the hospital), hospital acquired infection was present in 81 out of 365 HRV positive episodes (22%), with a first day of illness ranging from 3 to 242 days after admission. In 69 of 81 episodes (85.2%) HRV was detected as a mono-infection, compared to 179 out of 273 (66%) community acquired HRV infections ($p<0.001$). Even when the definition of hospital acquired infection was adjusted to a first day of illness five days or more after admission, still 20% of the HRV positive disease episodes were nosocomial related. No differences were observed in the age distribution in community and hospital acquired HRV infections. Although HRV-B was the least frequently detected HRV species, equal proportions of HRV-A and HRV-B infection were hospital acquired (33.3% resp. 38.5%), while HRV-C was in only 18.0% of the HRV positive episodes acquired during hospitalization. Due to the small number of HRV-B infections, these differences did not reach statistical significance. Using phylogenetic analysis, 41 clusters of three or more isolates with identical sequences could be identified during this study period. Nine of the 41 clusters were formed by three or more consecutively taken isolates from one patient per cluster.

Two clusters of EV68 were identified, corresponding with an upsurge of EV68 related respiratory tract infections detected in the autumn of 2010, as described previously [8]. We collected more detailed information about the wards patients were admitted to, first day of illness, date of admission and sample date in the remaining 30 clusters, to see whether the clustering of patients based on the virological analysis was supported by epidemiological data. In 8 clusters epidemiological information was suggestive of transmission of HRV on the same hospital ward (Figure 4.2).

Discussion

In this large hospital-based prospective cohort study into viral respiratory infections in children, HRV was the most commonly detected respiratory virus, in majority as HRV mono-infections. Also, high nosocomial infection rates were observed. HRV infection was associated with substantial respiratory illness, especially among children with pre-existing pulmonary disease.

Although the association of HRV with asthma is well established in recent years, the occurrence of HRV-infections in our study was associated with chronic respiratory illness in general [5,10,11]. Whether these children are more vulnerable for the acquisition of HRV, or whether they have more symptomatic illness related to HRV, remains to be elucidated. In patients with a chronic respiratory condition, only the need for oxygen was predictive for HRV detection; cough and shortness of breath was not only associated with the detection of HRV, but also with the detection of other respiratory viruses. Thus, respiratory symptoms alone are not sufficient to predict the presence of a HRV infection.

Several studies tried to relate viral load with the presence or absence of symptoms. Although a positive relationship between a higher viral load and more (serious) respiratory symptoms has been reported, others were not able to reproduce these findings [11-15]. Adequate quantification of HRV in respiratory samples is currently limited by several factors, as addressed in recent reports [16,17]. One major limitation is the lack of a reference standard for the quantification of all HRV types. Secondly, HRV viral load is affected by the sample type and the method of collection and detection [18]. Thus, although in our study no relationship between the relative amount of virus (expressed as Ct value) and the presence of clinical symptoms could be demonstrated, the interpretation of Ct value in relation to severity of illness remains to be determined and is probably, with the current limitations in quantification of HRV, hardly feasible.

HRV was detected year round, with the least frequent detection in February, July and August 2010. Although peaks in detection were seen in fall and spring, as is known from literature, HRV was still detected in around half of the samples positive for respiratory viruses in the remaining months, including the winter period [6,17,19]. However, as most studies suggest variation of HRV detection by season and year, our study period may well have been too short to fully understand the seasonality of HRV in our hospital.

Antibiotic use and duration of antibiotic therapy did not differ between the patients with and without a respiratory viral infection, suggesting that the decision to prescribe antibiotics was not influenced by the outcome of viral diagnostics, as has been observed elsewhere [20,21]. This might be explained by the concern of getting a bacterial co-infection. Indeed, antibiotic use was associated with submission of samples for bacteriological culture, assuming a suspicion of bacterial infection in those patients. However, although bacteria known to cause (secondary) respiratory infections in infants were more frequently cultured in patients with a respiratory viral infection compared to patients with no viral infection (*H. influenzae, S. pneumoniae*), the outcome of the bacteriological cultures did not influence

the use of antibiotics. Because the collection of samples for bacteriological culture was not standardized, but dependent on the judgment of the clinician and were taken in only a minority of patients, these results might have been biased and more studies are needed to confirm our findings.

The high prevalence of co-morbidities and the associated vulnerability of our patient population could have lowered the threshold for prescribing antibiotics. However, we did not find a positive association between antibiotic therapy and the presence of a chronic underlying illness. We did find a strong correlation between antibiotic therapy and pneumonia as clinical diagnosis. This may suggest that clinical signs and symptoms were more important in deciding on antibiotic therapy than patient characteristics and microbiological results.

Sequence analysis revealed a high diversity of different HRV serotypes, as reported before [22-24]. In our population, species HRV-A and HRV-C were detected in equal amounts, and dominated over HRV-B. We observed no species specific pattern of clinical illness, except that HRV-B was associated with a higher need for oxygen. Besides, patients with HRV-A and HRV-B had to stay in the hospital significantly longer than patients with HRV-C. Until now, studies that addressed the relationship between species and severity of illness reported HRV-B as being the least virulent compared to HRV-A and HRV-C [6,17]. However, recently Miller et al. also showed that patients with HRV-B were more likely to require supplemental oxygen and had a longer duration of stay in the hospital [19]. Also, patients with HRV-B tended to have higher disease severity scores. The reason for the different observations regarding HRV-B associated severity of illness is not clear. Our study and the study of Miller et al. included hospitalized children, whereas in a recent study of Lee et al, who showed a clear distinction in severity of illness between HRV-A/C and HRV-B, the majority of patients were not admitted to the hospital [6]. Besides, we observed a tendency for more chronic respiratory illness in patients with a HRV-B infection, possibly explaining the higher need for oxygen. These findings may suggest that HRV-B is associated with either asymptomatic carriage or mild respiratory disease, and that host factors like pre-existing pulmonary disease contribute to more serious, symptomatic respiratory illness caused by HRV-B. One of the most remarkable findings of our study is the frequency of hospital acquired HRV infection: in more than 20% of the HRV positive disease episodes, patients had a first day of illness more than 2 days after admission to the hospital. Phylogenetic analysis revealed several clusters of HRV with identical sequences; in more than 25% of these clusters epidemiological information was suggestive of transmission of the virus on specific wards. Although HRV-B is the least frequently found species in our study, almost 40% of HRV-B infections are acquired in the hospital. This suggests that the hospital environment is for some reason more favorable for transmission of HRV-B. Also, if HRV-B is indeed associated with asymptomatic carriage or mild respiratory disease, this would facilitate transmission within the hospital, only causing clear respiratory symptoms in patients with pre-existing pulmonary illness.

Outbreaks of rhinovirus infections have been described in long-term care facilities, but very little is known about the frequency of hospital acquired HRV infection [25,26]. Transmission mainly occurs via droplets or contact and although measures to prevent transmission were taken the moment HRV was detected in respiratory samples, infectiousness is probably highest in the early stages of disease, before the outcome of diagnostic tests is available and thus before infection control measures were taken. These findings advocates the implementation of infection control measures driven by the presence of respiratory symptoms instead of the outcome of a laboratory test. However, further investigations are needed to determine the contribution of health care workers, other patients and visitors in nosocomial transmission of HRV.

The present study has some limitations. Being a tertiary referral hospital could have biased our findings towards more serious illness, as is reflected by the characteristics of the included patients: more than 50% of the children had a chronic underlying illness, and even in the ones that had no respiratory virus detected, signs and symptoms of serious illness were present (e.g. mechanical ventilation). Furthermore, information on bacterial co-infection was based on cultures taken just around the time the viral sample was taken. Information on the presence of bacterial pathogens outside this timeframe was not included. Also, we had no information about the presence of so called atypical bacterial pathogens (*eg Mycoplasma* or *Chlamydophila*). However, given the high prevalence of HRV infection in our study population and the expected low prevalence of atypical pathogens, we assume that this limitation does not influence the main conclusions of our study [20,21].

In conclusion, HRVs are capable of causing serious respiratory disease in children hospitalized in a tertiary referral hospital, particularly in patients with pulmonary co-morbidities. Nosocomial infection occurs frequently. Most notably, HRV species B may have features that facilitate transmission within the hospital. Although antiviral therapy is not yet available for patients infected with HRV, the detection and identification of these viruses could help in explaining respiratory illness. Also, identifying HRV provides opportunities for implementing timely and accurately infection control measures to prevent further transmission. Our study illustrates the value of sequence analysis not only in gaining insight into the genetic diversity of rhinoviruses, but also as a tool in defining transmission routes within the hospital and in the detection of sources of nosocomial infection.

Acknowledgements

We greatly acknowledge the contribution to this study of Monique van Linschoten, who collected all clinical data. We thank Renze Borger en Reina van der Heide for their work in optimizing the HRV PCR and performing phylogenetic analysis.

Part of this work is presented at annual congresses of the European Society of Clinical Virology and the European Society for Pediatric Infectious Diseases.

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Abstract

Background

Comparative data on severity and treatment of seasonal, pandemic and post-pandemic influenza virus infections are scarce.

Objectives

To systematically analyze characteristics of hospitalized patients with influenza in the post-pandemic period compared to seasonal and pandemic influenza.

Study Design

Clinical and virological data of patients hospitalized in a tertiary referral hospital with post-pandemic influenza (2010- 2011) were compared with those during seasonal influenza epidemics (2007- 2009) and the influenza A(H1N1)pdm09 pandemic (2009- 2010).

Results

82 patients were admitted during the post-pandemic period, compared to 85 during the pandemic and 60 during seasonal influenza epidemics. No differences were observed in the occurrence of complicated illness and the need for intensive care. However, radiographic pneumonia was significantly more often diagnosed in patients with influenza A(H1N1)pdm09 compared to patients with seasonal influenza A (25% versus 71% in pandemic, p=0.004, and 55% in post-pandemic, p=0.047). Oseltamivir was more frequently prescribed in post-pandemic and pandemic patients compared to previous influenza seasons (48.9% resp. 76.5% versus 6.5%, p< 0.0001). During the post-pandemic period, patients with influenza B were significantly less often treated with oseltamivir compared to patients with influenza A (27.0% versus 48.9%, p=0.043), although the course of illness in patients with influenza B was comparable with influenza A. No upsurge of oseltamivir resistance was observed.

Conclusions

In our center, severity of illness was comparable for all influenza seasons, although more radiographic pneumonia was diagnosed in patients with influenza A(H1N1)pdm09. Despite the increased use of oseltamivir, no increase in oseltamivir resistance was detected.

Background

In March 2009 a novel influenza A H1N1 virus 'influenza A(H1N1)pdm09' emerged and rapidly spread around the world causing the first pandemic of this century. Although severe illness and death have been reported, it was mostly regarded as a relatively mild disease, with a course of illness comparable to seasonal influenza.[1-4] Historically, influenza in the immediate post-pandemic period has been known to be able to cause severe morbidity and mortality.[5] Indeed, some countries reported a more severe influenza season in 2010-2011 compared to the pandemic waves.[6] However, data based on systematic analysis of the impact of influenza in the post-pandemic period are scarce.

Objectives

In order to compare the characteristics of influenza in hospitalized patients in the post-pandemic period to those with seasonal and pandemic influenza, we systematically collected clinical information of patients hospitalized in a tertiary referral hospital with influenza from 2007-2011.

Study design

Study population

A retrospective observational study was conducted in all patients with influenza infection hospitalized in the University Medical Center Groningen (UMCG) from August 2007 till July 2011. Patients with acute respiratory illness were tested; only patients with real-time PCR (RT-PCR) confirmed influenza were included in the study. The UMCG is a large tertiary referral hospital with over 1300 beds in the northern region of the Netherlands. Patients were divided into three cohorts: patients with seasonal influenza (August 2007-May 2009), patients with pandemic influenza (June 2009-July 2010) and those with influenza during the first post-pandemic season (August 2010-July 2011). We compared clinical, epidemiological and virological data of patients with confirmed influenza A separately from those with influenza B infection.

Clinical data and definitions

Clinical information was gathered using a standardized questionnaire, including clinical symptoms, underlying chronic illness, medical complications, and treatment. Influenza vaccination history was initially included in the questionnaire, however because this was poorly documented in patients records, it had to be excluded for analysis. Complications were listed as pulmonary (pneumonia, respiratory insufficiency, pneumothorax, other pulmonary symptoms) or extra-pulmonary (renal failure, sepsis, neurological symptoms). Radiographic findings were classified into infiltrates, pleural effusion, interstitial abnormalities and pneumothorax. Bacterial co-infection was defined by isolation of a significant pathogen in respiratory or blood samples of a patient within 3 days before or after the detection of influenza. Time from onset of symptoms to admission and to sample date was calculated for each patient.

Laboratory methods

Nasopharyngeal swabs or nasopharyngeal aspirates were taken for the detection of respiratory viruses by a laboratory developed RT-PCR as has been described before.[7,8] In 11% of patients, sputum was used. Identification of influenza types and subtypes during 2007-2011 was performed as described elsewhere.[9-12] In short, RNA was isolated using the NucliSense EasyMag (bioMérieux, Lyon, France), or Magna Pure LC Total Nucleic Acid Isolation kit with external lysis protocol (Roche Diagnostics, Indianapolis,

Table 5.1. Primers used for screening and identification of influenza viruses used in this study (with adjustments from April 2011 onwards)

Target	Aim	Primer	Oligotide sequence 5′ => 3′ and labels
InfA	Screening	INFA-asense-TM	CAAAGCGTCTACGCTGCAGTCC
	04/18/11	infA-probe-2	FAM-TTTGTGTTCACGCTCACCGTGCC-BHQ1
InfB		INFA-sense-TM	AAGACCAATCCTGTCACCTCTGA
	Screening	INFB-sense-TM	GAGACACAATTGCCTACCTGCTT
		INFB-asense-TM	TTCTTTCCCACCGAACCAAC
		INFB-probe-TM	TET-AGAAGATGGAGAAGGCAAAGCAGAACTAGC-EDQ
	04/18/11	infb-NSfwdB	GRACAACATGACCACAACACAAAT
		infb-NSrevB	CACTCCARAATTCCTGCTTCAAA
		infb-NSprobeB	DRAGONFLY-CGGGAGCAACCAATGCCACCATAAA-BHQ2
H1N1	Identification	H1-RF1162	GAATAGCCCCACTACAATTGGGTAA
		H1-RF1163	GTAATTCGCATTCTGGGTTTCCT
		H1-RF1164	FAM-AAGATCCATCCGGCAACGCTGCA-BHQ1
	04/18/11	H1-fwd1	CCAAAGTATGTCAGGAGTGCAAAAT
		H1-fwd2	CCAAAGTATGTCAGGAGTACAAAAT
		H1-rev	CCTTCAATGAAACCGGCAAT
		H1-probe	FAM-TGGTTACAGGACTAAGGAACATCCCATCCA-BHQ1
H3N2	Identification	H3-RF1358	GATGTGTACAGAGATGAAGCATTAAACA
		H3-RF1359	TAGGATCCAATCTTTGTATCCTGACTT
		H3-RF1360	YY-AGCTCAACACCTTTGATCTGGAACCGG-BHQ1
		H3prb2	YY-AGCTCAACGCCTTTGATCTGGAACCGG-BHQ1
	04/18/11	H3-fwd	GGGAAAAGCTCAATAATGAGATCAG
		H3-rev	TTGGGAATGCTTCCATTTGG
		H3-probe	DRAGONFLY-TGCACCCATTGGCAAATGCAATTC-BHQ2
		H3-probe2	DRAGONFLY-TGCACCTATTGGCAAATGCAATTC-BHQ2
H1N1pdm09	Identification	MexFlu-H1-fwd	GGAAAGAAATGCTGGATCTGGTA
		MexFlu-H1-rev	ATGGGAGGCTGGTGTTTATAGC
		MexFlu-H1-pr	DRAGONFLY-TGCAATACAACTTGTCAGACACCCAAGGG-BHQ2
	04/18/11	SwN1-fwd	ACATGTGTGTGCAGGGATAACTG
		SwN1-rev	TCCGAAAATCCCACTGCATAT
		SwN1-probe	FAM-ATCGACCGTGGGTGTCTTTCAACCA-BHQ1
H1N1 H274Y	Resistance	FluAN1-H275F	CCGCCTCGTACAAAATCTTCAAGA
		FluAN1-H275R	CAGTGTCTGGGTAACAGGAACATT
		FluAN1-H275	VIC-CTCATAATGAAAATTG-MGBNFQ
		FluAN1-H275Y	FAM-CCTCATAATAAAAATTG-MGBNFQ
H1N1pdm09 H274Y	Resistance	panN1-H275a	TGCACACACATGTGATTTCACTAG
	04/18/11	panN1-275Hp	FAM-TTATCACTATGAGGAATG-BHQ1
		panN1-275Yp	DRAGONFLY-TTATTACTATGAGGAATG-BHQ2
		panN1-H275s	CAGTCGAAATGAATGCCCCTAA

USA). Both influenza A and influenza B were detected by generic RT-PCR assay targeting the matrix gene. Multiple primers were used for screening and subtyping (Table 5.1). All influenza A(H1N1) positive samples were subsequently screened for the presence of the H275Y mutation in the neuraminidase gene (N1 nomenclature), conferring full resistance to oseltamivir.[12]

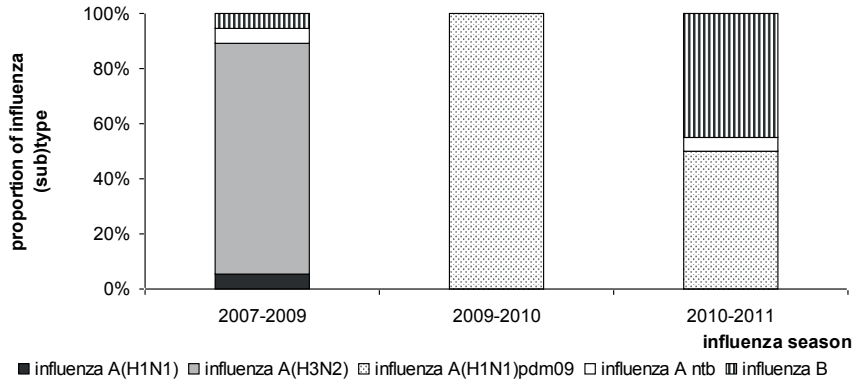
Statistical analysis

Statistical analyses were performed using SPSS version 18.0 (Chicago, USA). Interseasonal comparisons were tested using Mann Whitney U test and Fisher's exact test for continuous variables. Dichotomous variables were tested using $\chi 2$ test. The effect of age on outcome was analyzed using multinomial logistic regression, for which each cohort was divided in two groups: patients under the age of 15 and those above. P values ≤ 0.05 were considered as statistically significant.

Results

A total of 227 patients with confirmed influenza were included in the study: 60 patients during the pre-pandemic seasons (47 with influenza A, of which 33 with influenza A(H3N2)), 85 during the pandemic (all influenza A(H1N1)pdm09), and 82 in the post-pandemic period (45 with influenza A(H1N1)pdm09, 37 with influenza B) (Figure 5.1). For analysis, the pre-pandemic seasons (2007-2009) were compiled as no significant differences were observed with regard to patients' characteristics, course of illness and clinical outcomes (data not shown).

Figure 5.1. Influenza (sub)type distribution during influenza seasons 2007-2011



Influenza A ntb=influenza A, subtype not determined

Influenza A

Characteristics of patients with influenza A are summarized in Table 5.2. No significant differences were observed in gender ratio, although there was a tendency towards more male patients admitted during the pandemic and post-pandemic period (57.6% and 55.6% versus 42.7% during seasonal influenza, p=0.11 resp. p=0.30) Age distribution among patients with seasonal, pandemic and post-pandemic influenza A differed significantly (Table 5.3). Fourty percent of patients hospitalized with seasonal influenza A were aged under 4 years, significantly more than during the post-pandemic period (20%, p=0.03).

During the pandemic, a shift towards young adolescents was observed: almost 25% of patients with pandemic influenza A were aged 5-14 years, significantly more than during seasonal and post-pandemic influenza. In the post-pandemic period more patients aged 15-64 years were admitted (71%) compared to patients with seasonal influenza (38%, p=0.002) and with pandemic influenza (45%, p=0.004).

Table 5.2. Overview of influenza A cohorts as described in this study.

	seasonal influenza A	**pandemic influenza A**	**post-pandemic influenza A**
	(n = 47)	**(n = 85)**	**(n = 45)**
Influenza A subtypes (absolute numbers)			
• H1N1	7	0	0
• H3N2	33	0	0
• H1N1nv	0	85	41
• Non typable influenza A	7	0	4
Male gender (%)	42.6	57.6	55.6
Median age in years (IQR)	21.3 (1.1-59.5)	14.2 (4.6-48.5)	49.8* (14.9-59.5)
Underlying medical condition (%)	87.2**	70.6	80.0
Timespan, median in days (IQR)			
• From symptom onset to admission	2.5 (1.0-4.0)	2.0 (1.0-4.0)	1.0¶ (0.0-3.0)
• From symptom onset to sample date	4.0† (2.0-6.0)	2.0 (1.0-4.0)	2.0 (1.0-4.0)
• Hospital length of stay	8.0 (5.0-14.0)	6.0 (2.0-14.5)	6.0 (3.0-20.5)
Received oseltamivir therapy (%)	6.5	76.5§	48.9
Resistance to oseltamivir (absolute numbers)	4	4	1
Admitted to ICU (%)	25.5	29.4	22.2
Experienced complications (%)	38.3	49.4‡	40.0
Death (absolute numbers)	3	7	3

IQR=interquartile ranges

*) p=0.004 post-pandemic influenza A versus pandemic influenza A

**) p=0.03 seasonal influenza A versus pandemic influenza A

¶) p=0.03 post-pandemic influenza A versus seasonal influenza A

†) p=0.02 seasonal influenza A versus pandemic influenza A, p=0.008 seasonal influenza A versus post-pandemic influenza A

§) p<0.0001 pandemic influenza A and post-pandemic influenza A versus seasonal influenza A, p=0.001 pandemic influenza A versus post-pandemic influenza A

‡) p=0.2 pandemic influenza A versus seasonal influenza A

Table 5.3. Age distribution in patients with seasonal, pandemic and post-pandemic influenza A

	seasonal influenza A (n = 22)	pandemic influenza A (n = 44)	post-pandemic influenza A (n = 11)
0-4 years	40.4%	21.7%	20.0%*
5-14 years	6.4%	24.7%**	4.4%
15-64 years	38.3%	44.7%	71.1%†
> 65 years	14.9%§	3.5%	4.4%

*) p=0.03 post-pandemic influenza A versus seasonal influenza A

**) p=0.004 pandemic influenza A versus post-pandemic influenza A, p=0.009 pandemic influenza A versus seasonal influenza A

†) p=0.002 post-pandemic influenza A versus seasonal influenza A, p=0.004 post-pandemic influenza A versus pandemic influenza A

§) p= 0.02 seasonal influenza A versus pandemic influenza A

The course of illness in patients with influenza A was similar for all seasons. No significant differences were observed in the occurrence of complicated illness and the need for admission to the intensive care unit (ICU), also after adjustment for the differences in age distribution (Table 5.2). During all seasons, patients were admitted to the hospital within a median time of 3 days after onset of symptoms, independent of the presence of complicated illness or necessity for ICU admittance. No differences in duration of hospitalization were seen; only complicated illness was associated with a longer hospital stay ($p<0.0001$, data not shown). In patients admitted during the pandemic and post-pandemic period, respiratory samples for the detection of influenza were taken significantly more rapidly compared to patients admitted in previous influenza seasons.

Chronic underlying illness was significantly less present in patients with pandemic influenza A compared to those with seasonal influenza A (70.6% versus 87.2%, p=0.03). However, when adjusted for age, no significant differences of comorbidities were detected between the three influenza periods in patients over 15 years. In both the pandemic and post-pandemic period, comorbidites were significantly less present in patients under the age of 15: 59.1% with pandemic influenza A (p=0.025) and 54.4% with post-pandemic influenza A (p=0.044) compared to 86.4% of patients with seasonal influenza A.

The most remarkable differences however were seen in oseltamivir treatment: oseltamivir was more frequently prescribed in pandemic patients compared to previous influenza seasons (76.5% versus 6.5%, $p< 0.0001$). In the post-pandemic period, prescription rates diminished although still more patients received antiviral therapy compared to those with seasonal influenza A (48.9% versus 6.5%, $p<0.0001$). These differences remained after adjustment for the differences in age distribution.

During the pandemic, patients not treated with oseltamivir were significantly younger, had less chronic underlying illness, were admitted to the hospital later in the course of illness and had to stay hospitalized relatively shortly compared to those who were treated with oseltamivir. Also, patients not treated with oseltamivir were less frequently admitted to the ICU (Table 5.4). During the post-pandemic period however, these differences between patients with and without oseltamivir treatment were not observed. Despite the strong increase in oseltamivir treatment, no increase in oseltamivir resistance was detected. During the seasonal influenza period, all oseltamivir resistant influenza A were subtyped

as influenza A(H1N1), as was expected considering reports of emerging oseltamivir-resistant seasonal influenza A(H1N1) since 2007.[13] During the pandemic and post-pandemic period, four out of five patients developed oseltamivir resistance while being treated with oseltamivir; one patient was infected with primarily oseltamivir resistant influenza A(H1N1)pdm09.

Table 5.4. Characteristics and course of illness in patients with and without oseltamivir treatment during pandemic influenza

	oseltamivir treatment n=65	**no oseltamivir n=20**	**significance, p-value**
age (median, years)	26.4	2.9	p=0.001
underlying illness (% of patients)	78.5	45.0	p=0.004
ICU admittance (% of patients)	35.4	10.0	p=0.047
complications (% of patients)	53.8	35.0	NS
duration of illness at admission(median, days)	2.0	3.5	p=0.024
length of hospital stay (median, days)	6.0	2.0	p=0.006

ICU=intensive care unit
NS=not statistically significant

Chest radiographs were performed in 68%, 67% and 76% of patients with seasonal, pandemic and post-pandemic influenza A respectively. Radiographic abnormalities were reported in similar frequencies in pandemic and post-pandemic influenza A (60% and 59%), more than in seasonal influenza A (37.5%) although statistical significance could not be reached. Compared to seasonal influenza A however, radiographic pneumonia (25% in seasonal influenza A) was more often diagnosed in patients during the pandemic (71%, p=0.004) and post-pandemic period (55%, p=0.047). No significant differences were seen in isolated bacterial pathogens between all influenza seasons. Also, no difference was seen in the prescription rate of antibiotics: during all seasons approximately 70% of patients were treated with antibiotics.

Influenza B

In the study period, 50 patients were included with confirmed influenza B infection, 13 during pre-pandemic seasons, and 37 during the post-pandemic period. No differences were seen in patients' characteristics and course of illness between patients with post-pandemic influenza B compared to those with seasonal influenza B. The clinical characteristics of patients with influenza B were remarkably similar to those with influenza A (Table 5.5). However, during the post-pandemic period, patients with influenza B were significantly less often treated with oseltamivir compared to patients with influenza A (27.0% versus 48.9%, p=0.043). Patients with post-pandemic influenza B were admitted later in the course of illness (median 3 versus 2.5 days, p=0.028) and were also tested later for the presence of influenza (median 4 versus 3 days, p=0.006).

Table 5.5. Clinical characteristics, course of illness and treatment in patients with influenza B compared to seasonal and post-pandemic influenza A

	seasonal influenza A N=47 (%)	seasonal influenza B N=13 (%)	post-pandemic influenza A N=45 (%)	post-pandemic influenza B N=37 (%)
Underlying illness	41 (87.2)	10 (76.9)	36 (80.0)	31 (83.3)
ICU admittance	12 (25.5)	4 (30.8)	10 (22.2)	10 (27.0)
complications	18 (38.3)	6 (46.2)	18 (40.0)	17 (45.9)
Oseltamivir treatment	3 (6.4)	3 (23.1)	22 (48.9)	10 (27.0)*

*) p=0.043, post-pandemic influenza B versus post-pandemic influenza A

Discussion

Our study is one of the first to systematically assess the clinical, epidemiological and virological characteristics of patients with post-pandemic influenza. Influenza in the post-pandemic period, including both influenza A and influenza B, was in our center equally severe as the pandemic in terms of the number of patients admitted. The course of illness in patients with influenza A was comparable for all seasons, indicating no increased severity of influenza A(H1N1)pdm09 compared to other influenza A subtypes. Besides, patients with influenza B displayed similar clinical characteristics as those with influenza A. However, several aspects are noteworthy.

The age distribution of patients admitted with influenza differed significantly in the three study periods. Patients with seasonal influenza displayed the well known age distribution with relatively more infections in the young (<4 years) and the old (> 65 years) compared to pandemic and post-pandemic periods. During the pandemic, a shift was noticed towards the school-aged and adolescent population, as has been described by others.[2,14,15] The relative lower risk of infection among older individuals has been explained by the presence of cross reactive antibodies due to exposure to circulating descendants of the 1918 H1N1 pandemic virus before 1957.[16] The majority of hospitalized patients in the immediate post-pandemic period were significantly older compared to those admitted during the pandemic. Children might have been less susceptible for serious infection during the post-pandemic period because of a relatively high attack rate during the previous pandemic influenza season or to persisting vaccine-induced immunity.[17] In the Netherlands, vaccination strategy during the pandemic focused on risk groups and young children below the age of four. Vaccination coverage among children reached around 60% in the northern region of the Netherlands (personal communication B. Wolters, Municipal Health Service Groningen).

During all seasons patients were admitted relatively early in the course of illness. These findings suggest that serious illness is mainly due to effects of the influenza virus itself and not because of the occurrence of bacterial co-infection, although this is a well known complication of influenza. Still, more than two thirds of the patients during all seasons were treated with antibiotics. Although no significant differences were observed in severity of illness between the influenza seasons, more radiographic pneumonia was diagnosed during the pandemic and post-pandemic period. This is supported by in vitro data, which showed that influenza A(H1N1)pdm09 has an increased affinity for α2, 3-linked receptors on epithelial cells in the lower respiratory tract, in contrast to seasonal influenza subtypes.[18]

The majority of patients in our study had chronic underlying illness, regardless the season, emphasizing the impact of influenza in these high risk groups, and the importance of yearly influenza vaccination. Earlier reports recorded less comorbidities in patients admitted during the pandemic compared to other influenza seasons.[1,15] However, in our center, this was only observed in patients under the age of 15 years. During the post-pandemic period, a similar pattern was observed. These findings suggest that influenza A(H1N1)pdm09 can cause serious illness especially in previously healthy young patients. It probably also reflects the tertiary referral function of our hospital and might have biased our findings towards more complicated patients and more severe illness. However, the observed similarity in severity of illness between patients with seasonal and pandemic influenza is confirmed by a recent study in which the estimated burden of disease caused by pandemic influenza in the Netherlands, based on incidence, sequelae and mortality, was comparable with the burden of seasonal influenza.[19]

Another finding of our study is the frequency of oseltamivir treatment in patients with influenza infection. We observed a more than tenfold increase in the use of oseltamivir during the pandemic compared to seasonal influenza. This was probably at least partly due to national public health guidelines recommending treatment with oseltamivir in hospitalized patients, a phenomenon that very recently also has been described for the United States.[20] In contrast to the US-study however, where people above 65 years old were less likely to receive antiviral agents, we found that especially relatively healthy, young children who were already ill for a couple of days and required only short term admission, did not receive oseltamivir. These findings suggest that during the pandemic these patients were admitted out of cautiousness rather than because of the seriousness of illness. During the post-pandemic period, oseltamivir was less frequently prescribed in patients with influenza A, although still significant differences remained compared with seasonal influenza. The reasons for this remain unclear. Compared to the pandemic, the use of oseltamivir was much less advocated by national guidelines or professional standards. Besides, influenza A(H1N1)pdm09 was by then generally regarded as causing relatively mild disease, possibly accounting for more reluctance among physicians to start antiviral treatment. The frequent use of oseltamivir did not lead to an upsurge of oseltamivir resistance of the influenza virus, suggesting that other mechanisms than antiviral pressure are responsible for the occurrence of resistance. This is not unknown for influenza, as in recent years the emergence of drug resistant influenza strains have been described in the absence of antiviral drug pressure, e.g. adamantine resistant influenza A(H3N2) since 2003 and oseltamivir resistant influenza A(H1N1) since 2007.[21]

Influenza B caused illness similar to influenza A, regardless the season. This is rather remarkable as previous studies showed that influenza A(H3N2) was associated with highest annual rates of influenza associated hospitalizations (with pneumonia, respiratory and circulatory hospitalizations as discharge diagnoses) compared to influenza A(H1N1) and influenza B.[22] Despite similarity in severity of illness, patients with influenza B during the post-pandemic period were less treated with oseltamivir compared to the patients with influenza A. This might be explained by the observation that patients with influenza B were admitted later in the course of illness. Treatment with oseltamivir can shorten the duration of illness when given early (within 48 hours) in the course of illness.
A limitation of our study is the relative small amount of patients included, hospitalized in one single tertiary referral hospital. Larger studies in different patient populations are necessary to confirm our findings.

In conclusion, in our center, seasonal, pandemic and post-pandemic influenza showed many similarities with regard to patients' characteristics, severity of illness and clinical outcome. Influenza in the post-pandemic period led to an equally severe season in terms of number of patients admitted as compared to the pandemic. Although the use of oseltamivir became common practice, no increase in oseltamivir resistance was detected. Our findings particularly highlight the fact that influenza is an important cause of illness and death each year, and emphasizes the need for influenza vaccination.

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Summary

Background: Highly transmissible viruses like influenza are a potential source of nosocomial infections and thereby cause increased patient morbidity and mortality.

Aim: We assessed whether influenza virus sequence data can be used to link nosocomial influenza transmission between individuals.

Methods: Dutch A(H1N1)pdm09 positive specimens from one hospital (n=107) were compared with samples from community cases (n=685). Gene fragments of haemagglutinin, neuraminidase and PB2 were sequenced and subsequently clustered to detect patients infected with identical influenza viruses. The probability to detect a second patient was calculated for each hospital cluster against the background diversity observed in hospital and community strains. All clusters were further analysed for possible links between patients.

Findings: Seventeen A(H1N1)pdm09 hospital clusters were detected of which eight had a low probability of occurrence compared with background diversity ($p<0.01$). Epidemiological analysis confirmed a total of eight nosocomial infections in four of these eight clusters, and a mother and child combination in a fifth one. The nine clusters with a high probability of occurrence involved community cases of influenza without a known epidemiological link.

Conclusion: Our data indicate that, presuming a background sequence dataset is available, the detection of hospital sequence clusters that differ from dominant community strains can be used to select clusters requiring further investigation by hospital hygienists before a nosocomial influenza outbreak is epidemiologically suspected.

Introduction

Despite its overall mild appearance, the 2009 influenza A(H1N1) pandemic (A(H1N1)pdm09) resulted in a significantly increased demand on health services.[1,2] Outbreaks of nosocomial influenza can occur, facilitated by transmission from HCWs to patients and colleagues.[3,4] Ward closure is frequently performed when preventive measures fail to control virus spread, illustrating the problems in containing nosocomial outbreaks of viruses that are highly transmissible and require a low infectious dose like influenza viruses.[5-7] Consequently, identification of sources of nosocomial virus infection can direct outbreak control measures, leading to reduced patient morbidity and mortality, and preserving a functioning health care system.[7]

In this study we assessed whether influenza sequence data can be used to detect nosocomial influenza transmission, identify its sources and risk factors associated with nosocomial spread. Since April 2009, we have systematically sequenced clinical samples from A(H1N1)pdm09 positive cases for mutations previously associated with increased virulence and reduced antiviral susceptibility as part of our role in monitoring the emergence of the new influenza virus. We use these data to explore the potential for use in patient cluster detection. The sequencing protocol was implemented by several health care institutes like the University Medical Center Groningen (UMCG). We analyzed the national and UMCG sequence dataset for clusters of nosocomial influenza. A sensible sequencing strategy targeting antiviral resistance markers is discussed for retrospective as well as real-time characterization of nosocomial influenza infections in healthcare institutes during an epidemic.

Methods

Cases in the UMCG hospital

Between week 40 and week 53 in 2009, throat or nose swabs from patients as well as HCWs with symptoms of acute respiratory illness were tested by RT-PCR for the presence of influenza A(H1N1) pdm09 virus.[8, 9] Further characterization was done by sequence analysis as described below. Clinical and epidemiological data of hospitalized patients with influenza A(H1N1)pdm09 were gathered using a standardized case report form. Based on the previously reported two day (median) incubation period for A(H1N1)pdm09 virus infections, nosocomial infection was defined as development of respiratory illness two or more days after hospital admission.[10] The influenza viruses in this UMCG hospital dataset are referred to in the text as "hospital influenza viruses".

Cases from national surveillance

A background dataset consisting of all (n=685) sequenced A(H1N1)pdm09 positive samples obtained from the Dutch influenza GP sentinel surveillance system (n=103), and 582 non-sentinel samples that were collected between October 2009 and April 2010 were used to compare with hospital influenza viruses.[11] The influenza viruses in this background dataset are referred to in the text as "community influenza viruses".

Sequencing

Throat or nose swabs from A(H1N1)pdm09 positive cases were sequenced directly for surveillance of virulence and antiviral resistance markers, as described previously.[11] Briefly, RNA was extracted from the clinical specimen and transcribed into cDNA using ThermoScript ™ reverse transcriptase (Invitrogen) following target amplification using HotstarTaq Master Mix (Qiagen). PCR primers targeted the receptor binding site within haemagglutinin (HA) (nt 8–789), the neuraminidase inhibitor resistance markers in neuraminidase (NA) (nt 669–1323) and known virulence markers for influenza A viruses in the polymerase binding protein 2 (PB2) (nt 1684–2223). A viral load cut-off below Ct 25 in the Matrix RT-PCR assay was used for sequencing specimens obtained from HCWs. DNA sequences were analyzed using Bionumerics V6.5 (Applied Maths, Sint-Martens-Latem, Belgium).

Molecular data analysis

The character data from a concatenated HA (nt 58–713), NA (nt 702–1323) and PB2 (nt 1684–2218) nucleotide alignment was used to build a Maximum Parsimony network in BioNumerics.[12] This method was chosen for its ability to link A(H1N1)pdm09 positive cases to their suspected source of infection using a minimum number of "evolutionary events" based on the simplest, most parsimonious, explanation of an observation and free of specific evolutionary assumptions. Patient clusters were defined by 100% sequence identity of the sequenced A(H1N1)pdm09 gene fragments. As antiviral resistant influenza viruses can emerge during oseltamivir therapy of immunocompromised patients nucleotide variation at NA codon 275 encoding resistance marker H275Y was excluded from the cluster analysis.[11, 13]
Furthermore, hospital and community influenza virus datasets were compared for their Maximum Parsimony using character data and the evolutionary divergence over all sequence pairs using the Kimura 2-parameter model conducted in MEGA5.[14, 15] Standard error estimates were obtained by a bootstrap procedure (1000 replicates).

After sequence clustering, the resolution of the hospital sequence data was analysed by calculating the probability of observing a second patient with identical virus sequence for each virus strain that defined a hospital cluster ($n \geq 2$). This was done per gene fragment using the combined community and hospital virus sequence data sets as a measure for the total sequence diversity observed during the 2009 influenza epidemic, assuming that the proportions of sequence clusters in our dataset correspond with the proportions of clusters within circulating viruses in the Dutch population.

Epidemiological analysis

Influenza sequence clusters were analyzed further by reviewing the date of hospitalization, reported onset of illness and ward(s) where the person was hospitalized to find epidemiological links between patients. For HCW, reported onset of illness, absenteeism from work, presence of patient contacts during regular work and the ward or specialty where the HCW was working before illness was collected.

Results

Between October and December 2009, 288 of 1470 hospital respiratory samples (20%) tested positive for influenza A(H1N1)pdm09 virus. They were obtained from 121 patients (75 hospitalized patients and 46 out-patients) and 71 HCWs. Most prevalent symptoms were fever, cough and shortness of breath. Almost 30% of the patients had to be admitted to the Intensive Care Unit. In 7 patients (9%) the onset of symptoms occurred two or more days after admission to the hospital, suggesting hospital acquired infection. A total of 107 influenza A(H1N1)pdm09 positive hospital samples were sequenced: 43% of the out-patients (n=20), 83% of the hospitalized patients (n=62) and 35% of the HCWs (n=25), yielding sequence information on 101 NA fragments, 102 PB2 fragments and 69 HA fragments.

Molecular resolution in the hospital sequence dataset

To distinguish clusters of hospitalized patients, the hospital sequence dataset was compared with the national dataset to assess whether influenza viruses obtained from one hospital are either closely related or reflect community influenza virus diversity. This was achieved by using Maximum Parsimony networks built with influenza HA, NA or PB2 gene fragments, supplemented by a sequence diversity comparison using a character and a substitution based method (Figure 6.1&6.2). Both methods demonstrate that influenza viruses obtained from the hospital show similar diversity as the national dataset. This could allow the detection of patient clusters using hospital sequence data.

Figure 6.1. Maximum Parsimony network of influenza A(H1N1)pdm09 NA sequences obtained from the hospital (n=101; grey) and the community (N=601; white), supplemented by vaccine strain A/California/07/09 (black), showing that hospital A (H1N1)pdm09 viruses are dispersed similarly to community viruses. Genotypes (A-N) were assigned to clusters (N ≥ 2) of 100% identical hospital influenza sequences.

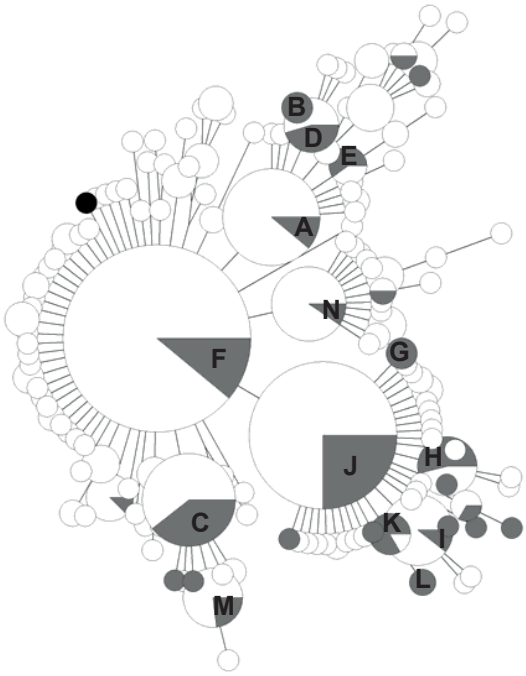
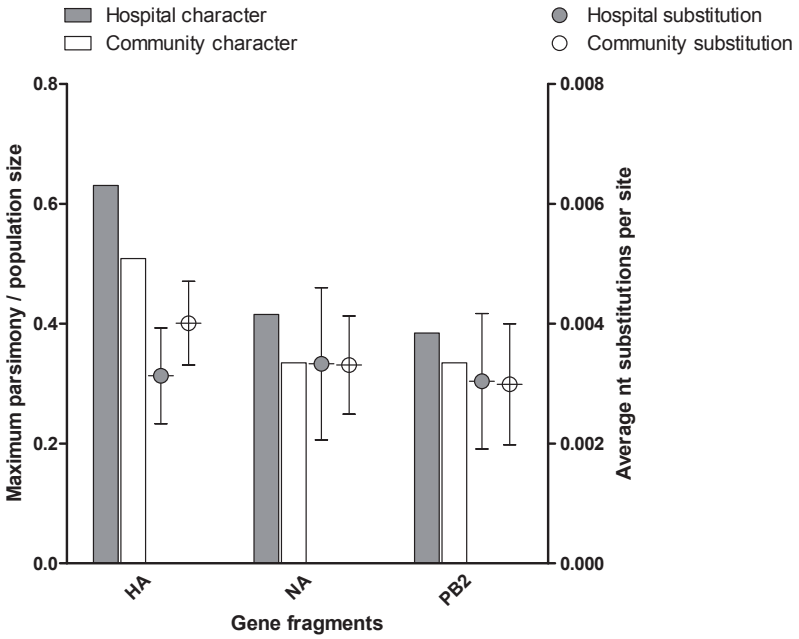


Figure 6.2. Influenza virus sequence diversities of the hospital and community datasets were compared using a character and substitution based method and expressed as the relative maximum pasimony per gene fragment (left y-axis) and the evolutionary divergence over alle sequence pairs per gene fragment (right y-axis), respectively. Both methods demonstrate similar sequence diversity for each gene segment present in the hospital dataset compared with the observed national diversity.



Molecular clustering

Initially, hospital sequence data were used without community data to detect patient clusters. Maximum Parsimony networks were constructed using 101 NA fragments, 96 PB2 fragments, 65 HA fragments, 96 combined NA and PB2 fragments, and 65 combined HA, NA and PB2 fragments to detect clusters (n≥2) of 100% identical influenza A(H1N1)pdm09 sequences. Clustering of hospital sequence data based on HA, NA or PB2 fragments identified 7, 14 and 7 sequence clusters, respectively. When combining the clustering results obtained using individual gene fragments, a total of 17 molecular distinct clusters were identified (Figure 6.3). Six of the seven patients with onset illness two or more days after hospitalization were present in four clusters. The seventh patient did not cluster with hospital and community sequences due to a silent mutation in NA codon 413.

A proportion of the hospital clusters was characterized by unique sequences compared with community sequence data. To enumerate the uniqueness of all detected hospital sequence clusters, a probability was assigned to each sequence cluster based on the frequency these variants were observed. Eight patient clusters were identified with a low probability ($p<0.01$) that these were expected based on random selection in a background diversity of hospital and community influenza strains (Figure 6.3).

Figure 6.3. Sequence clustering of hospital A(H1N1)pdm09 viruses identified a total of 17 molecular clusters based on the combined results obtained from the individual gene fragments. Sequence analysis of haemagglutinin (HA) fragments identified seven molecularly distinct clusters (genotypes A-G), of neuraminidase (NA) 14 clusters (NA genotypes A-N) and of PB2 seven clusters (PB2 genotyeps A-G). By combining these results, NA genotype C and F viruses are subdivided by addition of HA and/or PB2 sequence data, and NA genotype J viruses are subdivided by addition of HA data, yielding the 17 molecular distinct patient clusters. The probability of observing a second patient with an identical influenza gene fragment sequence is provided for all sequence clusters per separate and combined gene fragment (s). The left column shows the result of the epidemiological cluster investigation. Patients with epidemiological links are marked A, B or C, corresponding with the analysis of patient clusters in Figure 6.4. +Gene fragment sequence data present. *Patients with onset illness ≥ 2 days after hospitalization. HCW, health care worker.

Figure 6.3. ▸ next page

		Sequence clusters						Probability					
Cluster information		**HA**	(genotype)	**NA**	(genotype)	**PB2**	(genotype)	**p (HA)**	**p (NA)**	**p (PB2)**	**p (NA, PB2)**	**p (HA, NA, PB2)**	**Presence of p < 0.01**
community acquired	patient	+	A	+	A	+	A	0.0199	0.0598	0.1029	0.0313	0.0099	YES
	patient	+		+		+							
	patient			+		+							
	patient			+									
nosocomial cluster 1	patient A	+	B	+	B	+		0.0066	0.0050	0.1029	0.0067	0.0066	YES
	patient C	+		+		+							
	patient B *			+		+							
community acquired	patient	+	C	+	C	+	B	0.2185	0.0548	0.0224	0.0179	0.0199	NO
	patient	+		+		+							
	patient	+		+		+							
	HCW	+		+		+							
	patient			+		+							
	HCW			+		+							
	patient			+									
	HCW			+									
	HCW			+									
community acquired	patient	+	D	+		+	C	0.0265	0.0548	0.2662	0.0403	0.0331	NO
	patient	+		+		+							
	HCW	+		+		+							
	patient			+		+							
community acquired	patient			+	D	+		-	0.0183	0.2662	0.0179	-	NO
	patient			+		+							
	patient			+		+							
	HCW			+		+							
	patient			+									
nosocomial cluster 3	patient			+	E	+		-	0.0083	0.2662	0.0089	-	YES
	patient A *	+		+		+							
	patient B *			+		+							
community acquired	child	+	E	+	F	+	D	0.0497	0.2259	0.0067	0.0067	0.0099	YES
	mother	+		+		+							
	patient	+		+		+							
nosocomial cluster 2	patient A	+	C	+		+	C	0.2185	0.2259	0.2662	0.0559	0.0099	YES
	patient C *	+		+		+							
	patient B *	+		+		+							
	HCW			+		+							
	HCW			+		+							
	patient			+		+							
	HCW			+		+							
	patient			+		+							
	patient			+		+							
	patient			+		+							
	patient			+									
	patient			+									

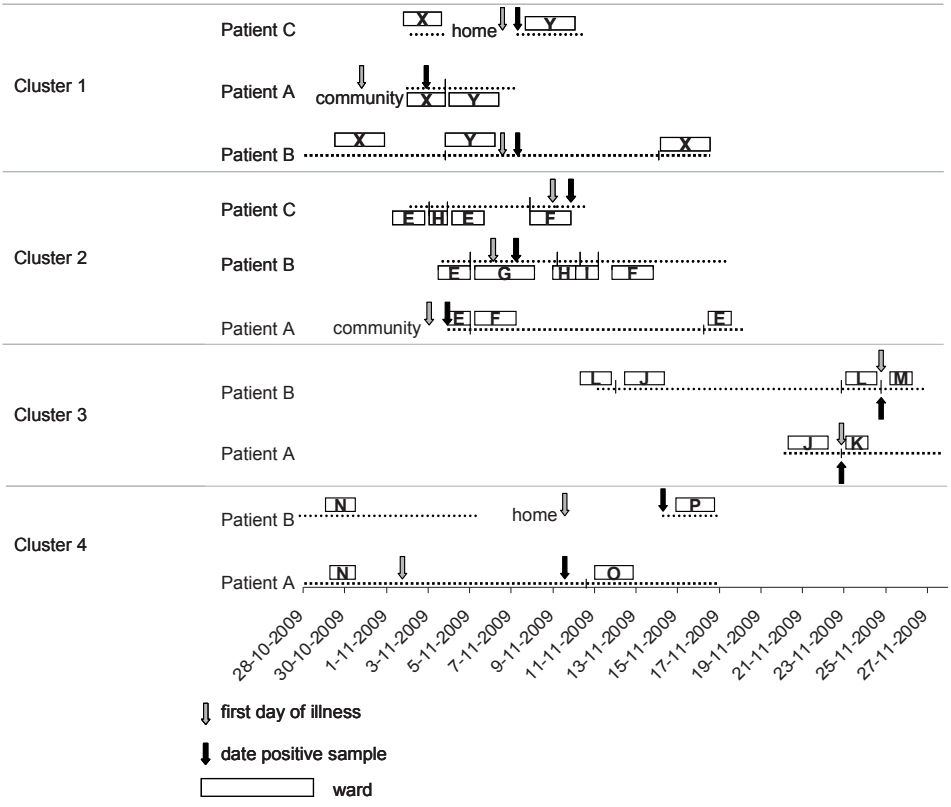
community acquired	patient patient HCW	+ + +		+ + +	G	+ + +	E	0.2185	0.0050	0.2416	0.0067	0.0099	YES
community acquired	child patient HCW patient mother patient patient	+ + +		+ + + + + + +	H	+ + + + +		0.2185	0.0216	0.2416	0.0201	0.0232	NO
community acquired	patient patient			+ +	I	+ +		0.2185	0.0316	0.2416	0.0179	-	NO
community acquired	HCW HCW HCW patient	+ +		+ + + +	J	+ + +		0.2185	0.1395	0.2416	0.1074	0.0265	NO
community acquired	patient patient HCW HCW patient patient patient patient patient patient patient patient patient HCW HCW	+	F	+ + + + + + + + + + + + + + +		+ + + + + + + +		0.0762	0.1395	0.2416	0.1074	0.0331	NO
community acquired	patient HCW patient patient patient	+ +	G	+ + + + +	K	+ + + +		0.0232	0.0100	0.2416	0.0089	0.0066	YES
nosocomial cluster 4	patient A * patient B	+		+ +	L	+ +		0.0232	0.0033	0.2416	0.0045	-	YES
community acquired	patient patient HCW			+ + +	M	+ +	F	-	0.0216	0.0201	0.0179	-	NO
HCW	HCW HCW	+		+ +	N	+ +	G	-	0.0349	0.0224	0.0291	-	NO

Epidemiological clustering

Epidemiological analysis of nine sequence clusters that had a clustering probability p>0.01 could not identify any epidemiological relations between the patients and/or HCWs within these clusters, except for the detection of a mother-child link within one cluster. Based on first day of illness, all patients within these nine clusters were infected with community acquired influenza virus infection.
Epidemiological analysis of the eight clusters that had a low probability (p<0.01) that these were expected based on random selection, was not able to identify any associations between the patients and/or HCWs within three out of eight clusters. One cluster included a mother and child, and epidemiological analysis of the remaining four clusters was suggestive for transmission of influenza virus between patients (Figure 6.3 and 6.4). These four clusters each contained one or more patients with a presumed hospital acquired influenza virus infection based on the first day of illness two or more days after admission.

1. Hospital cluster 1 contained three A(H1N1)pdm09 virus infected patients. Patient A, infected with community acquired influenza, was probably the source of infection for patient B and patient C. All three were on the same ward at the time patient A tested positive for influenza A(H1N1)pdm09 virus infection. Patient C was temporarily sent home, but was re-admitted with influenza like illness 4 days after leaving the hospital for the first time. Based on onset of symptoms compared to date of admission, patient C initially was considered as having a community acquired infection.
2. Hospital cluster 2 contained three patients with A(H1N1)pdm09 virus infection including two patients that had a first day of illness respectively 2 and 7 days after admission to the hospital. Patient A, infected with community acquired influenza, is regarded as the index patient for the other two patients.
3. Hospital cluster 3 consisted of three patients including 2 patients with hospital acquired influenza virus infection. As the third patient stayed at a different ward and was discharged before patient B was admitted, the actual source of infection is missing. Both nosocomial patients were on the same ward before becoming ill, but were already transferred to other wards when tested positive for influenza A(H1N1)pdm09 virus. Most likely the source of these two hospital acquired infections occurred in ward J.
4. Hospital cluster 4 consisted of two patients including one patient with a hospital acquired influenza virus infection. Both patients were on the same ward at the time patient A developed a respiratory illness caused by influenza A(H1N1)pdm09 virus. Patient B was discharged from the hospital but got ill 4 days after returning home, 8 days after the first day of illness of patient A. Patient B was re-admitted because of respiratory illness and subsequently tested positive for influenza A(H1N1) pdm09 virus infection. This re-admitted patient was not considered a patient with a hospital acquired infection in the initial comparison of first day of illness with date of admission. Like in cluster 3, the source of infection is missing and most likely was a patient, HCW or visitor from ward N.

Figure 6.4. Epidemiological analysis of four patient clusters each containing ≥ 1 patients with presumed hospital-acquired A(H1N1) pdm09 virus infection suggests that the source of nosocomial influenza virus infections within clusters 1 and 2 was a patient with community-acquired influenza. This analysis identified two additional nosocomial influenza patients (cluster 1, patient C; cluster 4, patient B) that initially were considered as having a community-acquired infection. An index case of nosocomial influenza virus infections within clusters 3 and 4 is missing, but might be associated with a specific ward. Separate wards have been assigned different letters.



Discussion

Retrospective analysis of sequence data combined with epidemiological data was performed to evaluate the usefulness of sequencing influenza gene fragments for the characterization of nosocomial influenza virus infections. Although characterization of nosocomial A(H1N1)pdm09 cases by a combined molecular and epidemiological approach has been reported[16, 17], the evaluation of this approach to guide the identification of nosocomial influenza clusters to our knowledge is not reported yet.

Our analysis identified nine nosocomial influenza infections during the 2009 influenza epidemic in a tertiary referral hospital, which comprises 15% of all the diagnoses during the study period. Although transmission from an index case to a hospitalized patient was observed four times, no onward transmissions could be demonstrated. In addition, the molecular and epidemiological analysis did not provide evidence for transmission between patients and staff. Our results highlight some important observations. First, patient transfer from a ward with influenza positive patients to another ward should be done with great care and follow up of the transferred patient. Secondly, follow up of recently discharged patients could identify possible spread of infection, which is highly relevant if antiviral resistant or virulent influenza virus variants are identified in the hospital. Finally, our analysis was able to link patients across wards in two clusters that would be difficult to detect using epidemiological data only.

Sequencing of HA fragments was less often successful than sequencing of the NA and PB2 fragments due to a reduced sensitivity for the HA fragment amplification, compared with NA and PB2 amplification (data not shown). This affected HA-based clustering as these sequences were missing for 52% of the hospital patients. Consequently, an unbiased comparison of HA with NA sequence data for the detection of patient clusters was not possible. The lower substitution rate for A(H1N1)pdm09 PB2 gene segments compared with NA segments, explains the discrepancy between the number of molecular clusters detected using NA (n=14) and PB2 (n=7) sequence data.[18] Nevertheless, the calculation of the probabilities that A(H1N1)pdm09 positive patients were infected by viruses with 100% identical gene fragments proved to be a useful selection. When comparing the NA sequences with GenBank (consulted on February 6, 2012), the five strains that defined NA clusters with $p<0.01$ all remained unique, while the strains that defined clusters with $p>0.01$ demonstrated on average 57 identical strains. This suggests that our observations did not result from the lack of resolution in the background data. It implies that during an influenza epidemic, commonly circulating influenza strains are detected at all geographical areas and can be used to express the uniqueness of a local patient cluster. Although sequence-based typing is possible in advanced clinical laboratories, routine application is relatively costly when considering its use for the sole purpose of identification of nosocomial infections. Therefore, we looked at the potential for using this approach on the basis of data collected for a different purpose, i.e. sequencing to detect markers of antiviral resistance. As antiviral treatment of immuno-compromised patients is associated with the emergence of antiviral resistance, monitoring for emergence of resistance is vital for both the patient as well as public health, and is increasingly common practice.[13] Hospital molecular diagnostics commonly utilize a targeted approach for the rapid identification of the primary oseltamivir resistance substitution H275Y in NA subtype 1, but our results demonstrate the possible added value of Sanger sequencing a larger region (>600bp).[19-21] Currently, sequence capacities and application of routine sequence analysis in clinical laboratories are increasing, especially in academic hospitals. Although the capacity to perform sequence analysis is not present in all hospitals, these hospitals could benefit from the knowledge generated on the characterization of influenza virus transmission chains in hospitals that are able to incorporate sequence analysis into routine clinical use.

Both retrospective and prospective approaches produce valuable insights in nosocomial virus spread that can direct outbreak control measures, leading to reduced patient morbidity and mortality.

Acknowledgements

We would like to thank Reina van der Heide and Felix Geeraedts for their help in performing the sequence analysis of UCMG influenza viruses. This study was funded by the Impulse Veterinary Avian Influenza Research in The Netherlands program of the Economic Structure Enhancement Fund.

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Abstract

Objective

Human rhinovirus (HRV) is associated with serious respiratory illness, particularly in patients with pulmonary comorbidities. HRV is increasingly considered as a nosocomial pathogen with a considerable burden of disease in certain groups of patients. However, little is known about the factors that determine and prevent nosocomial transmission of HRV. The objective of this study was to quantify patient-to-patient transmission of HRV in our hospital and to determine the impact of infection control measures.

Methods

Data on HRV detection, clinical symptoms and infection control measures were retrieved from a prospective project into respiratory infections in hospitalized children between October 2009 and January 2011. Data were used to inform a stochastic, individual-based, multi-ward model to assess the relative contributions of factors that drive HRV transmission dynamics: patient-to-patient transmission, introduction by newly admitted patients, visitors and health care workers and existing infection control measures.

Results

Given the yearly admission of 200 patients with a community acquired HRV infection, infection control policies reduce the number of hospital acquired HRV infections from 92, when no infection control measures at all are implemented, to 61 patients, when baseline infection control policies and HRV specific measures exist. Based on our model, a further reduction in nosocomial HRV infections is accomplished when all patients infected with HRV would be put in isolation as soon as they show symptoms of respiratory disease instead of based on a positive laboratory result or when a rapid point-of-care test could be implemented. However, the fraction of hospital acquired cases would still be 21% because of introduction of HRV from outside the hospital via visitors or health care workers (HCW). For our hospital, 80% of the hospital acquired HRV cases observed during one year would be due to a source other than patient-to-patient transmission (like introduction of HRV into the hospital by visitors or health care workers).

Conclusions

Infection control measures reduce the number of hospital acquired HRV cases. However, because of the continues introduction of HRV from the community into the hospital and the delay in implementation of appropriate infection control measures after a patient becomes infected, a considerable amount of hospital acquired HRV infection will persist despite the implementation of effective infection control measures.

Introduction

Human rhinoviruses are the most common cause of upper respiratory tract infections and are also associated with more severe clinical syndromes particularly in patients with pulmonary comorbidities.[1-5] Nosocomial viral infections occur frequently among pediatric patients, with respiratory tract infections as one of the most prevalent manifestations.[6-9] With the exception of a few recent publications, data on the frequency of hospital acquired HRV infections are scarce and mostly limited to the description of outbreaks of respiratory tract infections caused by HRV.[1,6,10,11] Currently, infection control measures are centered around the infected patient. Less attention is paid to visitors and health care workers, although working in or visiting a relative in a hospital when having symptoms of respiratory disease is not recommended. Also, HRV is usually not part of national guidelines on infection control within health care institutions.

Infection control measures have the goal to prevent transmission of pathogens from one patient to the other. Measures are focused on the main transmission routes of the pathogen, being either via contact with contaminated hands or surfaces, or via the air. Knowledge on the transmission dynamics of a pathogen is a prerequisite for the implementation of the appropriate infection control measures. In recent years mathematical modelling has been increasingly used to study the transmission dynamics of nosocomial infections, as well as the assessment of the effectiveness of control measures.[12-14] These models have mainly addressed the dissemination of bacterial pathogens such as Methicillin resistant *Staphylococcus aureus* (MRSA), Vancomycin resistant enterococci (VRE), and *Clostridium difficile*. Viral pathogens in the hospital setting have also been studied recently, with norovirus and influenza the most relevant examples.[15,16]

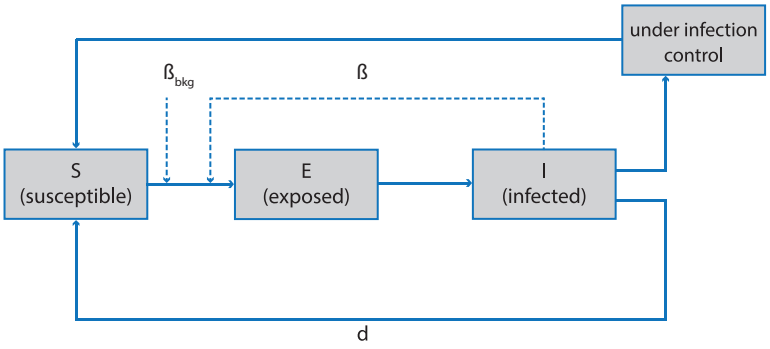
We present a mathematical model of nosocomial HRV transmission in a multi-ward hospital setting. By combining this model with HRV epidemiological data collected at a children's hospital, part of a large university hospital in northern Netherlands, we aim to answer the following two questions: firstly, what is the impact of different patient isolation strategies in stopping the nosocomial dissemination of HRV infections. Secondly, what is the relative contribution of two different transmission routes, namely direct patient-to-patient transmission, and introduction from the community by visitors and HCW.

Methods

We employ a stochastic SEIS simulation model that tracks the infection status of individual patients during their hospital stay. Patient admission, discharge and between-ward transfers follow typical movement patterns inferred from a large patient-location data set. The model is asynchronous and proceeds in discrete time steps of length $\delta t = 1$ day, motivated by the temporal granularity of the epidemiological and patient-location data.

Patients can be in one of three possible infection states: uninfected and susceptible to infection (S), infected but non-infectious (exposed: E), and infectious (I) (SEIS compartment transmission model, Figure 7.1). When susceptible patients become infected they enter the exposed state. After a length of time known as the exposed period, the patient becomes infectious. Infectious patients remain in this state for a length of time known as the infectious period, after which they recover from the infection. Due to the large diversity in circulating HRV serotypes we assume that HRV infection does not confer long-lasting immunity. Thus, patients return to the susceptible state after recovery from infection.

Figure 7.1. SEIS compartment transmission model: background transmission rate βbkg, patient-patient transmission rate β and infectious period d.



We consider two different transmission routes. Direct transmission involves the transmission of HRV between two patients in the same hospital ward, either due to direct or HCW-mediated patient-to-patient contact. We assume homogeneous mixing between patients within each ward. Background transmission accounts for all other transmission mechanisms and we assume that is mainly due to visitors and HCW becoming infected in the community, and subsequently transmitting the pathogen to hospitalized patients.

Two infection control strategies are modelled: baseline and HRV-specific isolation. Baseline isolation consists of placing of HRV-infected patients under isolation, during part or all of their infectious period, because of clinical or infection control considerations unrelated to the HRV infection. Conversely, HRV-specific isolation involves the isolation of laboratory confirmed HRV-infected patients according to existing HRV-specific isolation guidelines used in our hospital. HRV-specific isolation may depend on patient location and other characteristics and starts only after laboratory test results are obtained.
We evaluate five different infection control scenarios:

- The first scenario corresponds to only implementing baseline isolation (i.e. no HRV-specific isolation).
- The second scenario corresponds to implementing baseline isolation, as well as HRV-specific isolation for HRV-infected patients in ICUs and hematological wards with a positive PCR test result for HRV with a Ct value <30 (a randomly chosen value). These two scenarios are consistent with the existing isolation guidelines during the collection of epidemiological data.
- The third scenario corresponds to baseline isolation, as well as HRV-specific isolation for all HRV-infected patients (i.e. independent of ward and Ct value).
- The fourth scenario is similar to the third, except that in this case there is no delay between onset of symptoms and HRV-specific isolation (i.e. the time interval between onset of symptoms, availability of test results and patient isolation, is less than one day).
- The fifth scenario, which we used to counterfactually assess the overall impact of isolation measures, corresponds to no isolation.

Direct transmission is modelled as a density dependent process, whereas background transmission occurs with a constant daily probability per susceptible patient (i.e. a Poisson process with constant rate). The probability of a susceptible patient *i* becoming infected with HRV while located in ward *w* on day *t* can then be written as:

$$P_{i,w}^{SE}(t) = \beta \frac{I_w(t) - \alpha C_w(t)}{N_w(t)} \delta t + \beta_{bkg} \delta t$$

where β is the direct transmission rate, βbkg is the background transmission rate, α is the efficacy of isolation, I_w is the number of infected patients in ward *w*, C_w is the number or infected patients in ward *w* which are also under isolation, and N_w is the total number of patients in ward *w*. We assume that the effect of isolation is to reduce the direct transmission rate associated with an infected patient under isolation by a multiplicative factor (1-α).

The model is employed to calculate the proportion of all HRV-infected patients that are considered hospital associated, as well as the relative fraction of hospital-associated cases due to either direct or background transmission. We classify a HRV infection as hospital associated by employing the conventional cut-off approach: a hospital-associated case is defined as the onset of symptoms two or more days after hospital admission. To capture all stochastic variation we obtain our results from 1000 simulation replicates per infection control scenario and set of input parameters. Each simulation replicate starts with a number of patients (all susceptible) in each ward consistent with observed occupancy rates. Seeding occurs continuously by the admission of community-associated cases of HRV infection. These patients amount to a fraction ρ of all daily admissions. We run the model for a one year burn-in period, such that the initial transient has passed, and then we run the model for a further year during which we calculate the above mentioned metrics.

Epidemiological data

Data on HRV detection, clinical symptoms and infection control measures were retrieved from a prospective project into respiratory infections in children hospitalized in the Beatrix Children's Hospital between October 2009 and January 2011, as has been described in detail elsewhere.[1] The Beatrix Children's Hospital has 142 beds and is part of the University Medical Center Groningen, a tertiary referral hospital in het northern region of the Netherlands. Demographic, clinical and microbiological data were systematically collected from all children under 18 years of age, from whom respiratory samples were taken for the detection of 15 respiratory viruses (influenza A/B, respiratory syncytial virus A/B, coronavirus 229E/NL63/OC43, para-influenzavirus type 1-4, metapneumovirus, adenovirus, bocavirus and human rhinovirus). Samples were taken based on the clinical judgment of the physician. Clinical data were collected using a standardized case report form. Epidemiological data were gathered to determine whether the respiratory infection was community or hospital acquired, including infection control measures that were taken in the hospital to prevent further transmission of respiratory viruses. During the study period there was a change in the infection control guidelines associated with HRV-infected patients. This change splits the study period into an early and late stage. During the early stage, which runs from 1 October 2009 up to 20 June 2010, there were no HRV-specific isolation measures in place. During the late stage, which run from 1 July 2010 up to 31 December 2010, laboratory confirmed HRV-infected patients (Ct value <30) admitted in any of the intensive/high care wards, or hematological units, were placed under isolation until discharge or resolution of symptoms. The different infection control guidelines in the two stages in the study period are modelled by the first two infection control scenarios described in the previous subsection. Infection control measures consisted of a combination of droplet and contact precautions (gown, gloves and mask for health care workers during patient care, and patient in a single room) and were installed when HRV was detected.

Patients with HRV mono-infections (no other respiratory virus detected except HRV) and mixed infections (HRV detected together with one or more other respiratory viruses) were included in the model. One unique patient could be included with more than one disease episode, which was defined as a period of time starting with a first day of illness and ending with full recovery from respiratory symptoms or discharge, during which one or more samples were taken for the detection of respiratory viruses. Each disease episode generated one record in the final data set. Each record includes information on: patient date of birth and sex, first day of illness (i.e. date of symptoms onset), sampling date, admission and discharge dates, location, presence and duration of symptoms (for the following signs and symptoms: cough, dyspnoea, need of oxygen, fever, and intubation), isolation data (type, and start/end dates), presence of other respiratory virus (which), HRV genotype, and Ct value associated with PCR detection of HRV.

Detection of HRV

All respiratory samples (mainly nasopharyngeal swabs or nasopharyngeal aspirates) were tested for the presence of HRV by a laboratory developed real-time PCR test (RT-PCR) as described elsewhere.[1] The Ct value (the number of amplification cycles needed for a PCR to become positive) was used as an estimate for the amount of HRV present in the samples.

Patient location data

We extracted patient location data from the University Medical Center Groningen hospital information system. These data cover the one year period 6 January 2011 – 5 January 2012 and consist of 365 snapshots of the Beatrix Children's Hospital patient population. Each snapshot, generated daily at approximately 13:30 hours, consists of a list of records corresponding to all currently hospitalized patients. Each record contains a unique patient identification number, date and detailed patient location information (ward, room, bed and medical specialty). By comparing consecutive snapshots, we determined between-ward patient movements, as well as ward admission and discharge rates, length of stay, discharge/transfer probabilities and daily ward change in number of occupied beds.

Model parameterisation

Most of the model parameters can be sampled from probability distribution functions directly estimated as relative frequencies from the epidemiological and patient location data sets. A comprehensive list is shown in Table 7.1. We note that the length of the exposure period is sampled from the probability distribution function reported in Lessler et al.[17] We assume that the exposure period equals the incubation period.

Direct (β) and background (βbkg) transmission rates are free parameters of the model. We estimate their values by minimising the average relative error *E* between observed and model-predicted proportion of hospital-associated HRV infections:

$$E(\beta,\beta_{bkg}) = \frac{1}{2}\left[\frac{\left|f^{HA}_{model}(\beta,\beta_{bkg};early) - f^{HA}_{data}(early)\right|}{f^{HA}_{data}(early)} + \frac{\left|f^{HA}_{model}(\beta,\beta_{bkg};late) - f^{HA}_{data}(late)\right|}{f^{HA}_{data}(late)}\right]$$

In the above expression f^{HA}_{data} is the fraction of hospital-associated cases according to the data, and f^{HA}_{model} is the model-predicted fraction of hospital-associated cases for a given value of the free parameters; early/late refer to stage of the epidemiological studie period, wich in turn determines the isolation scenario used by the model.

From the data: $f_{data}^{HA\ (early)} = 0{,}26$ and $f_{data}^{HA\ (late)} = 0{,}25$. Minimization was performed by a simple scan of the two-dimensional parameter space.

Table 7.1. Summary of model parameters. o.p.d.f: observed probability distribution function.

Epidemiological data	
parameter	Notes
Admission rate of HRV community cases	Directly calculated from data
Exposed period	Sampled from published incubation time distribution [17]
Infectious period	Assumed to be equal to duration of coughing
Fraction of cases under baseline isolation	Directly calculated from data
Delay onset symptoms-baseline isolation	Sampled from o.p.d.f
Duration of baseline isolation	Sampled from o.p.d.f
Delay onset symptoms-sampling	Sampled from o.p.d.f
Delay sampling-HRV-specific isolation	Sampled from o.p.d.f
Direct transmission rate	Minimisation of average relative error
Background transmission rate	Minimisation of average relative error
Patient location data	
Parameter (per ward)	Notes
Lengt of stay	Sampled from o.p.d.f
Initial occupancy	Sampled from o.p.d.f
Daily change in occupancy	Sampled from o.p.d.f
Destination after discharge	Sampled from o.p.d.f
Admission and discharge rates	Sampled from o.p.d.f

Results

Epidemiological data

From October 2009 till January 2011, 339 HRV positive disease episodes were included. Hospital acquired infection, based on the first day of illness and date of admission to the hospital, was present in 88/339 (26.0%) episodes divided into 58/226 (25.7%) in the early stage of the study and 26/106 (24.5%) in the late stage (Table 7.2). The transition stage reflects the period in which infection control policy changes were implemented. In 238/339 (70.2%) disease episodes information on infection control measures was present. In the early stage of the study, with no HRV specific infection control policies, 84 patients were however in isolation because of other reasons (84/129=65%).

Table 7.2. Overview of the number of HRV cases (either community associated (CA) or hospital associated (HA)) and infection control measures.

	Early	Transition	Late	Total
Community associated	168	3	80	251
Hospital associated	58	4	26	88
Total	226	7	106	339
With isolation data	129	3	107	238
Isolated	84	1	57	142

Early: 01.10.2009-20.06-2010; transition 21.06.2010-31.06.2010; late: 01.07.2010-31.12.2010.

Model predictions

Figure 7.2 shows the contour curves of the relative error between the data and the model predictions. These curves quantify the relative error between the model and the epidemiological data. The free parameters direct (β) and background (βbkg) transmission rates are estimated minimizing the average relative error between observed and model-predicted proportion of hospital acquired cases (depicted as the yellow dot in figure 7.2). Figure 7.3 shows the heat map of the model predicted fraction of hospital acquired cases as a function of direct (patient-to-patient) and background (introduction by visitors, health care workers) transmission rate. This describes how the model behaves for each combination of the free parameters β and β_{bkg}.

Figure 7.2. Contour curves for relative error between the data and the model, quantifying the relative error between the model and the epidemiological data. The free parameters direct (β) and background (βbkg) transmission rates are estimated minimizing the average relative error between observed and model-predicted proportion of hospital acquired cases. The yellow dot represents the minimum average relative error between observed and model-predicted proportion of hospital acquired cases, corresponding to the actual epidemiological situation.

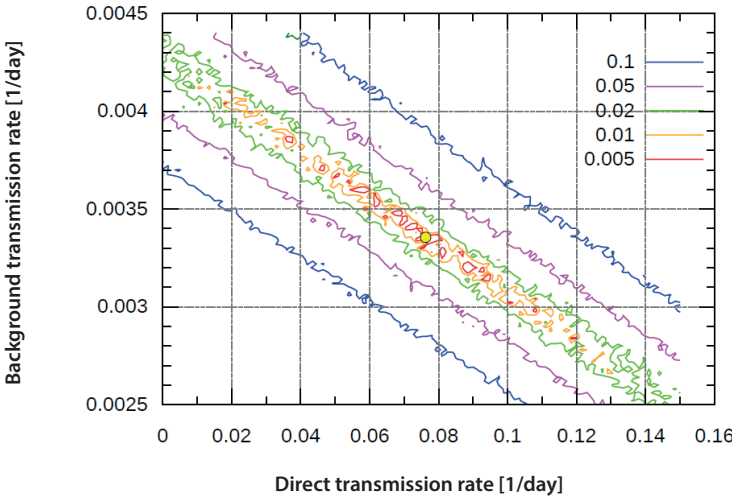
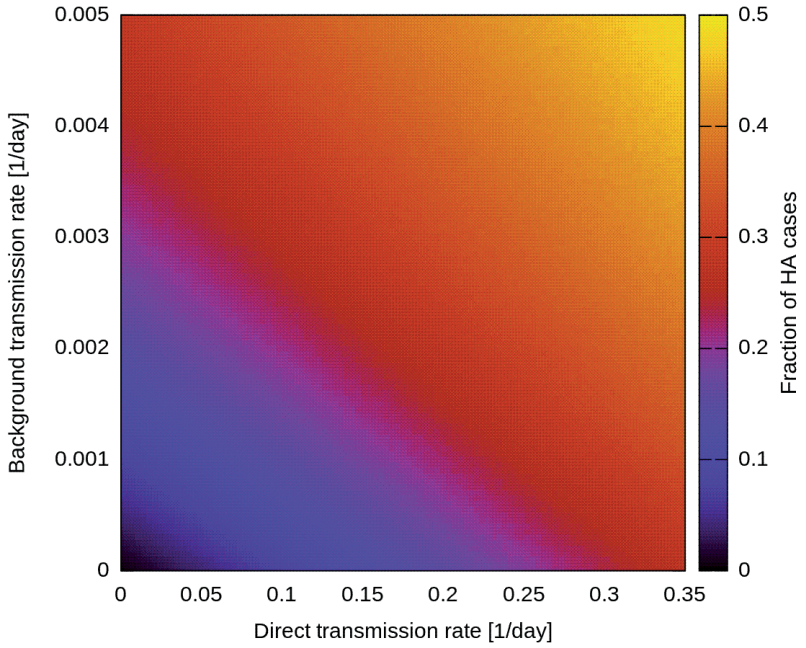
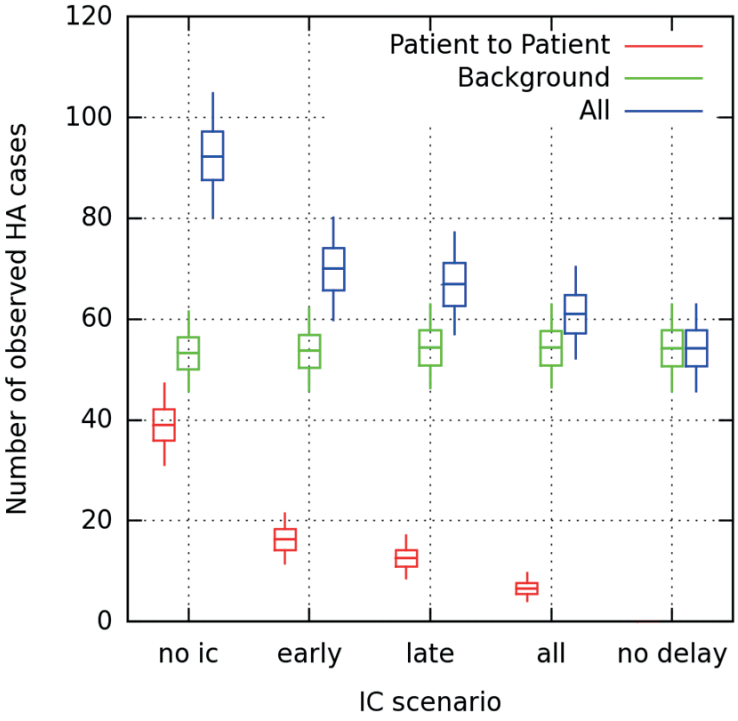


Figure 7.3. Heat-map of model-predicted fraction of hospital acquired cases as a function of direct (patient-patient) and background (introductions by visitors, health care workers) transmission rate, describing how the model behaves for each of the combination of the free parameters β and β_{bkg}.



The model predicted number of observed hospital acquired cases, based on the admission of 200 patients with community associated HRV infection, for the five different infection control scenarios is given in figure 7.4. The lowest number of hospital acquired HRV cases is reached when all patients with HRV are put in isolation the moment they show symptoms of infection (the "all (no delay)" scenario). Approximately 54 patients will develop a hospital acquired HRV infection given an influx of 200 community associated infections, corresponding to a fraction of hospital acquired cases of 21.4% (52/252 patients). Our current policy, i.e. installment of infection control measures based on a positive laboratory result, leads to an increase in the number of nosocomial HRV infections from 54 to 61 patients (corresponding to a fraction of hospital acquired cases of 23.3%). In the imaginary situation that no infection control measures at all are in place (not for HRV but neither for other reasons), the number of hospital acquired cases would increase to 92 (fraction of hospital acquired HRV infections of 31.6%).

Figure 7.4. Model predicted number of hospital acquired cases for five different infection control scenarios as depicted in the method section of the paper (blue boxes). The contribution of patient-to-patient transmission (red boxes) and background transmission (green boxes) for the given number of hospital acquired cases is also given.



The model was further used to predict the fraction of hospital acquired cases due to patient-to-patient and background transmission (Figure 7.4 and Table 7.3). Overall, the model predicated contribution of background transmission to the number of hospital acquired cases varied between 58% (given the scenario that no infection control is implemented at all) and 100% (given the scenario that all patients are put in isolation the moment they show symptoms). As expected, infection control measures lower the amount of hospital acquired cases due to patient-to-patient transmission. However, even when all HRV infected patients are in isolation, direct transmission still contributes to nosocomial HRV transmission because of the delay in the implementation of infection control measures. The combination of infection control measures for all HRV infected patients plus no delay between onset of symptoms and implementation of infection control measures reduces the amount of hospital acquired cases due to direct transmission to zero. As this does not effect the contribution of background transmission on nosocomial infection, still a substantial number of hospital acquired cases will occur. As the "early" and "late" scenarios reflect the two stages of our study, between 77% en 81% of hospital acquired HRV infections were in our case due to introduction from outside the hospital by visitors or health care workers (corresponding with approximately 55 patients given an influx of 200 patients with community acquired HRV infection).

Table 7.3. Fraction of hospital acquired cases due to direct (patient-to-patient, fDirect) and background (visitors, health care workers, fBackground) transmission for the five different scenarios (with 90% confidence interval).

Scenario	fDirect	fBackground
Early	0.232 (0.181-0.287)	0.768 (0.713-0.819)
Late	0.190 (0.139-0.243)	0.810 (0.757-0.861)
All	0.108 (0.065-0.154)	0.892 (0.846-0.936)
All (no delay)	0.000 (0.000-0.000)	1.000 (1.000-1.000)
No isolation	0.421 (0.359-0.480)	0.579 (0.520-0.641)

Discussion

To our knowledge, our study is one of the first to try to unravel the transmission dynamics of nosocomial HRV infections by combining epidemiological methods and diagnostic results with mathematical modeling techniques in order to gain more insight into the impact of infection control on nosocomial infections. Our study focuses on the relative contribution of three factors that drive HRV transmission within our children's hospital in order to get to a more quantitative assessment of the infection control policy and the lessons we can learn from this: patient-to-patient transmission, introduction from outside the hospital and delay in implementation of infection control measures.

Several conclusions can be drawn based on the model predicted impact of infection control measures on hospital acquired HRV infections. First, adding HRV specific infection control measures to existing infection control policies would only slightly reduce the number of hospital acquired HRV infections. A further reduction in nosocomial HRV infections would be accomplished by reducing the delay between the onset of symptoms and the implementation of infection control measures. In our case, patients are placed in isolation based on a positive laboratory results instead of clinical symptoms, thus creating a timeframe in which a patient is not isolated while already able to spread the virus to others. This period should be as short as possible. The period of delay can be divided into four sections: (1) the time between the first day of illness and the actual collection of material for viral diagnostics, (2) the time between the collection of the material and the arrival at the laboratory, (3) the time to result and (4) the time between result and actual isolation of the patient. Rapid diagnostic tests will shorten section 3 and, in case of point-of-care testing at the bedside of the patient, section 2. However, rapid diagnostics will not influence the time between onset of symptoms and the collection of respiratory material, or the actual implementation of infection control measures. Rather, the implementation of infection control measures based on symptoms will reduce section 1. Thus, rapid diagnostic tests (or point-of-care tests) in combination with an infection control policy based on clinical syndromes instead of a positive laboratory result would benefit a further reduction in the occurrence of nosocomial HRV infections. The time between a positive laboratory result and the actual implementation of infection control measures relies on close collaboration between the laboratory and infection control nurses, clinical microbiologists and clinicians.

Another important finding in this study is the relative contribution of sources of nosocomial HRV infection other than patient-to-patient transmission. In our situation, with either no HRV specific infection control guidelines or only if the patient is on specific high risk wards, approximately 80% of the hospital acquired HRV cases is due to introduction of HRV from outside the hospital. Family members of the patient and health care workers can carry HRV into the hospital, and transmit the virus by close contact with the

patient. Also, public areas dedicated to parents of admitted children could lead to transmission of the virus from one visitor to the other, thus putting also other patients than their own children at risk for infection. These findings strongly suggest that infection control policies should also take into account people surrounding the patient in order to reduce the prevalence of nosocomial infections. Although for other, mostly enteric, viruses persistence of the virus in the environment has a role in prolongation of an outbreak, the role of contaminated environmental surfaces in the transmission of HRV is not clear.[18] Infectious virus can be transferred from surfaces to fingertips, however a loss of infectivity is reported by 24 hours after deposition.[19] Education of visitors on the prevention of transmission of pathogens, stressing the importance of hand hygiene and the advice to retain from visiting the hospital when signs and symptoms of infection are present, are examples of the measures that could be taken. Also, health care workers should be educated about the necessity to stay at home while experiencing respiratory or influenza like illnesses. The role of HCW in nosocomial outbreaks has been described before.[20,21] A recent paper assessing the impact of impact of 2009 influenza A (H1N1) pandemic on health care workers showed that health care workers with influenza like illness often worked while ill.[22] Education and support of health care workers were described to be of critical value.

Our study has some limitations. Several assumptions had to be made because specific clinical and epidemiological data were lacking: we had no information on HRV positivity and infection control status of asymptomatic patients or patients with subclinical symptoms. Only when the physician suspected respiratory illness and decided to take samples for viral diagnostics, patients were included in the study. We assumed a period of infectiousness which is distributed equal to the length of coughing for disease episodes in the data, not for other symptoms. Also, a lot of patients were already in isolation because of other reasons (e.g. the presence of other respiratory viruses or multi drug resistant bacteria or because of immune suppression that made protective infection control measures necessary). We assumed that these isolation measures were sufficient to prevent transmission of HRV to others and that baseline infection control policies did not change over time. We have no information about the infection control status of so called susceptible, non-HRV infected patients. Some of these patients are probably in isolation, which most likely reduces the probability of them acquiring HRV infection. Because this information is not available, the model did not take into account these different probabilities of acquiring nosocomial HRV infections of susceptible patients. The model is based on epidemiological and patient transfer data from our hospital. Therefore, the results are dependent on our particular patient population and type of hospital.

In conclusion, infection control measures reduce the number of hospital acquired HRV cases. However, because of the importance of introduction of HRV from outside the hospital and the delay in implementation of appropriate infection control measures after a patient becomes infected, a considerable amount of hospital acquired HRV infection will persist despite effective infection control policies. All measures attempting to reduce this delay are highly recommended and directly influence the outcome of infection prevention. Infection control measures should also focus on people surrounding the patient, not only on the infected patient itself. Besides, strategies should be developed in which highly sensitive (molecular based) and rapid point-of-care diagnostic tests play an important role.
Combining epidemiological methods and mathematical modeling techniques improve the understanding of nosocomial HRV transmission dynamics, which contributes to the implementation of appropriate infection control interventions.

Aknowledgements

J. Arends, MD., medical microbiologist at the University Medical Center Groningen, is greatly acknowledged for providing patient transfer data.

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Abstract

Background

Sequence based information is increasingly used to study the epidemiology of viruses, not only to provide insight in viral evolution, but also to understand transmission patterns during outbreaks. However, sequence analysis is not yet routinely performed by diagnostic laboratories, limiting its use in clinical practice.

Objectives

To describe the added value of sequence based information available within 3 days after the detection of norovirus in fecal samples of patients and personnel during a suspected outbreak on a hospital ward. Results were used to guide the implementation of appropriate infection control measures, in particular closure of the ward.

Study Design

Observational study

Results

Norovirus infection was detected in seven patients and two health care workers on an oncology ward of the children's hospital. Six of seven patients had a hospital acquired infection defined as a first day of illness more than two days after admission. After notification of the first two patients, supplementary infection control measures were taken to prevent further spread. Despite these measures, three additional patients with norovirus infection were identified. Characterization of the noroviruses of 5 out of 7 patients was available within 7 days after the notification of the first patient. Four different genotypes were detected, providing evidence for multiple introductions of different norovirus strains with only a few secondary cases rather than ongoing nosocomial transmission. Therefore, we maintained the already implemented infection control interventions without closure of the ward.

Conclusions

Sequence based information available in real-time is helpful for understanding norovirus transmission in the hospital and facilitates appropriate infection control measures during an outbreak.

Background

In recent years, the use of sequence based typing techniques for viral surveillance has become more common. Virus characterization and typing may be used to explain clinical illness and guide treatment (as in resistance profiling).[1-3] Apart from this, sequence based information is increasingly used as a tool to define transmission routes in outbreaks[4-6]

Since most laboratories have not incorporated sequence analysis in their daily routine testing, information on typing and characterization of viruses is mostly available retrospectively and useful in understanding transmission of, on the basis of epidemiological data, suspected outbreaks.[4] Shortening the timeframe by which sequence based information becomes available during an actual outbreak might benefit the unraveling of nosocomial transmission and thus may guide the implementation of appropriate infection control measures. We describe a suspected norovirus outbreak on a hospital ward, where sequence analysis results were made available immediately after the detection of norovirus and enabled us to decide on the necessary measures to prevent further spread.

Objectives

To describe the added value of providing sequence based information in real-time, available immediately after detection of norovirus in fecal samples of patients and personnel during a suspected outbreak on an oncology ward of the children's hospital. Sequence analysis results were used to understand transmission routes and to guide infection control measures.

Study design

Study population

Fecal samples of all patients with gastrointestinal symptoms (diarrhea, vomitus) admitted on the oncology ward of the Beatrix Children's Hospital from January 14th until January 30th, were collected for the detection and characterization of norovirus. Personnel with gastrointestinal complaints were also asked to submit fecal samples. Only fecal specimen from patients or personnel with gastrointestinal symptoms were analyzed, no asymptomatic persons were included. First day of illness, date of admission to the hospital ward, sample date and date of discharge were collected for all norovirus positive patients. Patients who tested norovirus positive, were isolated and health care workers were advised to wear gloves and a gown during patient care. Additional measures consisted of emphasizing the importance of hand hygiene using soap and water or appropriate alcohol-based hand wash, extra cleaning of relevant surfaces on the ward using hypochlorite, providing information about norovirus and the risk factors for transmission to parents and caregivers, and cohorting patients and staff. Staff with gastrointestinal complaints were not allowed to work until symptoms had resolved.
Ethical approval of the institutional review board was not required.

Laboratory methods

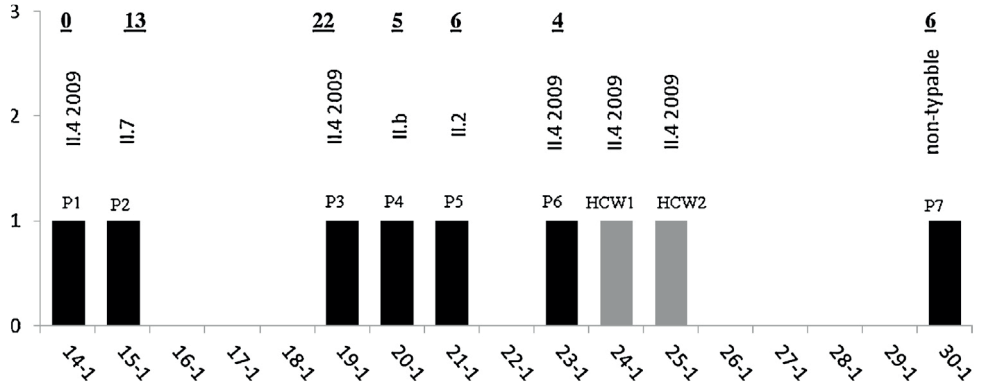
Stool samples were suspended in NucliSens easyMAG lysisbuffer (2x volume of feces, bioMérieux, Marcy l'Etoil, France). After centrifugation, 100 µl was used as input for RNA/DNA extraction using NucliSens easyMAG extraction buffer and magnetic silica (bioMérieux). cDNA was synthesized using 10 µl of extracted RNA in an iCycler thermocycler (Biorad, California, USA). All fecal samples were tested for the presence of seven enteric viruses (norovirus, rotavirus, parechovirus, enterovirus, adenovirus, bocavirus and astrovirus) using a multiplex real-time PCR as has been described elsewhere.[7]
All reactions were performed with Phocine Distemper Virus as an internal control in a total volume of 25 µl containing 6.25 µl 4x Fast Virus reactionmix (Life Technologies, California, USA), 30 pmol of each primer, 10 pmol of probe and 10 µl of genomic RNA template.[8] Characterization of norovirus was done by amplification and sequencing of a 285 nucleotide fragment of the ORF1 gene using an automated genotyping tool (www.noronet.nl).[9,10]

Multiple alignment was performed using BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium) and phylogenetic trees were constructed using the neighbor-joining method with complete deletion of missing data.

Results

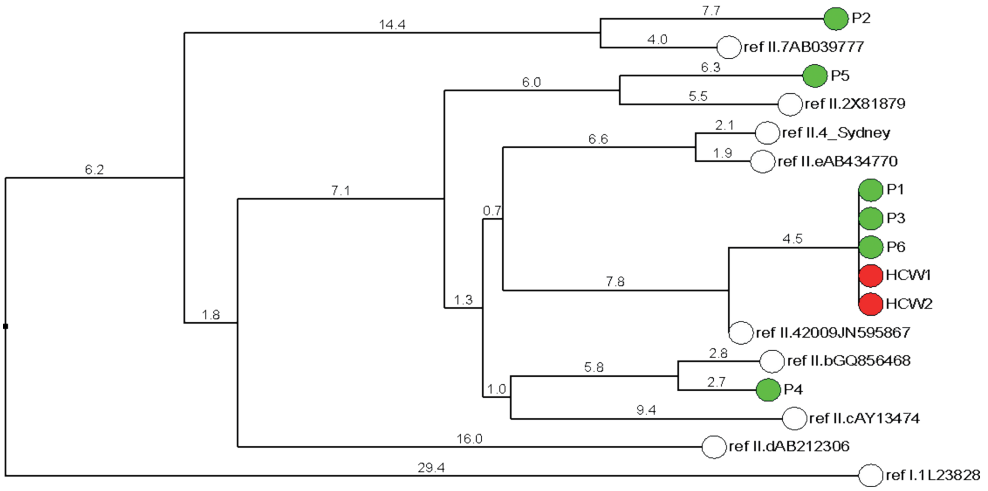
From January 14th till January 30th, norovirus was detected in seven patients and two health care workers (Figure 8.1). In six of the seven patients (P2-P7), norovirus infection was hospital acquired: gastrointestinal symptoms occurred between four and 22 days after admission. The first patient, P1, was admitted with a community acquired norovirus infection and was placed in isolation at the moment norovirus was detected in fecal material (one day after admission). After notification of the first two patients (P1-P2), the importance of hand hygiene (i.e. washing hands with water and soap) was stressed. Despite this, another three patients were detected with norovirus infection within three days (Figure 8.1, P3-P5). Meanwhile, gastrointestinal complaints in health care workers of the same ward and possibly also in caregivers became known. Because of the suspicion of ongoing norovirus transmission on the ward, additional infection control measures were taken: use of appropriate alcohol hand rubs, rigorous environmental cleaning, education of parents/caregivers, excluding ill staff from working and separating staff to infected and non-infected patients. After identification of the sixth norovirus positive patient (P6), the necessity of closure of the ward to stop further transmission was discussed. However, within half a day after detection of P6, sequence analysis results of the noroviruses detected in P1-P5 became available. Four different genotypes were found: norovirus genotype II.4 2009 (P1 and P3), genotype II.b (P4), genotype II.7 (P2) and genotype II.2 (P5). This strengthened us in our policy to maintain the infection control measures that were already implemented, without closing of the ward, despite the notification of patient P6. During the following days, norovirus was detected in fecal samples of two health care workers and one patient (P7). After patient P7, no additional patients or health care workers were found to be infected with norovirus.

Figure 8.1. Timeline of norovirus positive patients (in black, numbered P1-P7) and health care workers (in grey, HCW1-HCW2), based on date of sample collection. The different norovirus genotypes are given above each bar. The time after admission to onset of symptoms is depicted in bold numbers for each patient.



In addition to the sequence analysis results of the first five patients, we were able to characterize norovirus in patient P6 (genotype II.4 2009) and in both health care workers (both norovirus genotype II.4 2009) (Figure 8.2); the detected norovirus in patient P7 could not be genotyped. A cluster of norovirus II.4 2009 was found (three patients and both health care workers); the remaining patients were all infected with different norovirus genotypes

Figure 8.2. Neighbor Joining tree of the norovirus strains identified in this study. The positive patients are depicted in green (P1-P6), the health care workers in red (HCW1-2). Reference strains are named "ref" followed by the various norovirus genotypes.



Discussion

Our report of a norovirus pseudo-outbreak on an oncology ward in our children's hospital shows that results from sequence analysis, made available in real-time and immediately after the detection of norovirus, were essential for understanding the actual nosocomial transmission of norovirus, in a season when admission of different patients with norovirus occurs frequently. The sequence based information supported us in deciding on infection control measures to prevent further spread.

Norovirus is the most common cause of gastroenteritis worldwide and known for its ability to cause outbreaks, especially affecting health care institutions as nursing homes and hospitals.[11] It causes substantial morbidity and mortality in vulnerable people (the young, the elderly and immunocompromised patients).[12,13] In addition, norovirus outbreaks have significant impact on hospital resources, notably because of the necessity to close wards.[14-16]

Timely detection of norovirus with immediate implementation of appropriate infection control measures is one of the key factors in the containment of an outbreak.[15,17] The detection of seven patients with norovirus infection within two weeks on one oncology hospital ward should lead to rigorous infection control interventions, regardless of the norovirus genotypes. However, knowing that the rise in infections is in fact due to several introductions of different genotypes of norovirus instead of ongoing transmission of the same virus between patients, helps deciding on high impact measures like closure of a ward. Apart from this, rapid diagnostic tests for detection and characterization of pathogens help regulating adequate admission policies and provide the opportunity to act pro-actively in prevention of further spread within the hospital.

The technique of sequence analysis is commonly used in clinical laboratories, for example antiviral resistance profiling of HIV, but to our knowledge not many have incorporated characterization of viruses like norovirus into their standard diagnostic workflow. Costs, hands-on time of technicians and unknown clinical benefit are often used as arguments hampering the implementation of sequence analysis in the daily routine of diagnostic laboratories. In our laboratory setting, a workflow is implemented, which enables performance of sequence analysis routinely on a weekly basis. Students are trained to assist the technicians in this routine. As illustrated by this report, the potential benefits of having sequence based information available in real-time, should be taken into account when addressing only the costs of performing sequence analysis routinely; the closing of a ward is also expensive for a hospital. The developments in next-generation sequencing are moving fast forward, which also can make real-time sequencing become more feasible in the near future.

In conclusion, we have shown that adding real-time sequence based information to the classical epidemiological tool of clustering in place, time and person, provides essential information for understanding and controlling transmission routes of norovirus infections within a hospital.

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The benefit of molecular viral diagnostics

Molecular diagnostic techniques are a common tool within the daily routine in microbiological laboratories, especially in the field of clinical virology. [1,2] The potential benefits of molecular diagnostics are threefold. First, the use of molecular tests may benefit clinical management of a patient with signs and symptoms of an infection. By providing a rapid and accurate diagnosis, it has been hypothesized that unnecessary and additional laboratory testing and imaging can be prevented. If indicated, antibiotic treatment can be discontinued and proper antiviral therapy (if available) can be started which may all reduce the length of hospital stay. Secondly, the timely detection of a viral pathogen may benefit "in-hospital infection control": appropriate infection control measures can be installed in a timely manner in order to prevent nosocomial transmission. A third potential benefit of molecular diagnostic techniques is the opportunity to provide accurate epidemiological information to public health authorities through a more detailed surveillance of viral pathogens.
The presented studies in this thesis focus on the first two benefits of molecular diagnostic tests. Firstly, the value of molecular diagnostic tests in clinical management of the patient: the association of detected virus with clinical syndromes and the effect of finding a virus on clinical care. Secondly, the applicability and added value of molecular tests in in-hospital infection control to understand the transmission patterns of viral pathogens within the hospital, thereby supporting the timely implementation of appropriate infection control measures.

The benefits of molecular viral diagnostics for patient management

The advantage of molecular tests in explaining patterns of clinical illness was illustrated by the presented studies on Enterovirus 68 (EV68). EV68 is a member of the genus Enterovirus species D together with a small group of related viruses, like EV70 and EV94. In contrast to other enteroviruses, the biological properties of EV68 resemble those of human rhinoviruses. EV68 is acid labile and grows efficiently at an optimum temperature of 33°C, the temperature of the upper respiratory tract (chapter 3). Although until 2010 only sporadically detected worldwide, EV68 gained much interest in recent years because of its association with outbreaks of respiratory illness with varying severity, as illustrated by the description of an upsurge of infections of EV68 in the autumn of 2010 in the Northern part of the Netherlands in chapter 2. Although much of the clinical data associated with the detection of EV68 are based on observational studies in selected study populations, the virus can be regarded as an emerging respiratory pathogen, with a spectrum of clinical disease ranging from asymptomatic carriage to severe respiratory illness requiring hospitalization, with rare fatal cases (chapter 3). Also, the reviewed and our data suggest that the presence of a chronic respiratory disease might be a risk factor for EV68 infection.[3] The use of applied molecular diagnostic tests greatly enhanced the ability to detect EV68 in respiratory material, thereby facilitating the understanding of patterns of clinical illness observed by clinicians. Furthermore, the use of sequence analysis techniques made it possible to link clinical and epidemiological observations to distinct phylogenetic clustering of the virus in 2010 compared to 2009, suggesting sequence variation as a possible contributing factor to more and severe infections caused by EV68, as is shown in chapter 2. Interestingly, the global emergence of the virus was initially not recognized, but as a result of collaboration with colleagues from the CDC in the USA and from Japan, we were able to show the occurrence of the virus in different places of the world during a certain time period.[4]
Chapter 4 addressed the clinical and epidemiological features of infection with another important respiratory virus, the human rhinovirus (HRV), in hospitalized children in our university hospital. The development of sensitive and rapid molecular techniques markedly improved the detection rate of HRV, thereby also revealing a high genetic diversity with over 150 serotypes described so far.[5] The presented study in this thesis focused on the relationship between the detection of HRV and clinical symptoms,

the underlying chronic illness, patient management and nosocomial transmission patterns. HRV was the most frequently detected virus in respiratory samples from 644 patients with 904 disease episodes during a 15 month study period. In particular, HRV was detected in patients with chronic pulmonary illness (asthma, congenital pulmonary illness). These patients had significantly more complaints of coughing and shortness of breath and a higher need for oxygen, compared to children without HRV. All detected HRV were further characterized by sequence analysis, which revealed a predominance of species HRV-A and HRV-C over species HRV-B. No species-specific patterns of illness could be identified, although HRV-B was more often associated with a need for oxygen. This study showed that the detection of HRV can be associated with serious respiratory illness, especially in patients with a pulmonary underlying illness and underlines the additional value of molecular diagnostics in understanding disease patterns.
We also examined the influence of detecting HRV in patients with respiratory illness on clinical management, more specifically on the use of antibiotics. Regardless of the outcome of viral diagnostics, antibiotics were given in more than half of the patients. The prescription of antibiotics was strongly associated with the clinical diagnosis of pneumonia, and was neither influenced by the outcome of bacterial cultures nor detected viruses or the presence of underlying illness. These findings suggest that for the clinician, signs and symptoms may be more important in deciding on antibiotic therapy than patient characteristics and microbiological results.

Chapter 5 focused on a pandemic influenza virus. In 2009 a new variant of influenza A H1N1, called H1N1pdm09, rapidly spread around the world, resulting in the first influenza pandemic of the 21th century.[6] Historically, influenza in the immediate post-pandemic period has been associated with severe morbidity and mortality.[7] In the presented study a comparison between seasonal, pandemic and post-pandemic influenza is made in patients hospitalized in our medical center. Seasonal, pandemic and immediate post-pandemic influenza showed many similarities with regard to patients' characteristics, severity of illness and clinical outcome. Influenza in the post-pandemic period led to an equally severe influenza season in terms of number of patients admitted compared to the pandemic. Treatment with oseltamivir was much more frequently given during the pandemic and post-pandemic period, however, no increase in oseltamivir resistance of influenza A H1N1pdm09, as detected by molecular characterization of the influenza viruses, was seen. Although clinical and epidemiological data in patients with influenza virus suggested that the influenza virus itself was most probably the cause of respiratory illness, still more than two thirds of patients during all influenza seasons were treated with antibiotics.

The benefits of molecular viral diagnostics for infection control

The influenza A H1N1 pandemic also made clear the importance of a rapid, accurate diagnosis of influenza virus infection for preventing nosocomial transmission. Patients, health care workers or visitors infected with the influenza virus can spread the virus to vulnerable patients, causing nosocomial outbreaks with increased patient morbidity and mortality.[8,9] Appropriate and timely implemented infection control measures are necessary to prevent transmission of influenza virus within the hospital. The added value of characterization of the influenza virus by sequence analysis in the detection of clusters of patients with influenza is shown in chapter 6. By combining sequence based information with epidemiological data several clusters of nosocomial influenza virus infection could be identified, some of which would not have been detected based on available epidemiological data on their own.

To obtain knowledge on the nosocomial spread of HRV infections the same approach was used. In the prospective study among hospitalized children, almost a quarter of the HRV positive disease episodes were acquired when the patient was already in the hospital for several days because for other reasons (chapter 4). Phylogenetic analysis revealed several clusters of HRV with identical sequences; in more than

a quarter of these clusters epidemiological information was suggestive of intra-hospital transmission. Furthermore, although HRV-B was the least frequently found species in our study, almost 40% of HRV-B infections were acquired in the hospital, suggesting that the hospital environment is for some reason more favorable for transmission of HRV-B and that perhaps more stringent infection control measures could be necessary when HRV-B is detected.

We further theoretically explored the factors that drive nosocomial transmission of HRV by mathematical modeling (chapter 7). Data on HRV detection, clinical symptoms and infection control measures were retrieved from the prospective project into respiratory infections in hospitalized children and were used in an agent-based, multi-ward, stochastic mathematical model to assess the relative contributions of factors that drive HRV transmission dynamics: patient-to-patient transmission, introduction by visitors and health care workers and existing infection control measures. Based on model predictions, timely implementation of infection control measures (i.e. the moment the patient displays symptoms of respiratory infection) in combination of infection control policies which include HRV specific measures would reduce the number of hospital acquired HRV cases almost by half. However, still a considerable amount of hospital acquired HRV infections would exist because of introduction of HRV from outside the hospital via visitors or health care workers. For our hospital, up to 80% of the hospital acquired HRV cases observed during one year would be due to a source other than patient-to-patient transmission. Infection control policies should also focus on people surrounding the patient, not only on the infected patient itself. Besides, strategies should be developed in which sensitive (molecular based) and rapid point-of-care diagnostic tests play an important role. This study illustrates that combining epidemiological methods and mathematical modeling techniques improve the understanding of nosocomial HRV transmission dynamics, which contributes to the implementation of appropriate infection control interventions.

The use of sequence based data in the surveillance of (hospital acquired) viral infections and in infection control is not a common practice and is mainly used to characterize pathogens when an outbreak is already over in order to understand on hindsight the possible transmission routes, as is also shown in chapter 4 and chapter 6.[10-12] In chapter 8 the benefit of providing sequence based information in real-time, immediately after detection of a pathogen, is illustrated. In this chapter a norovirus pseudo-outbreak is described on a children's oncology ward. Rapid characterization of the detected norovirus in 5 out of 7 patients was available within 7 days after notification of the first patient. Four different genotypes were detected and this provided evidence for multiple introductions of different norovirus strains with only a few secondary cases rather than ongoing nosocomial transmission. This rapidly available information strengthened us not to close the hospital ward, but to maintain the already installed infection control measures. During the outbreak, gastrointestinal complaints in health care workers of the same ward and possibly also in caregivers became known, again stressing the importance of visitor- or healthcare mediated transmission in nosocomial infections. Adding real-time sequence based information to the classical epidemiological tool of clustering in place, time and person, provides essential information in order to understand and to control transmission routes of norovirus within a hospital setting.

Concluding remarks and future prospective studies

The outcome of a molecular test should, as with every diagnostic method, be translated into clinical signs and symptoms of the patient in order to determine whether a positive test clarifies the etiology of the illness of the patient. As developments in molecular diagnostics go rapidly, clinical data related to these tests are lagging behind. Clinical studies addressing patterns of disease and patients characteristics associated with the outcome of molecular diagnostics are necessary to fully understand the clinical value

of these tests. This is a common feature of every new technology or treatment option, but is seems that within molecular technologies, there are some hurdles to be taken. These studies should include data to answer the questions whether the use of molecular diagnostic methods improve patients outcome and whether the use of these techniques benefits the health care system (length of stay, drug prescription, infection control practices, costs).

An important factor in the optimal integration of molecular diagnostic tests in patient management is the role of the clinician. Effort should be put into the education of health care providers about the value and utility of available molecular tests. Results of clinical studies and cost effectiveness analyses should be shared with and disseminated to physicians. Also, the reasons for, for example, continuing empirically started antibiotic treatment despite an alternative viral diagnosis should be explored: is it a lack of knowledge on the clinical value of a molecular test, a lack of confidence that a viral diagnosis indeed explains the patient's illness or the lack of implementation of molecular tests in clinical guidelines?

Optimal integration of molecular tests in clinical care implies that results of these tests should be known the moment the patient is cared for. The ability to provide results 'in real time' maximizes the impact of molecular tests on clinical care and infection control.[13] Easy to use tests which can be performed on demand with short turnaround time without losing sensitivity and specificity are needed for the optimal integration of molecular technologies in clinical care (like for instance with the currently used GeneXpert® or Filmarray® assays). Equally important is probably the value of molecular tests in excluding viral infection and disease, thereby focusing clinical care and preventing the implementation of unnecessary infection control practices. Rapid test results with high positive as well as high negative prognostic value are needed in order to be able to predict on a more solid basis which care is needed for whom.

Rapid characterization of viruses provides information on epidemiological linkage between patients, not on hindsight but during clinical care. As such, sequence based information is not only useful in gaining insight in transmission routes, but also serves as input for actual infection control policies. This creates the possibility to focus human and material resources in a more efficient way. However, to maximize the impact of real time sequencing on in-hospital infection control, information on circulating strains of viruses outside the hospital is necessary: this enables analysis of possible transmission routes and clusters of infections within the hospital against a known background of circulating virus types. Patients travel along networks of health care associated institutes, moving to and from homes for (elderly) care, regional and university hospitals and rehabilitation centers, carrying their different viruses with them. Collaboration and data sharing between regional laboratories are necessary to gain insight on circulating types of viruses and to use this information for unraveling transmission patterns on a regional scale. We call this initiative in our region REGIOTYPE, which compares to (inter)national networks like VIRO-TYPENED and Noronet.[14,15]

Much of the discussions about the integration of molecular diagnostic tests in patient management and infection control is dominated by costs. The benefits and thus cost savings for patient management and infection control are insufficiently taken into account. Cost effectiveness studies are currently needed to properly address the impact of a timely and accurate diagnosis on all aspects of patient care: do the benefits of molecular techniques in clinical care and infection control outweigh the costs of performing the tests (the "euro-hour concept": costs of molecular tests relative to the hours of unnecessary treatment or isolation measures waiting for test results compared to standard diagnostic procedures).

Although antiviral therapy for respiratory viruses is currently limited, much effort is put in the development of potent antivirals against picornaviridae, most notably rhinovirus and enterovirus.[16,17] These developments towards newly available antiviral therapy for highly prevalent viruses, like HRV, should be taken into account when cost effectiveness studies are being performed.

In conclusion, molecular diagnostic methods have gained a solid position in clinical virology. The value of a rapid, accurate diagnosis on patient management and infection control is becoming clearer in recent years. More data, especially resulting from cost effectiveness studies, are needed to overcome current questions regarding costs and clinical value of molecular tests in clinical virology. For this, many clinical and epidemiological data associated with results of molecular tests are needed. To enable an optimal integration of molecular diagnostic techniques in patient management education of health care providers on the proper use and interpretation of these tests is crucial. As such, a "diagnostic stewardship" model could be developed aimed at the most accurate and cost effective diagnostic algorithms given clinical and epidemiological questions related to viral infections.

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Introductie

De diagnostiek naar virale infecties kent een relatief korte geschiedenis. Het duurde tot aan het einde van de 19e eeuw voordat het bestaan van virussen in een laboratorium werd aangetoond.[1,2] Het besef dat virussen levende cellen nodig hadden voor vermenigvuldiging en dat infectie van een cellijn met een virus een cytopathisch effect tot gevolg had, leidde tot het gebruik van celkweken bij de detectie van virussen in klinische materialen. Naast het feit dat niet alle virussen te kweken waren, duurde het vaak (te) lang voordat een resultaat bekend was. De ontwikkeling van snellere diagnostiek met behulp van specifieke antistoffen tegen virusantigeen maakte het mogelijk vaker een relatie te leggen tussen specifieke virusinfecties en klinische symptomatologie.[3,4] Bovendien werd het gebruik van specifieke antistoffen ingezet bij de zoektocht naar preventieve methodes om dodelijke ziekten als pokken te voorkomen: succesvolle vaccinatiestrategieën waren het gevolg. Aan de overtuiging dat (virale) infecties voorgoed tot het verleden behoorden, kwam begin jaren tachtig abrupt een eind met de ontdekking van het HIV-virus als verwekker van het immuun deficiëntie syndroom bij jonge homoseksuele mannen.[5] In dezelfde periode dat de HIV epidemie zich over de wereld verspreidde, deden moleculaire technieken hun intrede in de klinische diagnostische virologie. De ontdekking van de polymerase chain reactie (PCR), waarbij zeer kleine hoeveelheden genetisch materiaal gedetecteerd kon worden door exponentiële vermenigvuldiging, leidde tot een doorbraak in de diagnostiek van virale infecties.[6] Moleculaire technieken speelden een belangrijke rol in het ontwarren van de replicatiecyclus van HIV en in de ontwikkeling van anti-retrovirale middelen. Tevens bleek dat progressie van ziekte samenhing met de hoogte van de virale load in het bloed (en het CD4 + getal).[7] Het monitoren van de virale load met behulp van moleculaire technieken werd een belangrijk middel om prognose van ziekte te bepalen en het effect van therapie te vervolgen. Zo vormden moleculaire technieken niet alleen een middel om virussen te detecteren, maar werden ze ook een essentieel onderdeel van de patiëntenzorg.

Moleculaire diagnostiek van virale infecties

De afgelopen twintig jaar is er veel ontwikkeling geweest in moleculaire diagnostische methoden. Inmiddels zijn moleculaire testen een onmisbaar onderdeel geworden van de routine diagnostiek in een microbiologisch laboratorium, vooral op het gebied van de klinische virologie.[8,9] Door de hoge sensitiviteit en specificiteit van moleculaire testen vooral in het begin van klinische ziekte is de tijdige en adequate detectie van virale pathogenen verbeterd. Het ontwikkelen van multiplex PCR, waarbij meerdere targets in een test zijn geïncorporeerd, maakte het mogelijk bij specifieke klinische syndromen te kijken naar verschillende virale verwekkers. Naast de detectie van virussen zijn kwantitatieve moleculaire technieken belangrijk in het bepalen van progressie en prognose van ziekte. Het monitoren van de virale load en het bepalen van resistentie bij onvoldoende respons op adequate antivirale therapie is een belangrijk toepassingsgebied van moleculaire methoden. De meest recente ontwikkelingen gaan richting zogenaamde "point-of-care" testen: snelle, makkelijk te gebruiken moleculaire testen die dichtbij de patiënt kunnen worden uitgevoerd.

De technische ontwikkelingen rondom moleculaire diagnostische methoden gaan snel. Dit roept vragen op met betrekking tot de klinische toepasbaarheid van moleculaire testen: wat betekent een testuitslag voor de zorg rondom een patiënt, niet alleen voor wat betreft therapie en aanvullende diagnostiek maar ook voor wat betreft maatregelen ter voorkoming van verdere verspreiding van virussen. Om tot een optimale integratie van moleculaire testen in de patiëntenzorg te komen, moeten dergelijke vragen gericht op klinische toepasbaarheid beantwoord worden.

De voordelen van moleculaire virale diagnostiek

De voordelen van moleculaire diagnostische methoden zijn drieledig. Ten eerste kan het gebruik van moleculaire testen de zorg rondom patiënten met tekenen van infectie verbeteren. Een snelle en accurate diagnose van een virale infectie voorkomt de inzet van onnodige extra diagnostiek (laboratoriumonderzoek, beeldvorming), maakt het mogelijk empirisch gestarte antibiotische therapie te staken, eventueel antivirale therapie te starten en kan zorgen voor een verkorting van de ligduur in het ziekenhuis. Ten tweede maakt een tijdige detectie van een virus het mogelijk adequaat en snel maatregelen te treffen ten behoeve van infectiepreventie in het ziekenhuis om zo verdere verspreiding van een virus te voorkomen. Typering van virussen helpt om mogelijke transmissieroutes van virussen in het ziekenhuis te begrijpen, zodat infectiepreventiemaatregelen daar kunnen worden ingezet waar ze het grootste effect hebben in het stoppen van verdere verspreiding. Als laatste spelen moleculaire methoden een belangrijke rol in de openbare gezondheidszorg. Moleculaire technieken maken een meer gedetailleerde surveillance van virale pathogenen mogelijk. Ook zijn moleculaire technieken belangrijk bij de identificatie en detectie van nieuw opkomende virussen zoals MERS-Coronavirus.[10]
De gepresenteerde studies in dit proefschrift richten zich op de voordelen van moleculaire diagnostische methoden voor de directe patiëntenzorg en voor infectiepreventie bij respiratoire en enterale virusinfecties.

Voordelen van moleculaire diagnostische methoden voor de patiëntenzorg
De ontwikkelingen in moleculaire diagnostische testen hebben ertoe geleid dat een palet aan nieuwe virussen is ontdekt in respiratoir en fecaal materiaal. Voor sommige virussen wordt de relatie tussen detectie van het virus en klinische symptomatologie erkend. Voor andere virussen is de klinische betekenis nog onduidelijk en onderwerp van debat. Hoewel er steeds meer informatie beschikbaar komt over de relatie tussen de detectie van een virus en symptomen van ziekte, laten de meeste studies tot nu toe zien dat detectie van een virus niet leidt tot bijvoorbeeld het stoppen van empirisch gestarte antibiotische therapie. Naast de behoefte aan aanvullende studies naar klinisch relevante uitkomstmaten (mortaliteit, complicaties) is het van wezenlijk belang dat clinicus de betekenis van een positieve test in het verklaren van ziekte bij de patiënt leert begrijpen.

In dit proefschrift worden de voordelen van moleculaire technieken in het verklaren van ziekte beschreven aan de hand van studies naar Enterovirus 68 (EV68), rhinovirus (HRV) en influenzavirus.
EV68 behoort tot het genus Enterovirus groep D. In tegenstelling tot andere enterovirussen is EV68 zuur labiel en groeit optimaal bij een temperatuur van 33°C, de temperatuur van de bovenste luchtwegen. De biologische kenmerken van EV68 lijken daarmee erg op die van rhinovirus (hoofdstuk 3).
De detectie van EV68 is tot aan 2010 slechts sporadisch beschreven. De laatste jaren staat EV68 echter in de belangstelling als oorzaak van uitbraken van respiratoire infecties waarbij de ernst van de ziekte varieert van asymptomatisch dragerschap tot aan ernstige respiratoire ziekte met opname in het ziekenhuis als gevolg. Hoewel de meeste gegevens die tot nu toe gepubliceerd zijn, gebaseerd zijn op observationele studies in geselecteerde populaties kan EV68 als een opkomend pathogeen worden beschouwd. Door het gebruik van moleculaire testen is de detectie van EV68 in respiratoir materiaal vergemakkelijkt, waardoor het mogelijk wordt klinische ziektebeelden beter te begrijpen, zoals wordt geïllustreerd door de toename van respiratoire infecties door EV68 in het najaar van 2010 in het noorden van Nederland (hoofdstuk 2). Typering van EV68 met behulp van sequentie-analyse liet zien dat er sprake was van twee fylogenetische clusters, wat suggereert dat variatie in sequenties mogelijk heeft bijgedragen tot de plotse toename van EV68 infecties.

In hoofdstuk 4 worden de klinische en epidemiologische kenmerken van HRV infecties bij kinderen opgenomen in ons universitair medisch centrum beschreven. De detectie van HRV is sterk gestimuleerd door de ontwikkeling van gevoelige moleculaire testen, waarbij tevens duidelijk werd dat HRV een enorme genetische diversiteit kent met inmiddels meer dan 150 beschreven serotypes. In de in dit proefschrift beschreven studie is de relatie bestudeerd tussen de detectie van HRV en klinische symptomatologie, patiëntkenmerken en antibiotisch beleid bij kinderen. HRV was het meest frequent gedetecteerde virus in respiratoire materialen van 644 patiënten met 904 ziekte-episodes gedurende een periode van 15 maanden. De detectie van HRV was geassocieerd met de aanwezigheid van chronische longaandoeningen (astma, aangeboren longafwijkingen). Deze kinderen hadden significant meer klachten van hoesten en benauwdheid met daarbij een hogere zuurstofbehoefte vergeleken met kinderen zonder HRV. De gedetecteerde HRVs zijn getypeerd waarbij species HRV-A en HRV-C de meerderheid vormden. De data lieten geen verband zien tussen species en klinische symptomatologie, hoewel HRV-B meer geassocieerd was met verhoogde zuurstofbehoefte. Antibiotica werden in meer dan de helft van de patiënten voorgeschreven, ongeacht de uitkomst van virale diagnostiek. Het voorschrijven van antibiotica was sterk geassocieerd met de klinische diagnose pneumonie en hing niet samen met de uitkomst van de bacteriële kweek of de aanwezigheid van onderliggend lijden. Deze data suggereren dat voor de behandelaar klinische symptomen wellicht belangrijker zijn in de afweging om antibiotische therapie voor te schrijven dan microbiologische diagnostiek en patiëntkenmerken.

Hoofdstuk 5 heeft als onderwerp het pandemische influenzavirus. In 2009 verspreidde een nieuwe variant van influenza A H1N1 zich snel over de hele wereld, resulterend in de eerste influenza pandemie van de 21e eeuw. Historisch gezien wordt ook influenza in het eerste post-pandemische seizoen vaak gekenmerkt door een hoge morbiditeit en mortaliteit. De in dit proefschrift gepresenteerde studie vergelijkt de in ons universitair medisch centrum opgenomen patiënten met seizoensgriep (2007-2009), pandemische influenza (2009-2010) en post-pandemische influenza (2010-2011). Er werden veel overeenkomsten gezien in ernst van ziekte, patiëntkenmerken en klinische uitkomsten tussen de verschillende influenzaseizoenen. Het aantal patiënten dat moest worden opgenomen met influenza was in het post-pandemische seizoen gelijk aan het aantal tijdens de pandemie. Oseltamivir werd veel vaker gebruik tijdens de pandemie en het post-pandemische seizoen, maar leidde niet tot meer oseltamivir resistentie (gedetecteerd met behulp van sequentie-analyse). Hoewel klinische en epidemiologische data suggereerde dat het influenzavirus zelf de meest waarschijnlijke oorzaak was van de respiratoire ziekte, werd meer dan tweederde van de patiënten behandeld met antibiotica.

<u>Voordelen van moleculaire diagnostiek voor infectiepreventie</u>
De influenza A H1N1 pandemie liet ook het belang zien van een tijdige en accurate diagnose in het voorkomen van nosocomiale transmissie. Patiënten, medisch personeel of bezoekers met influenza kunnen het virus verspreiden naar anderen, waardoor nosocomiale uitbraken kunnen ontstaan met verhoogde morbiditeit en mortaliteit bij patiënten. Tijdige implementatie van adequate infectiepreventie maatregelen zijn nodig om transmissie van influenza virus te voorkomen. De toegevoegde waarde van sequentie analyse in de detectie van clusters van patiënten met influenza wordt beschreven in hoofdstuk 6. Door het combineren van epidemiologische data met informatie gebaseerd op sequentie analyse van gedetecteerde influenza virussen konden verschillende clusters van nosocomiale infecties worden geïdentificeerd. Sommige daarvan zouden op basis van alleen de epidemiologische data niet zijn gevonden.

Dezelfde aanpak is gebruikt om inzicht te krijgen in nosocomiale HRV infecties. In de in dit proefschrift gepresenteerde prospectieve studie onder kinderen opgenomen in ons ziekenhuis (hoofdstuk 4) bleek bijna een kwart van de HRV positieve ziekte-episodes in het ziekenhuis te zijn opgelopen. Op basis van

fylogenetische analyse konden meerdere clusters van identieke HRV sequenties worden aangetoond; in meer dan 25% van deze clusters was ook de epidemiologische informatie suggestief voor nosocomiale transmissie van HRV. Opvallend was dat, hoewel species HRV-B de minst frequent aangetoonde species was, bijna 40% van de HRV-B infecties in het ziekenhuis waren opgelopen. Dit suggereert dat de ziekenhuisomgeving om wat voor reden ook gunstig zou kunnen zijn voor transmissie voor HRV-B.

In hoofdstuk 7 zijn we dieper ingegaan op de factoren die nosocomiale transmissie van HRV kunnen beïnvloeden. De klinische en epidemiologische data uit de prospectieve studie onder kinderen dienden als input voor een stochastisch mathematisch model. Dit model werd gebruikt om de invloed van patiënt-op-patiënt transmissie, introductie van HRV door bezoekers en ziekenhuispersoneel, en infectiepreventie maatregelen op de hoeveelheid nosocomiale HRV infecties te bepalen. Berekeningen gebaseerd op dit model lieten zien dat infectiepreventie maatregelen, welke een combinatie waren van algemene en HRV specifieke maatregelen, zorgden voor een halvering van nosocomiale HRV infecties, vooral door een afname in patiënt-op-patiënt transmissie. Verdere reductie van het aantal nosocomiale HRV infecties zou te bewerkstelligen zijn door het zo kort mogelijk maken van de tijd tussen eerste symptomen van respiratoire infectie en start van de isolatie. Dit zou kunnen worden gerealiseerd door bijvoorbeeld de inzet van snelle point-of-care testen. Wanneer alle patiënten met een HRV infectie meteen bij de eerste ziektedag in isolatie zouden worden gelegd, zou echter nog steeds een aanzienlijk aantal nosocomiale HRV infecties ontstaan. Dit is toe te schrijven aan introductie van HRV in het ziekenhuis door bronnen anders dan geïnfecteerde patiënten zoals bezoekers en ziekenhuispersoneel. In ons ziekenhuis zou, gebaseerd op het model, circa 80% van de nosocomiale HRV infecties toe te schrijven zijn aan deze introductie van buitenaf. Dit maakt duidelijk dat infectiepreventiemaatregelen niet alleen gericht moeten zijn op de geïnfecteerde patiënt, maar dat de focus ook moet worden gelegd op bezoekers en ziekenhuispersoneel. Deze studie illustreert hoe het combineren van epidemiologische data met mathematische modelleringtechnieken het mogelijk maakt nosocomiale transmissie van virussen beter te begrijpen.

Het gebruik van op sequentie analyse gebaseerde informatie bij de surveillance van (nosocomiale) virale infecties en in infectiepreventie is nog geen gemeengoed. Het wordt tot op heden vooral gebruikt om achteraf te begrijpen hoe transmissie tijdens een uitbraak is verlopen, zoals in hoofdstuk 4 en hoofdstuk 6 wordt getoond. In hoofdstuk 8 laten we het voordeel zien van het leveren van op sequentie analyse gebaseerde informatie op het moment dat er een uitbraak is, meteen volgend op de detectie van in dit geval norovirus. In dit hoofdstuk wordt een norovirus pseudo-uitbraak beschreven op een kinderoncologie afdeling. Karakterisering van het virus was voor 5 van de 7 patiënten bekend binnen een week na de melding van de eerste patiënt. Vier verschillende genotypen werden aangetoond, wat suggestief was voor verschillende onafhankelijke introducties van buitenaf in plaats van voortgaande nosocomiale tranmissie van norovirus. Op basis van deze aanvullende informatie is besloten niet over te gaan tot het sluiten van de afdeling om verdere transmissie te voorkomen, maar om de al ingestelde intensieve infectiepreventiemaatregelen te handhaven. Tijdens de uitbraak werd bekend dat ook personeel en bezoekers gastro-intestinale klachten hadden, wat opnieuw het belang duidelijk maakt van de rol van personeel en bezoekers in nosocomiale transmissie van virussen. Het toevoegen van "real-time" typeringsinformatie aan de klassieke epidemiologische methode van clustering in plaats, tijd en persoon, levert essentiële informatie op voor een beter begrip en adequate controle van nosocomiale transmissie van norovirus infecties.

Conclusie en toekomstperspectieven

De voordelen van moleculaire diagnostische methoden zijn meer dan alleen detectie, kwantificering en karakterisering van een pathogeen. Moleculaire testen komen de patiëntenzorg ten goede en versterken infectiepreventie. Door een snelle en accurate diagnose helpen moleculaire testen bij het verklaren van ziekte. Dit kan het klinisch handelen rondom een patiënt positief beïnvloeden: juiste antivirale therapie kan worden voorgeschreven, onnodige antibiotische therapie kan worden gestopt en aanvullende laboratoriumtesten en diagnostiek kunnen worden verminderd. Daarnaast zijn moleculaire methoden van belang in het begrijpen van transmissie van virussen in het ziekenhuis, informatie die als input kan dienen voor adequate infectiepreventie.

Tot op heden zijn moleculaire testen nog niet optimaal geïntegreerd in de klinische patiëntenzorg en infectiepreventie. Inzicht krijgen in de factoren die een optimaal gebruik van moleculaire diagnostische methoden hinderen, is noodzakelijk.

Het resultaat van een moleculaire test moet, zoals bij elke diagnostische methode, vertaald worden naar de symptomatologie van de patiënt om te bepalen of een testuitslag de ziekte van de patiënt kan verklaren. De ontwikkelingen in de moleculaire diagnostiek gaan snel en klinische gegevens gerelateerd aan uitkomsten van moleculaire diagnostiek lopen hierbij achter. Klinische studies gericht op ziektebeelden en patiënt karakteristieken zijn nodig om de toepasbaarheid en waarde van de moleculaire diagnostiek ten volle te begrijpen en te benutten. In deze studies moeten ook gegevens verzameld worden die antwoord kunnen geven op de vraag naar de effecten van moleculaire diagnostiek op klinische uitkomstmaten, ligduur, het voorschrijven van antimicrobiële middelen en infectiepreventiemaatregelen.

Een belangrijke factor bij de optimale integratie van moleculaire diagnostische methoden in klinische patiëntenzorg is de behandelaar. Resultaten van klinische studies en kosteneffectiviteitanalyses moeten worden gedeeld met en verspreid onder clinici. Zij moeten uitleg krijgen over de waarde en de bruikbaarheid van moleculaire testen. Daarnaast moet worden onderzocht wat de redenen zijn voor het bijvoorbeeld continueren van empirisch gestarte antibiotische therapie ondanks de aanwezigheid van een alternatieve diagnose van virale infectie: is dit door een gebrek aan kennis over de klinische betekenis van een moleculaire testuitslag, een gebrek aan vertrouwen dat een virale diagnose inderdaad de ziekte van de patiënt verklaart of zijn moleculaire testen niet (goed genoeg) geïmplementeerd in klinische richtlijnen?

Optimale integratie van moleculaire testen in patiëntenzorg is alleen mogelijk als de uitslagen van deze testen bekend zijn op het moment dat de patiënt de zorg ontvangt. De mogelijkheid om resultaten "in real time" te genereren leidt tot een zo groot mogelijke impact van moleculaire testen op de klinische patiëntenzorg en de infectiepreventie. Makkelijk te gebruiken testen die random kunnen worden ingezet met een korte doorlooptijd, zonder dat dit ten koste gaat van sensitiviteit en specificiteit, zijn nodig om tot deze optimale integratie te komen. Dergelijke testen zijn niet alleen van belang in het aantonen van virale infecties; het uitsluiten ervan is waarschijnlijk net zo belangrijk. Dit leidt tot een meer gerichte klinische behandeling en voorkomt het instellen van onnodige infectiepreventie maatregelen. Het snel typeren van virussen levert informatie op over transmissieroutes tussen patiënten, niet achteraf, maar op het moment dat patiënten zorg ontvangen. Op sequentie analyse gebaseerde informatie is niet alleen nuttig om inzicht te krijgen in nosocomiale transmissie van virussen, maar is ook belangrijke input voor infectiepreventiebeleid, juist op het moment dat de patiënt nog in het ziekenhuis ligt. Dit maakt het mogelijk menskracht en materiaal efficiënt en effectief in te zetten.

Voor een juiste interpretatie van typeringsgegevens van virussen in het ziekenhuis is inzicht in en informatie over circulerende virusvarianten buiten het ziekenhuis noodzakelijk: zo kunnen clusters van identieke virussen in het ziekenhuis geanalyseerd worden tegen een bekende achtergrond van circulerende virusvarianten. Patiënten met hun verschillende soorten virussen verplaatsen zich langs netwerken van gezondheidszorginstellingen, van verpleeghuizen naar regionale ziekenhuizen, revalidatiecentra of universitaire centra en weer terug. Door deze zorgnetwerken is samenwerking tussen regionale laboratoria en het uitwisselen van gegevens noodzakelijk om inzicht te krijgen in de variatie aan circulerende virustypes en om deze informatie te gebruiken bij het doorgronden van transmissieroutes. Binnen onze regio noemen we dit initiatief "REGIOTYPE", als lokale aanvulling op het (inter)nationale TYPENED en Noronet netwerk.[11,12]

De discussie over het gebruik en de integratie van moleculaire testen in klinische patiëntenzorg en infectiepreventie wordt vaak gedomineerd door kosten. De voordelen, en dus kostenbesparingen, van het gebruik van moleculaire testen worden hierin vaak niet meegenomen. Om de impact van snelle en accurate moleculaire testen op alle aspecten van de patiëntenzorg goed te kunnen bepalen, zijn kosteneffectiviteitstudies noodzakelijk: wegen de voordelen van moleculaire testen voor patiëntenzorg en infectiepreventie op tegen de kosten van het uitvoeren van de testen (het "euro-uur" concept: de kosten van moleculaire diagnostiek ten opzichte van met de tijd dat patiënten (ten onrechte) in isolatie liggen of onnodige therapie ondergaan in afwachting van de testuitslag).

Hoewel antivirale therapie voor respiratoire virussen op dit moment beperkt beschikbaar is, zijn er veel ontwikkelingen vooral voor picornavirussen (rhinovirus, enterovirus).[13] Deze ontwikkelingen moeten worden meegenomen in kosteneffectiviteitanalyses, zeker wanneer het gaat om veel voorkomende virussen als rhinovirus met daardoor een hoge directe en indirecte ziektelast.

Concluderend kan worden gesteld, dat moleculaire diagnostische methoden een stevige positie hebben ingenomen in de klinische virologie. De waarde van een snelle, accurate diagnose voor de patiëntenzorg en de infectiepreventie in het ziekenhuis wordt de laatste jaren steeds duidelijker. Meer data, vooral gebaseerd op kosteneffectiviteitstudies, zijn nodig om vraagstukken rondom kosten en klinische betekenis van moleculaire diagnostiek in de klinische virologie te beantwoorden. Om tot een optimale integratie van moleculaire diagnostische methoden in de klinische patiëntenzorg te komen, is educatie van medisch personeel over het juiste gebruik van moleculaire testen en een juiste interpretatie van de uitslagen cruciaal.

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Curriculum Vitae

English

Janette Rahamat-Langendoen was born on the 26th of January 1970 in Delft, the Netherlands. She grew up in Naaldwijk and after graduating from high school, she went to the Erasmus University in Rotterdam to study medicine. After obtaining her M.D. degree in 1996 she obtained a residency in the Department of Obstetrics and Gynecology at the Erasmus University Medical Center. During that year she became interested in public health and decided to leave the hospital for the Municipal Health Services (GGD). She focused on the prevention and control of infectious diseases and became a consultant in communicable diseases in 2001. In 2004 she got the opportunity to join the Department of Epidemiology at the National Institute of Public Health and the Environment (RIVM), the Netherlands. Together with a few colleagues she was responsible for the early warning of threats caused by infectious diseases. To complete her knowledge on infectious diseases she decided to become a medical microbiologist and she started her training in 2008 at the University Medical Center Groningen. During the first months of her training she got the opportunity to start with her research at the Division of Clinical Virology, which resulted in this thesis, under the supervision of prof. dr. H.G.M. Niesters.

Dutch

Janette Rahamat-Langendoen is op 26 januari 1970 geboren te Delft. Ze groeide op in Naaldwijk en na het afronden van de middelbare school begon ze met de studie geneeskunde aan de Erasmus Universiteit in Rotterdam. In 1996 behaalde ze haar artsenbul en begon als arts-assistent niet in opleiding op de afdeling Gynaecologie en Verloskunde van het toenmalige Dijkzigt Ziekenhuis. Gedurende dat jaar groeide haar interesse voor de openbare gezondheidszorg en besloot ze het ziekenhuis in te ruilen voor de GGD. Ze richtte zich vooral op de bestrijding van infectieziekten en voltooide in 2001 haar opleiding tot arts infectieziektebestrijding. In 2004 kreeg ze de mogelijkheid te beginnen op de afdeling Epidemiologie van het Rijksinstituut voor Volksgezondheid en Milieu (RIVM). Samen met enkele collega's was ze verantwoordelijk voor de tijdige signalering van dreigingen veroorzaakt door infectieziekten. Om haar kennis met betrekking tot infectieziekten meer compleet te maken besloot ze arts-microbioloog te worden; in 2008 begon ze met haar opleiding in het Universitair Medisch Centrum Groningen (UMCG). Tijdens de eerste maanden van haar opleiding kreeg ze de mogelijkheid om binnen de sectie Klinische Virologie te starten met haar promotieonderzoek onder leiding van prof. dr. H.G.M. Niesters, wat heeft geleid tot dit proefschrift.

Dankwoord

Dit proefschrift was er niet gekomen zonder de hulp van een groot aantal mensen. Enkelen wil ik in het bijzonder bedanken.

In de eerste plaats mijn promotores prof. dr. H.G.M. Niesters en prof. dr. E. Hak.
Beste Bert, jij hebt de afgelopen jaren dit onderzoek mogelijk gemaakt; je denkt niet in belemmeringen maar in kansen, dat waardeer ik enorm! Het is prettig met jou samenwerken.
Beste Eelko, dank voor de epidemiologische ondersteuning en je snelle reacties op vragen en concept versies van artikelen.

Daarnaast wil ik dr. E. H. Schölvinck, co-promotor, bedanken. Beste Liesbeth, jouw inbreng vanuit de kliniek is van grote waarde geweest voor dit onderzoek, heel veel dank daarvoor!

Ik wil hier ook mijn dank uitspreken aan de leescommissie, prof. dr. G.J.J. van Doornum, prof. dr. A. Voss en prof. dr. A.L.W. Huckriede, voor de beoordeling van mijn manuscript.

Zonder de hulp en ondersteuning van de analisten van de sectie Klinische Virologie van het UMCG was dit onderzoek niet mogelijk geweest. Zelfs in het weekend zijn jullie bereid geweest materialen in het kader van mijn onderzoek te verwerken, heel veel dank daarvoor! In het bijzonder wil ik Renze Borger bedanken, voor het geduld en het meedenken gedurende het hele traject. En natuurlijk dank aan "onze" studenten: jullie bijbaantje, het doen van sequentie-analyses, heeft veel input voor dit onderzoek geleverd.

Caroline, ontzettend bedankt voor alles wat je hebt gedaan, jij wist altijd een oplossing voor kleine en grote praktische problemen.

In het algemeen wil ik de organisaties waar ik heb gewerkt en waar ik nu werk, danken voor de mogelijkheden en kansen die ik heb gekregen en nog steeds krijg. Ik waardeer dit zeer.

Tot slot, Imro, Juliëtte en Ivo. Imro, we hebben dit samen gedaan. Ik ben ongelooflijk trots op wat wij hebben gepresteerd, ik ben blij dat jij er bent. Juliëtte en Ivo, bedankt voor jullie begrip en relativerende woorden (zonder dat jullie er erg in hadden dat het relativerend was!), jullie zijn een bijzonder stel.

Notes

Notes