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Human influenza viruses have caused significant pandemics and epidemics throughout history and continue to be a relevant health problem in humans. These viruses express a remarkable genomic and antigenic plasticity due to high mutation rates and evade host immunity through rapid antigenic drift. Influenza clinical studies are thriving due to the availability of new powerful molecular diagnostic tools and the recent emergence of a novel pandemic strain and antiviral-resistant viruses. Molecular techniques are essential to increase our knowledge on virus characteristics, clinical manifestations, and host-pathogen interactions in a growing number of high-risk patients. In the next paragraphs, we summarize and discuss our findings on the implementation and clinical evaluation of different molecular methods intended for the rapid detection and genetic characterization of influenza virus infections and outbreaks in the clinical setting. We evaluated virus excretion duration, antiviral resistance development, and host immune responses in determining virus-associated symptoms and outcome in hematology-oncology patients.
Molecular diagnosis of respiratory virus infections in children

The studies in chapters 2 and 3 concern the clinical evaluation of influenza and other respiratory viruses in children using molecular diagnostic methods. Respiratory virus infections are the most important cause of morbidity in children worldwide [1-3]. Influenza signs and symptoms were previously defined using conventional diagnostic methods in hospitalized children and are therefore limited towards sampling and disease severity. New molecular tools provide the opportunity to re-evaluate the occurrence and clinical spectrum of different respiratory viruses including influenza viruses. In chapter 2, we used multiplex real-time PCR to investigate the relative incidence of respiratory virus infections in children sampled ≤48 hours of hospital presentation and evaluated virus-specific clinical correlations in young children. We confirmed the results of other recent molecular studies that showed a high incidence of respiratory viruses in 82% of children presenting to the hospital with ARTI [4, 5]. Among children with single virus infections, the relative incidence of influenza virus (10%) was lower compared to respiratory syncytial virus (43%) and human rhinovirus (33%) as described by others [4, 5, 9]. Influenza-like illness, defined as an acute febrile respiratory tract infection, was observed in ~90% of influenza cases and ~25% of other single virus infections. Our findings corroborate with previous studies that ILI is a poor predictor of laboratory-confirmed infection and varies during the course of the influenza season [6-9]. The accuracy of symptom-based influenza diagnosis was limited because other virus infections shared similar symptoms. Moreover, presenting symptoms could not differentiate between different virus infections due to common shared signs and symptoms, a high frequency of mixed viral infections and relative incidence differences. Low numbers and the relatively mild influenza presentations were probably due to a low virus activity during 2006 and 2007 winter seasons and do not allow conclusions to be generalized to other seasons. From the findings of our study, we conclude that PCR diagnostics are required to firmly establish a virus-specific diagnosis in the clinical setting and to guide antiviral treatment. Future molecular studies should re-evaluate the relative incidence and clinical evaluation of different respiratory viruses in a prospective study design using a more complete virus panel. Recent temporal clusters of acute flaccid paralysis and cranial nerve dysfunction associated with a newly recognized respiratory virus (enterovirus D68) serve as a clinical reminder that molecular diagnostics should include new respiratory viruses to fully comprehend the clinical impact of respiratory virus infections and to improve clinical management [10].
Severe influenza virus infections in children

The combined clinical descriptions in chapters 2 and 3 support the general knowledge that influenza infections among children in the hospital setting are often mild but that life-threatening events do occur \cite{11,12}. In chapter 3, we describe two children with severe influenza-associated encephalopathy (IAE), multiple organ failure and shock. Fortunately, this clinical entity is extremely rare but it remains poorly defined and not universally recognized. The children were infected with influenza A (H3N2) Fujian 2002 lineage virus drift variants (Fujian/411/02-like and Wyoming/003/03-like) which were associated with a high activity and frequent pediatric complications during the 2003-2005 seasons \cite{13,14}. The two children in this study had clinical manifestations that were similar to severe IAE cases in Japan \cite{15}. Previous studies report occasional detection of influenza RNA in cerebrospinal fluids but neuro-invasion remains controversial \cite{15– 18}. In an attempt to confirm or refute virus dissemination and replication in different organs, we performed pathological and molecular studies following parental permission for autopsy and developed a new M2 mRNA PCR as a molecular confirmation for viral replication. In this study, pulmonary pathology findings were compatible with primary viral pneumonia \cite{19} and active viral replication was confirmed by immunohistochemistry and M2 mRNA PCR. Similar pulmonary findings are reported to accompany other fatal cases of influenza-associated encephalopathy in Japan \cite{20,21}. In our case, the brain and other organs contained influenza RNA but lacked evidence of viral replication by immunohistochemistry and M2 mRNA PCR. The low-level presence of influenza RNA and absence M2 mRNA, suggested a residual deposition of genomic RNA from replication elsewhere (lungs). Influenza RNA in the brain may be the result of an increased permeability of the blood-brain barrier as described by others \cite{18}. Recent studies confirm that influenza RNA can be detected in the blood of patients with severe illness \cite{22,23}. The lack of virus replication in the brain implicates pro-inflammatory cytokines in the pathogenesis \cite{24}. Unfortunately cytokine diagnostics were not performed in serum or cerebrospinal fluid in our study. Accumulating evidence in literature indicate that elevated cytokines in serum and CSF (IL-6, IL-10, TNF-α, sTNF-R1 and IL-6) are correlated with onset and adverse outcomes of IAE \cite{25,26}. An ongoing inflammatory reaction will not sufficiently be contained by antiviral agents, therefore additional high-dose steroids and plasmapheresis may be considered in similar cases \cite{27}. Future studies should evaluate if virus RNA and high levels of cytokines in serum and CSF can serve as markers to predict clinical severity and outcome. Routine annual influenza vaccination is not offered to children in the Netherlands but may be considered during clinical relevant drift seasons.
Molecular detection of influenza outbreaks in nursing homes

Nursing homes continue to experience common influenza outbreaks that are associated with a high morbidity among elderly residents despite nationwide vaccination programmes [28, 29]. Rapid diagnostic influenza outbreak confirmation is highly important to implement timely infection control measures. Control measures include (re)vaccination of elderly and health care workers, active surveillance, transmission precautions and early antiviral treatment [30]. The study in chapter 4 evaluated different sampling techniques, logistical support and laboratory diagnostic methods to optimize diagnostic confirmation of influenza outbreaks in nursing homes. Nasopharyngeal swabbing was better tolerated, more practical and equally sensitive compared to nasopharyngeal washings and more sensitive than throat swabs (mean difference PCR cycle threshold value 4.7, \( P = 0.005 \)). The sensitivities of virus cultures (54%) and immunoassays (38% using nasopharyngeal swabs) were low compared to PCR. Sampling methods and symptom duration likely determined the sensitivity of immunoassays as described by others [31]. Immunoassays remain attractive for their convenience, speed and positive predictive value, but negative results always require PCR confirmation. A recent Norwegian study confirmed our findings that nasopharyngeal swabs are the sampling method of choice and provided new evidence that nylon flocked swabs could improve the sensitivity compared to rayon swabs (mean difference CT 2.3, \( P = <0.017 \) ) [32]. Outbreak team logistical support shortened diagnostic intervals but this remains to be confirmed by others. We now recommend the use of nasopharyngeal nylon flocked swabs, real-time PCR and logistical support for the rapid confirmation of influenza outbreaks in nursing homes. Remarkably, the majority of elderly influenza cases were vaccinated (82%) whereas the viruses always matched the corresponding vaccine strains. These findings are in line with other studies which show that influenza outbreaks continue to occur in nursing homes with high vaccine coverage rates and vaccine match [33–35]. A probable explanation for these ‘vaccine failures’ is that standard-dose influenza vaccines have a low effectiveness in elderly people (~45–70% protection) [29, 36]. Recent studies demonstrate that high-dose vaccines or mid-season boosting result in higher antibody responses, more protective titres and a 25% reduction of laboratory-confirmed infection [37, 38]. High-dose immunogenic vaccines may be considered among frail elderly people in nursing homes and new studies should confirm their immunological and clinical effectiveness. The exciting discovery of human broadly-neutralizing or Fc-effector mediated human antibodies that target the hemagglutinin stalk hold great promise for the future development of more universal influenza vaccines [39, 40].
**Prolonged influenza virus infection in the immunocompromised host**

Influenza antiviral treatment is associated with a lower risk of pneumonia and mortality among high-risk patients in the clinical setting\(^{[41, 42]}\). Until 2007, oseltamivir-resistant viruses were rare and deemed incapable of circulating due to compromised viral fitness and transmissibility\(^{[43-45]}\). The emergence of oseltamivir-resistant seasonal A (H1N1) viruses during the 2007-2008 season was of great concern and the origin remained unclear\(^{[46-48]}\). In chapter 5, we describe the results of a study to identify clinical sources of drug-resistant influenza viruses. Earlier studies reported anecdotal immunocompromised patients who developed drug-resistant viruses during therapy but these incidences were considered extremely rare\(^{[49-52]}\). During a 3-year period, we demonstrated that 8 adult hematology-oncology patients with lymphopenia manifested prolonged influenza virus excretion and frequent development of drug-resistant viruses (67% of eligible patients) in a single medical center. Complete viral clearance correlated with lymphocyte reconstitution. We hypothesized that immunocompromised patients with prolonged viral excretion due to (functional) lymphopenia often develop resistant virus. Later animal and human clinical studies now support this hypothesis\(^{[53]}\). Recent studies show that immunocompromised patients are more at risk to develop resistant viruses compared to immunocompetent patients\(^{[54, 55]}\). Large studies confirm that hematology-oncology patients often manifest high levels of prolonged virus excretion\(^{[56]}\) and develop resistant viruses in 45-58% of cases\(^{[52, 57]}\). We are concerned that antiviral resistance and viral LRTI are more common in hematology-oncology patients\(^{[58]}\). Independent risk factors for influenza LRTI include age, lack of (early) antiviral treatment, profound lymphopenia and HSCT donor mismatch\(^{[41, 59-61]}\). Future studies should further evaluate host risk factors predisposing for the development of drug-resistant viruses and viral LRTI. Our study findings confirm that antiviral resistance monitoring is important. New real-time PCR assays are now available to improve early diagnostics to guide antiviral treatment and clinical management\(^{[62]}\). Oseltamivir treatment seems to prevent viral LRTI\(^{[41, 42]}\) but antiviral protection is hampered by drug-resistant viruses\(^{[53, 63, 64]}\). Alternative treatment regimens using available drugs (high-dose oseltamivir, combined oseltamivir with inhaled zanamivir, and triple-combination antiviral drug) may raise the genetic barrier but appear to lack superiority to standard-dose oseltamivir\(^{[65-69]}\). We recommend that future studies should concern the development of new antiviral agents and alternative routes of drug administration (e.g. intravenous zanamivir). In addition, the clinical role of host immune responses remains to be elucidated.
Hospital transmission of oseltamivir-resistant influenza virus

Before 2008, it was assumed that oseltamivir-resistant influenza viruses could not circulate and cause relevant illness due to compromised transmissibility and attenuated pathogenicity. In chapter 6, we provide new evidence that H275Y oseltamivir-resistant A (H1N1) viruses readily transmitted between patients in a hospital setting. Later epidemiological studies confirmed a global spread of H275Y influenza A/Brisbane/59/2007-like antigenic drift variants which replaced the wild-type A/Solomon Islands/3/2006 virus. Previous assumptions that NAI-resistance invariably compromised virus fitness were no longer valid. A scientific explanation for this unabated spread of H275Y virus remained elusive until genetic studies demonstrated that permissive NA gene mutations (R222Q, V234M, D344N and D354G) restored deficient virus fitness by improving NA folding, surface expression and sialic acid affinity. In our study, H275Y virus retained significant pathogenicity in high-risk patients with lymphopenia who manifested viral LRTI (3 patients) and associated mortality (2 patients). Lymphopenia is a known risk factor for viral LRTI and mortality. Patient 4 manifested a remarkable relapse of viral LRTI and ARDS during sustained profound lymphopenia. Physicians should remain vigilant and may consider re-administration of antivirals to patients with sustained profound lymphopenia to prevent relapses of severe viral LRTI and ARDS. We underscore that antiviral susceptibility monitoring is important to guide influenza treatment and control. Unfortunately, oseltamivir treatment is hampered by drug-resistant viruses due to treatment failures. Inhaled zanamivir is effective for mild but not for severe influenza LRTI due to the risks of bronchospasms and clogging of ventilator tubes. The lack of alternative antiviral treatment options is of great concern and we recommend new clinical evaluations of investigational intravenous zanamivir and development of new antiviral agents and multidrug regimens. Our study describes the new clinical implementation of computational phylogenetic analysis using appropriate virus controls for the unequivocal molecular confirmation of oseltamivir-resistant influenza A (H1N1) virus outbreaks in the clinical setting. Later studies have used similar computational phylogenetic assays to confirm the emergence and spread of new oseltamivir-resistant influenza A(H1N1)pdm09 viruses in hematology-oncology wards in the USA and in the UK. Future antiviral resistance surveillance studies should monitor permissive and antiviral resistance mutations in the clinical and community setting.
Figure 1. Maximum-likelihood tree of concatenated HA and NA genes of A(H1N1)pdm09 hospital outbreak strains, unlinked clinical strains and community surveillance strains in Wales and the United Kingdom.

The tree was rooted on A/California/07/2009 and bootstrap values are displayed in brackets. Oseltamivir-resistant viruses are in bold marked with #. OT = oseltamivir treatment.

Source: adapted from [84].
Molecular surveillance of influenza A(H1N1)pdm09 virus

In chapter 7, we evaluated the accuracy of mass spectrometry-based comparative sequence analysis (MSCSA) to monitor virulence and oseltamivir-resistance markers in 70 surveillance specimens and 35 selected clinical specimens obtained during the 2009 H1N1 pandemic. MSCSA and Sanger sequencing results revealed a high concordance (nucleotides >99%, SNPs ~94%) and MSCSA may therefore be used to screen for influenza virulence markers. All surveillance specimens had wild-type virulence marker sequences in PB2, PB1-F2 and NS1 genes and stop codons in PB1-F2 and NS1 genes. Remarkably, PB2 gene lacked 627K or 701N mammalian signature changes that facilitate replication at low temperatures [85, 86]. Recent studies unveil that PB2 gene G590S and Q591R compensate for reduced polymerase activities [87]. Reverse genetics studies show that PB2-627K and PB2-701N do not increase replication and pathogenicity [88, 89]. Restored NS1 and PB1-F2 expression or PB1-F2-N66S mutation do not appear to alter virulence in the current genetic background [90, 91]. New mutations (PB2-T271A, PB2-H357N, PA-A36T, PB2-E158G and PB2-T558I) may increase polymerase activity, replication kinetics and pathogenicity and should be monitored [92-95]. We conclude that genetic surveillance should include new polymorphisms and should not rely on known virulence and resistance markers [87]. In our study, real-time PCR detected H275Y

![Figure 2. Emergence of V241I and N369K permissive mutations in circulating A(H1N1)pdm09 viruses.](source: adapted from [108].)
oseltamivir-resistant A(H1N1)pdm09 virus in 19/35 clinical specimens. MSCSA only detected H275Y in fully mutant virus populations (4/4) but not in mixed populations (0/15) and is not suitable to screen for resistance markers in the clinical setting. Other studies report similar anecdotal H275Y A(H1N1)pdm09 viruses [96–103] with a compromised fitness [75]. In 2010-2011, small H275Y community clusters appeared in the UK and USA among cases with no prior oseltamivir treatment exposure [104, 105]. Later epidemiological studies uncovered large widespread H275Y clusters in Australia [106] and Japan [107] caused by viruses with new permissive NA gene mutations (V241I, N369K) that emerged in 2010 (Figure 2) [108]. New permissive mutations are now present in >99% of circulating A(H1N1)pdm09 viruses and enhance NA gene expression/activity and restore H275Y virus replication/transmission fitness [108, 109]. Fortunately, wild-type viruses still appear to outcompete H275Y viruses (Figure 3) [107]. Epidemiological antiviral resistance monitoring is important and may uncover new relevant polymorphisms and unexpected large clusters [100, 107, 110]. Future studies may evaluate the use of improved next generation (deep) sequencing methods for whole-genome influenza sequencing using standardized data analysis pipelines [111–114].

**Figure 3.** Circulating H275Y virus was replaced by wild-type A(H1N1)pdm09 virus in Hokkaido, Japan. Source: adapted from [107].
Host immune responses dictate influenza outcome in hematology-oncology patients

In chapter 8, we evaluated the role of humoral and cell-mediated immune responses in determining A(H1N1)pdm09 virus symptoms and viral clearance in six hematology-oncology patients with prolonged viral excretion. The clinical role of host immune responses remains unclear since immune monitoring studies are lacking in humans. The results in chapters 5 and 8 suggest that prolonged viral excretion and viral clearance are correlated with T-cell lymphopenia and influenza virus-specific T-cell responses respectively. Humoral responses were not correlated with viral clearance since three patients manifested ongoing viral excretion during seroprotective HI titers. Our findings corroborate with virus challenge studies in healthy volunteers that correlated virus-specific T-cells with influenza host immunity and with human CMV studies that correlated virus-specific T-cells with viral clearance. CD4+ and CD8+ influenza virus-specific T-cell responses circulate for only ~30 days after infection and are associated with recent active infection. We evaluated host protective immune responses during prolonged influenza virus excretion. Clinical protection with sustained mild symptoms was associated with the presence of CD8+ T cells in two patients and with additional CD16+ FcIgG cell-mediated immunity in one case. Four patients developed severe viral LRTI during profound T-cell lymphopenia and (transient) absence of ADCC. More knowledge on protective immune responses is awaited to improve the clinical management of high-risk patients. New antiviral strategies may include favipiravir or NAI combination treatments but the clinical relevance is unclear. We further evaluated possible adverse innate and adaptive cell-mediated immune responses to elucidate correlates of viral ARDS immunopathogenesis during prolonged viral excretion. Previous studies suggested that T-cell responses are important determinants of influenza immunopathology. In this study, viral ARDS manifested during remarkable profound T-cell lymphopenia and coincided with innate cell reconstitution. These findings concur with recent animal models which show that innate cell recruitment elicit a viral cytokine storm and immunopathology. Recent studies show that severe human influenza infections manifest hypercytokinemia. New treatments using sphingosine analogs effectively temper immune pathology in animal models and may be promising in humans. We conclude that a wide range of host immune responses determine
influenza outcome and viral clearance. This study is limited by a low number of patients and we therefore recommend large immune monitoring studies to confirm the clinical role of different immune responses and to evaluate new treatment options.

Figure 4. The percentages of virus-specific CD8+ (a) and CD4+ (b) influenza (H1N1)pdm09 virus-specific T-cells in individual patients at different time points after infection onset.

Source: adapted from [119].
Conclusion

The studies described in this thesis demonstrate that influenza molecular diagnostic assays are indispensable in the clinical setting. Reverse-transcriptase PCR methods allow for the sensitive and accurate identification of single and mixed respiratory virus infections including human influenza viruses. New molecular diagnostic assays are helpful to explore and redefine relevant virus characteristics, clinical manifestations and epidemiology. Prospective studies are awaited to characterize and establish the relative incidence of respiratory virus infections including influenza virus infections in different patient groups and clinical settings. We demonstrate that improved diagnostic sampling, specimen logistics, and laboratory diagnostics can optimize the clinical management of individual patients and the rapid implementation of control measures. Our studies provide evidence that new molecular diagnostic assays can detect virus expression, resistance mutations and virulence markers, and that computational phylogenetics provide accurate and practical confirmation of virus outbreaks in the clinical setting. In this thesis we show that influenza-infected hematologoncology patients are at risk to develop antiviral-resistant virus during prolonged viral excretion and that resistant viruses are transmissible and retain pathogenicity. We performed human immune monitoring studies which suggest that T cells are important for clinical protection and viral clearance. Our study findings indicate that ADCC may provide important clinical protection and that influenza immunopathology is mediated by innate immune cells. Future studies should evaluate the clinical effectiveness and efficacy of high-dose influenza vaccines and new antiviral agents including favipiravir. The research and clinical development of hemagglutinin stem-only ‘universal’ influenza vaccines \[129, 130\], anti-HA stalk monoclonal-antibody treatments \[131\] and novel immunomodulating therapeutic approaches (e.g. sphingosine analogs) is highly anticipated \[127, 128\]. The results from this thesis demonstrate that molecular tools revolutionize influenza laboratory diagnostics and improve our clinical understanding of this continuously evolving virus. The inherent viral genetic variability and antigenic plasticity is a continuous incentive for new research to keep up with relevant mutations and to outsmart the virus.
References


