Jairo Gooskens

Molecular and Clinical Insights into Seasonal and Pandemic Influenza

Molecular and Clinical Insights into Seasonal and Pandemic Influenza

Jairo Gooskens

Colophon

Thesis The study described in this thesis was conducted at the Department of Medical Microbiology of Leiden University Medical Center in Leiden, the Netherlands. Research collaborations were established with the Department of Virology and the National Influenza Center at Erasmus Medical Center in Rotterdam, with the Public Health Services GGD Hollands Midden in Leiden and with the Section of Virology at the RIVM National Institute for Public Health and the Environment in Bilthoven.

Cover *The Stoclet Frieze* by Gustav Klimt (1862–1918) is a series of three mosaics. The mosaics 'Expectation', 'The Tree of Life' and 'Fulfilment' portray the passionate flow of interconnected human existence and collective intelligence. The swirling branches of 'The Tree of Life' express the beauty and perpetuity of the cycle of life. Gustav Klimt was made an honorary member of the Viennese Academy of Fine Arts in 1917. It is widely assumed that he was struck by the influenza epidemic or pandemic of 1918 after reaching artistic and creative fulfilment.

Design & print Ralph Boer (ralphboer@icloud.com)

ISBN/EAN 978-90-824257-1-0

Molecular and Clinical Insights into Seasonal and Pandemic Influenza

Proefschrift

ter verkrijging van
de graad Doctor aan de Universiteit Leiden
op gezag van Rector Magnificus prof. mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op woensdag 2 december 2015
klokke 15.00 uur

door

Jairo Gooskens
geboren 7 oktober 1974
te Oranjestad, Aruba

Promotiecommissie

Promotor	Prof. dr. A.C.M. Kroes
Overige leden	Prof. dr. L.G. Visser
	Prof. dr. G.F. Rimmelzwaan, Erasmus Universiteit Rotterdam
	Prof. dr. E. de Jonge
	Dr. E.C.J. Claas

Liefde is waar het allemaal om gaat.

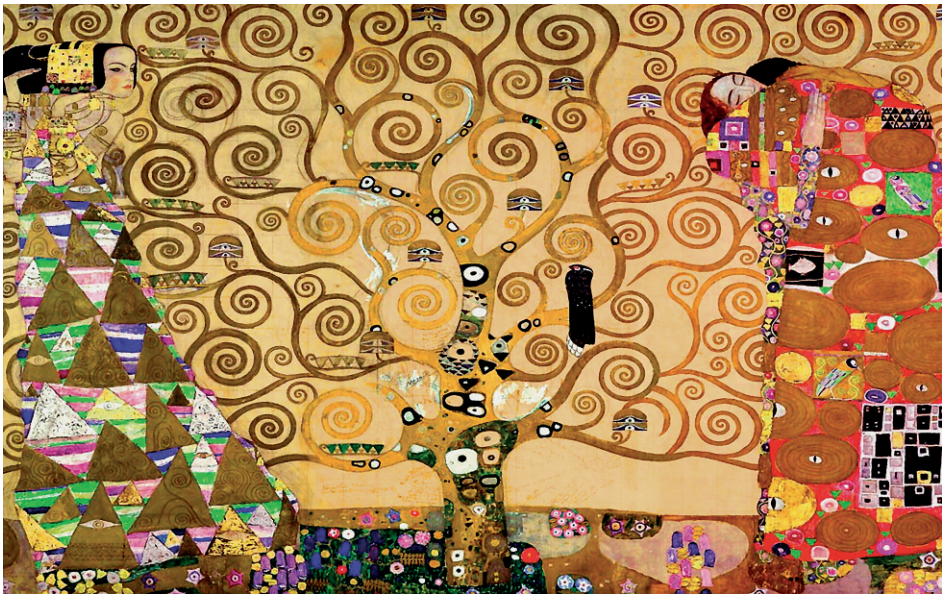
voor Sophia

Table of contents

Chapter 1	Introduction and outline of this thesis	**9**
Chapter 2	Clinical evaluation of viral acute respiratory tract infections in children presenting to the emergency department of a tertiary referral hospital in the Netherlands *BMC Pediatrics 2014; 14: 297*	**33**
Chapter 3	Severe influenza resembling hemorrhagic shock and encephalopathy syndrome *Journal of Clinical Virology 2007; 39: 136–40*	**51**
Chapter 4	Rapid molecular detection of influenza outbreaks in nursing homes *Journal of Clinical Virology 2008; 41: 7–12*	**63**
Chapter 5	Prolonged influenza virus infection during lymphocytopenia and frequent detection of drug-resistant viruses *The Journal of Infectious Diseases 2009; 199: 1435–41*	**77**
Chapter 6	Morbidity and mortality associated with nosocomial transmission of oseltamivir-resistant influenza A(H1N1) virus *JAMA 2009; 301: 1042–6*	**95**
Chapter 7	Mass spectrometry-based comparative sequence analysis for the genetic monitoring of influenza A(H1N1)pdm09 virus *PLoS One 2014; 9: e92970*	**107**
Chapter 8	Host immune responses dictate influenza A(H1N1)pdm09 infection outcome in hematology-oncology patients *Bone Marrow Transplantation 2015; Advance online publication.*	**123**
Chapter 9	General discussion	**135**
	Samenvatting (Summary in Dutch)	**160**
	List of publications	**167**
	Curriculum Vitae	**168**
	Acknowledgements	**169**

Chapter 1

Introduction and outline of this thesis



The early history of influenza epidemics

Epidemics of febrile acute respiratory tract infection (ARTI) have been known throughout recorded history as 'febris catarrhalis epidemica', referring to descriptions by Hippocrates in 412 BC [1]. Since the late 15th century, these epidemics were characterized as mild self-limiting illnesses of the upper respiratory tract which could progress to severe bronchitis or pleuritis with old age predisposing towards mortality [2]. In the 18th century, the French word 'la grippe' and Italian word 'influenza' became widely adopted in Europe. The etymology of 'influenza' lies with the description 'influenza di catarro', referring to medieval beliefs that unfavourable astrological or miasmic influences resulted in these disease outbreaks [3].

Accurate descriptions of influenza epidemics appeared in the 18th century. The rapid spread among all layers of society prompted Grant (1782) and Johnson (1789) to postulate airborne or contact transmission [1, 4]. Medics remained divided on the role of contagionism, until the germ theory was proven by Snow (1855), Pasteur (1876) and Koch (1890). A large influenza epidemic in 1889 received global media attention and was the first recognized pandemic in a modern connected world **(Figure 1)** [5]. Expeditious studies by Pfeiffer at the laboratory of Koch theorized that a bacterium *Haemophilus influenzae* was the etiologic agent. Ironically, the assumption did not fulfil Koch's postulates and a 'contagium vivum fluidum' was not considered since the 1889 pandemic predated the discovery of viruses in plants (Ivanovsky, 1892; Beijerinck, 1898), animals (Loeffler, 1898) and humans (Reed, 1901).

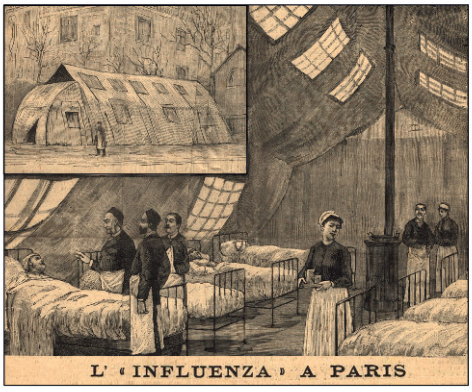


Figure 1. The 1889 influenza pandemic media reports predated modern photojournalism.
Source: adapted from Le Petit Parisien, Dimanche 12 Janvier 1890. Described in [5].

Virus discovery

The bacterium *H. influenzae* was discredited as the cause of human influenza infections by clinical and comparative pathology studies in the wake of the 1918 influenza pandemic [6]. Accumulating data indicated that an unknown agent caused influenza infection and a wide range of pneumonic bacterial co-infections [6–8]. High mortality rates during the 1918 pandemic renewed the expeditious pursuit of a causative agent [9, 10]. The search for a viral agent was fuelled by leading medical journals stating 'in the course of evolutionary processes there suddenly is liberated a form of infectious agent against which large numbers of people offer little or no resistance and which is transmitted readily from person to person' [11].

Berkefeld virus filters **(Figure 2)** were important to affirm that a 'contagium vivum fluidum' transferred influenza infection among birds, mammals and humans. Filter passing agents transferred fowl plague among chickens (1901, Centanni and Lode), 1918 pandemic influenza among humans (1918, Selter and Nicolle), and swine or human influenza among pigs and ferrets (1931, Shope; 1933, Smith) [12-16]. The work by Smith (1935) and Dochez (1936) provided clear evidence of a viral etiology by showing that virus filtrates of human influenza infection specimens could serially be cultivated in embryonated eggs or chick embryo tissue and cause influenza infection in ferrets and human volunteers [17-18]. The discovery of virus culture and hemagglutinin inhibition intensified scientific studies on influenza virus characteristics, clinical manifestations, epidemiology, vaccine development and host immunity [18, 19].

Figure 2. Berkefeld 'bacterial water' or 'virus' filter.
Source: adapted from [20].

Virus characteristics

The virus family *Orthomyxoviridae* consists of enveloped viruses with a single-stranded negative sense segmented RNA genome and include *Influenzavirus A, Influenzavirus B, Influenzavirus C, Isavirus, Thogotovirus* and *Quaranjavirus.* Human influenza infection is mainly caused by influenza A and B viruses. Influenza A viruses have the unique capability to exchange gene segments with avian and mammalian strains which allow new subtypes to emerge against which humans have no pre-existing immunity. The pandemic potential of a new influenza A virus is determined by its tropism for 2,6-linked sialic acid (SA receptor) expressed in the human respiratory tract required for efficient human-to-human transmission.

Influenza A virus has a 13kb genome with 8 segments which encode 14 proteins **(Figure 3)** [21-23]. Virus subtypes are based on 18 hemagglutinin (HA) and 11 neuraminidase (NA) surface glycoproteins which mediate sialic acid receptor binding or cleavage. Matrix protein (M1) provides envelope rigidity and aids assembly. Matrix transmembrane proteins (M2, M42) allow proton influx to uncoat virus particles during entry. Nucleoprotein (NP) forms structure complexes with viral RNA and polymerase enzymes (PB1, PB2, PA) modulate transcription and "cap snatching" of host cell mRNA. The nuclear export protein (NEP/NS2) mediates host cell transport of ribonucleoprotein complexes. PB1-F2 proteins mediate host cell apoptosis and nonstructural protein 1 (NS1) disrupts type I interferon antiviral signaling and antigen presentation. PB1-N40 and PA-X act as negative virulence regulators.

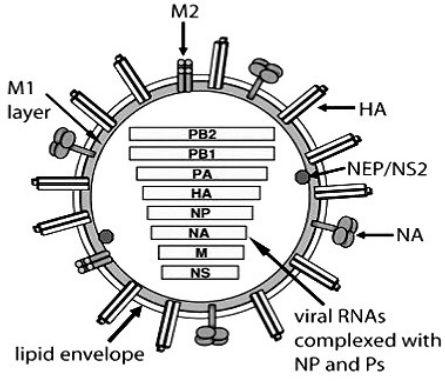


Figure 3. Influenza virus structure and genome segments.
Source: adapted from [24].

Laboratory diagnostics

Conventional influenza virus laboratory diagnostics include cell culture, antigen detection or serum hemagglutination inhibition tests (HI). Trypsin-based cell culture has low sensitivity and speed but is useful for virus subtyping and phenotypic antiviral susceptibility testing. Antigen detection by immunofluorescence or enzyme immunoassay (EIA) is fast but the sensitivity may be limited. Serum antibody detection by complement fixation test, enzyme-linked immunosorbent assay (ELISA) or HI **(Figure 4)** is useful to confirm recent influenza infection but not for rapid diagnosis.

Polymerase chain reaction (PCR) tests provide sensitive and specific detection of a wide range of respiratory viruses. Real-time PCR developed at Leiden University Medical Center provide a 100 to 1000 fold higher sensitivity compared to cell culture [25]. Sampling and logistical support may enable rapid diagnostic results to improve clinical management and outbreak control in health care settings. New molecular assays may also improve the detection of antiviral drug-resistance genes and virus strain subtyping [26].

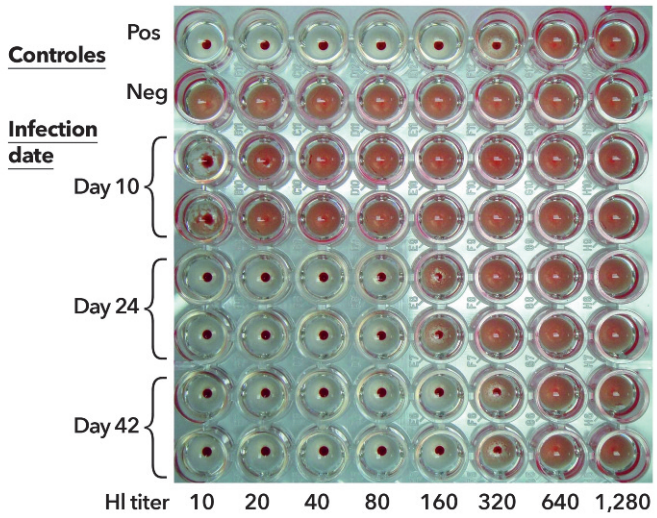


Figure 4. Serum HI tests confirm seroconversion against influenza A (H1N1) virus.
Source: adapted from [27].

Clinical spectrum

Influenza virus attachment and replication occurs in ciliated epithelial and goblet cells in the human airway tracts [28]. After a 2-day incubation period, most cases develop a 5-7 day mild and self-limiting upper respiratory tract infection (URTI) with fever, malaise and myalgia (Figure 5) [29]. Molecular studies confirm that virus excretion peaks on day 2 and decreases steadily until day 8 or 9 of illness [30-32]. Proinflammatory cytokines (eg IL-6) are associated with the development of systemic symptoms including fever and other mild systemic symptoms [33].

Influenza infection may be complicated by different types of pneumonia [34-37]. Rare severe primary viral pneumonia with an acute onset <5 days is poorly defined [34, 35]. Mixed or secondary bacterial and fungal co-infections can emerge after virus-associated host cell apoptosis of ciliated bronchial epithelial cells [34, 36, 37]. Other complications include viral lower respiratory tract infection (LRTI), acute respiratory distress syndrome (ARDS), non-pulmonary organ involvement (e.g. brain, kidneys) and cardiovascular events [38, 39].

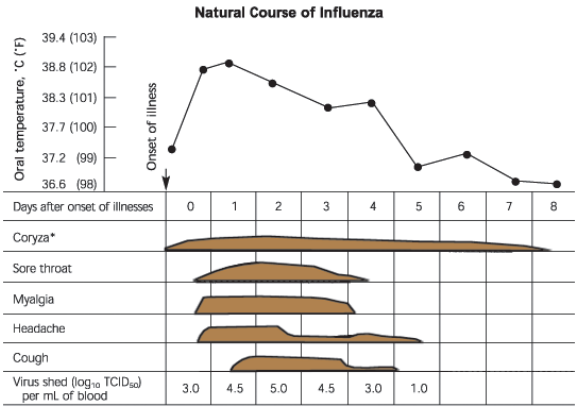


Figure 5. The natural course of uncomplicated influenza ARTI.
Source: adapted from [29].

Influenza pandemics and seasonal epidemics

Influenza A virus pandemics are the result of antigenic shift and occur ~3 times each century. Recorded pandemics include 1889 H3N8, 1918 H1N1, 1957 H2N2, 1968 H3N2 and 2009 H1N1 **(Figure 6)** [40-43]. High mortality rates during the pre-antibiotic era compared to later pandemics (1957 onwards) underscore the importance of antibiotics and developed health care systems. Extreme mortality rates during the 1918 pandemic **(Figure 7)** remain enigmatic and were probably due to a low pre-existing immunity in humans and high virulence [44, 45]. Current seasonal epidemics are caused by A/H1N1pdm09, A/H3N2, B/Yamagata and B/Victoria viruses. Annual recurrence is caused by frequent antigenic variation and host immune evasion due to mutations acquired during replication with a low-fidelity RNA polymerase. Excess morbidity and mortality is a hallmark of influenza epidemiology and supports annual virus surveillance and vaccination of high-risk patients [46].

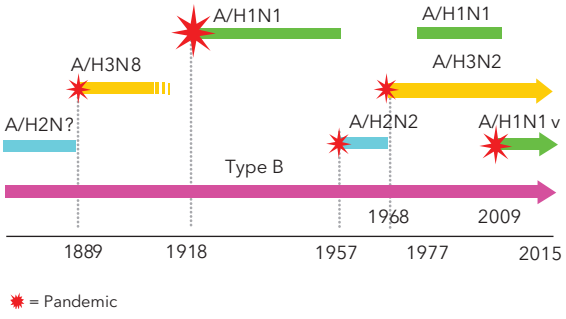


Figure 6. Influenza pandemics and epidemics.
Source: adapted from [43].

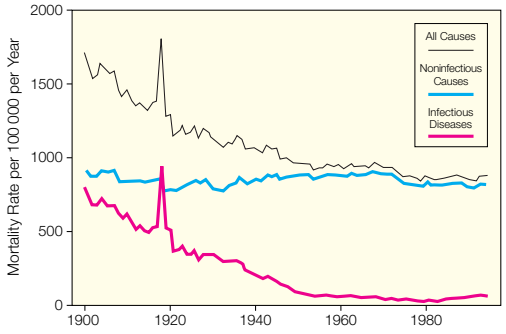


Figure 7. Extreme mortality rates during the 1918 pandemic.
Source: adapted from [44].

Virus surveillance

In 1947, the World Health Organisation (WHO) launched global influenza surveillance plans 'to collect and share information on epidemics, strain type and vaccine composition' [47]. National surveillance networks weekly assess influenza-like illness reported by primary health care providers to monitor virus activity and to collect specimens for virus surveillance **(Figure 8)** [48]. The WHO now promotes hospital-based surveillance to assess clinical relevant virus changes to improve prevention and control measures [49–51]. Annual surveillance is important for the genetic and antigenic characterization of circulating influenza viruses to detect new antigenic clusters and subtypes with pandemic potential. Antigenic clusters that are genetically divergent at key epitopes are selected for vaccine composition **(Figure 9)** [52]. Molecular tools are increasingly used to monitor virus epidemiology, genetic divergence, antiviral drug-resistance and virulence genes [53].

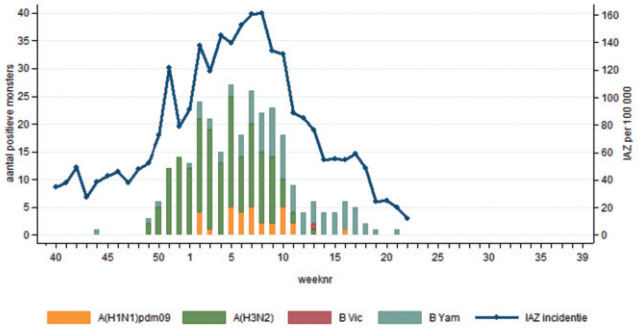


Figure 8. The Dutch epidemiology of influenza-like illness during the 2014/2015 season.
Source: adapted from [48].

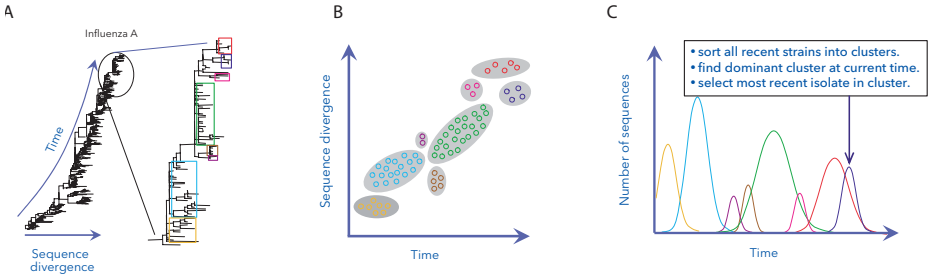


Figure 9. Influenza virus sequence divergence (A) and vaccine strain selection (B, C).
Source: adapted from [52].

Antiviral treatment

Three classes of influenza antiviral agents exist. Adamantanes (amantadine and rimantadine) are M2 channel blocking agents that are now obsolete due to poor effectiveness and common resistance. Neuraminidase inhibitors (NAIs: oseltamivir, peramivir, zanamivir) are effective agents that block the NA enzyme and prevent host cell viral release **(Figure 10)** [54, 55]. A new viral RNA polymerase inhibitor (favipiravir) is not yet approved but is a promising candidate for influenza combination therapy in patients who are most at risk [56, 57].

NAIs initiated <48 hours of onset reduce symptoms by ~1 day in healthy adults [58]. NAIs lower the risk of LRTI in healthy adults but the efficacy is low as the numbers needed to treat are high [59, 60]. Non-randomized observational studies show that a 5-day NAI treatment lowers the risk of LRTI and mortality significantly in hospitalized and immunocompromised patients [61-68]. Severe influenza pneumonia is associated with higher virus levels compared to mild infection and ratifies an extended NAI treatment duration [69].

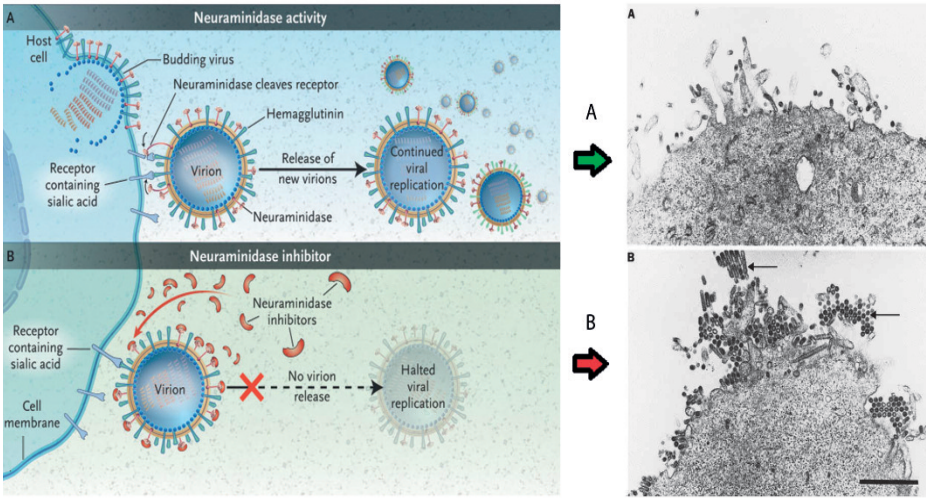


Figure 10. Schematic view and electron micrographs showing continued viral replication during absence of NAIs (A) and halted viral replication during presence of NAIs (B).
Source: adapted from [54, 55].

Antiviral resistance and permissive mutations

Influenza resistance mutations can emerge during antiviral treatment [70]. Oseltamivir and zanamivir resistance mutations may encode highly reduced inhibition (HRI) (>100 fold) or reduced inhibition (RI) (10-100 fold) IC_{50} over wild-type virus (Table 1) [71]. NAI resistance mutations modify the NA active site involved in cleavage of sialic acid binding structures. Fluorometric assays can measure virus NA activity and inhibition by oseltamivir carboxylate and zanamivir to determine IC_{50} fold-changes and phenotypic susceptibility [72]. NA gene active site modifications were deemed unlikely to emerge due to compromised viral fitness. In 2007, NA gene H274Y mutated oseltamivir-resistant A/Brisbane/59/07 (H1N1) virus emerged and circulated dominantly (Figure 11A) [73]. Emergence of permissive mutations restored deficient NA folding, surface expression and sialic acid affinity and accommodated the resistance mutation (Figure 11B) [74-76]. Oseltamivir-resistant A (H1N1) virus became extinct in 2009 and was replaced by wildtype A(H1N1)pdm09 virus.

Table 1. Emergence of influenza A virus NAI resistance mutations in the clinical setting.

Influenza subtype	NA mutation	Phenotype in NA inhibition assays	
		Oseltamivir	Zanamivir
A(H1N1)	H274Y	HRI	S
	Q136K	S	HRI
A(H1N1)pdm09	N294S	HRI	S
	H274Y	HRI	S
	I222R	RI	RI
A(H3N2)	N294S	HRI	S
	R292K	HRI	RI
	Q136K	S	RI
	E119V	HRI	S

HRI, highly induced inhibition; RI, reduced inhibition; S, sensitive. Source: adapted from [71].

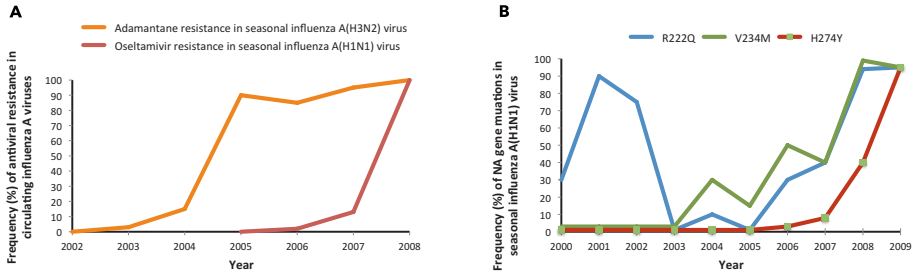


Figure 11. Emergence of influenza antiviral resistance (A) and A(H1N1) virus permissive mutations (B). Source: adapted from [73, 76].

High-risk patients

Influenza-infected high-risk patients are more prone to develop severe complications and adverse outcomes [77]. High-risk patients include the elderly, aged ≥65 years **(Figure 12)** and patients with pre-existing chronic cardiac or pulmonary conditions, cardiovascular disease, diabetes mellitus, renal disease, immunosuppression, and obesity **(Table 2)** [78, 79]. During the 2009 H1N1 pandemic, extreme obesity (BMI ≥40) was recognized as a new independent risk factor for the development of influenza complications [80]. Elderly people aged ≥65 years are at risk to develop influenza complications due to frequent co-morbidities and a functional decline of the adaptive immune system (immunosenescence). The annual influenza mortality rate is estimated at a minimum of 1 in 1500 elderly persons [81]. Nursing home residents are particularly at risk to develop associated morbidity and mortality [82]. Frequent severe influenza outbreaks in nursing homes demand effective preventive and control measures and early diagnostics to guide early clinical management [83].

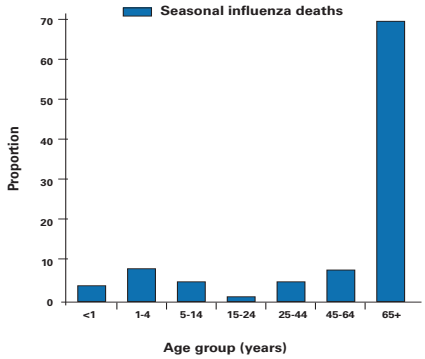


Figure 12. Age distribution of seasonal human influenza mortality in the human population.
Source: adapted from [78].

Table 2. Increased mortality in high-risk patients with influenza A (H1N1)pdm09 virus infection.

Charateristic	Nonsevere outcome, no. (%) of patients *n = 1171*		Death			
			No. (%) of patients *n = 72*		RR (95% CI) *	
Pre-existing heart disease	80/1049	(7.6)	16/65	(24.6)	3.5	(2.1–5.9)
Diabetes mellitus	82/1047	(7.8)	13/65	(20.0)	2.7	(1.5–4.7)
Renal disease	51/1044	(4.9)	7/64	(10.9)	2.2	(1.1–4.7)
Immunosuppression	93/1041	(8/9)	17/64	(26.6)	3.3	(2.0–5.5)
Lung disease (including asthma)	309/738	(41.9)	30/52	(57.7)	1.8	(1.1–3.1)

Source: adapted from [79].

Vaccination

Influenza viruses frequently mutate and evolve into phylogenetic and antigenic variants [84]. Annual subtype dominance varies but A (H3N2) viruses appear more common due to more common mutations and antigenic variation (Figure 13 A, B). Multiple observational studies show that vaccines lower the risk of influenza infection, hospitalization and mortality in high-risk patients but confirmatory randomized controlled trials are lacking [85-89]. Vaccines lower the risk of laboratory confirmed influenza in community-dwelling elderly [87-89], and of pneumonia and associated mortality in institutionalized elderly [90]. Serum HI titers ≥40 after vaccination provide ~50% (up to 70%) clinical protection against homologous virus infection and are traditionally considered seroprotective (Table 3) [91, 92].

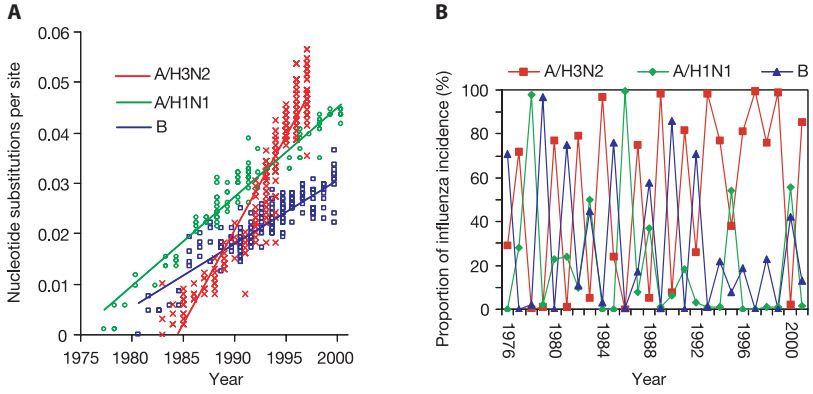


Figure 13. Influenza virus evolution and genetic changes (A) with subtype dominance (B).
Source: adapted from [84].

Table 3. Relationship between serum HI titers and influenza virus infection in volunteers.

	Titre of serum HI antibody to A/Scotland/74 (H3N2) virus					
	≤10	20-30	40-60	80-120	160	Total
Number of volunteers	19	9	7	16	24	75
Number of infected *	14	3	2	1	1	21
Infection (%)	74	33	29	6	4	28

* Infection proved by vrus isolation 3 days after infection and/or a fourfold or greater rise in serum HI antibody

Source: adapted from [92].

Host immune responses

Innate and adaptive immune responses are important for influenza infection control **(Figure 14, 15)**[93, 94]. Host Toll-like receptors, RIG-I receptors and NLRP3 recognize virus pathogen-associated molecular patterns (PAMPs) and activate interferon (IFN) responses[93, 95]. Infected epithelial cells produce CCL2 that attracts monocytes, natural killer (NK) cells and memory T-cells. T cells, macrophages, NK cells, neutrophils limit viral spread by destruction of infected cells or phagocytosis and produce proinflammatory cytokines (e.g. IL-6, TNF-α). Adaptive immune responses and memory development result from antigen presentation by dendritic cells[93, 96]. Plasma B cells produce virus-specific antibodies whereas T cells regulate immunity and virus-infected cell killing. The precise role of innate and adaptive host immune responses in determining symptoms and viral clearance is unclear.

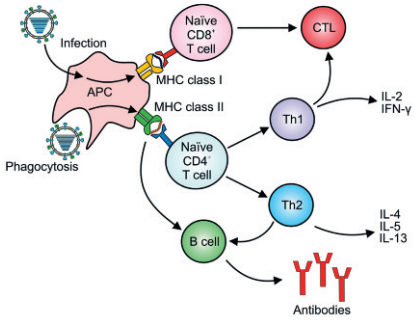


Figure 14. Induction of influenza humoral and cell-mediated adaptive immunity.
Source: adapted from [93].

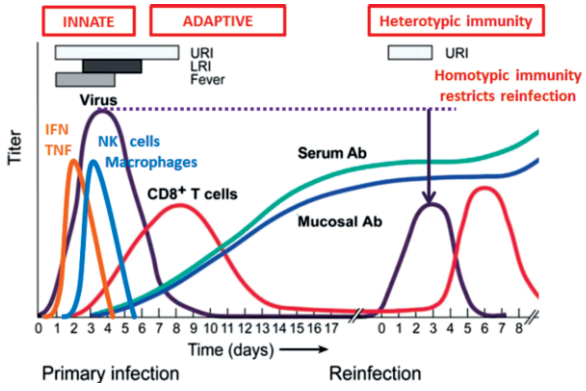


Figure 15. Host immune responses during influenza virus infection.
Source: adapted from [94].

Hematology-oncology patients with influenza

Influenza-infected hematology-oncology patients are more likely to develop LRTI compared to other immunocompromised patients [97]. Risk factors for viral LRTI include age, lack of (early) antiviral treatment, profound lymphopenia and hematopoietic stem cell transplantation (HSCT) donor mismatch [98, 64-66]. Severe immunodeficiency is associated with high level prolonged virus excretion and with frequent development of resistant virus during antiviral treatment **(Figure 16)** [99-103]. Hematology-oncology patients develop a wide clinical spectrum ranging from mild to severe virus-associated symptoms [104, 105]. Adverse outcomes often occur early after HSCT [98]. Immune monitoring studies are awaited to elucidate the role of host immune responses in determining virus-associated symptoms and viral clearance among an expanding numbers of HSCT patients **(Figure 17)** [106, 107].

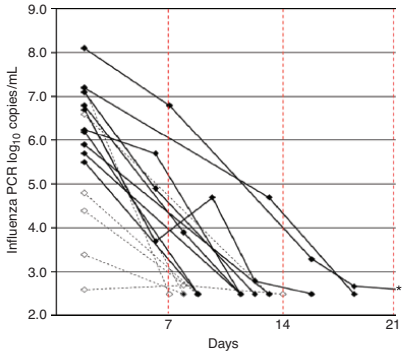


Figure 16. Influenza virus levels in severe (♦) vs moderate (◊) immunodeficient patients.
Source: adapted from [99].

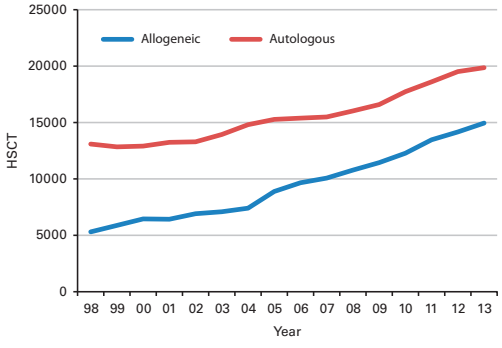


Figure 17. Rising numbers of hematology-oncology patients in Europe from 1998 to 2013.
Source: adapted from [106].

Outline of this thesis

This thesis focuses on a variety of subjects related to the molecular diagnosis and clinical consequences of seasonal and pandemic influenza virus infections in a wide range of patients. The main objectives were (1) to evaluate influenza real-time PCR diagnostic methods and clinical aspects of seasonal and pandemic influenza virus infection, (2) to investigate the applicability and accuracy of mass spectrometry-based molecular techniques and real-time PCR to detect influenza virus resistance and virulence genes, and (3) to correlate the role of different host immune responses with virus-associated symptoms and viral clearance in immunocompromised patients with prolonged influenza virus excretion.

The studies described in this thesis are presented in the following chapters:

Chapter 2 describes the relative incidence of respiratory virus infections in children presenting to the hospital and virus-specific clinical correlations in young children.

In **chapter 3**, two children with fatal influenza virus-associated pneumonia, encephalopathy and multiple organ failure are described and we performed molecular and pathological studies to confirm or refute virus dissemination and replication in other organs.

Chapter 4 is a study that compared influenza immunoassay and PCR methods on nursing homes specimens and evaluated the efficacy of Public Health Service outbreak team support.

Chapter 5 describes the clinical manifestation and antiviral resistance development in immunocompromised patients with ≥14 days prolonged influenza virus excretion.

In **chapter 6**, a phylogenetic relationship was performed among oseltamivir-resistant influenza A (H1N1) viruses in a patient cluster to assess nosocomial virus transmission.

Chapter 7 describes the detection of influenza virus resistance and virulence markers in routine clinical specimens using mass spectrometry-based comparative sequence analysis.

Chapter 8 describes the role of host immune responses in determining influenza virus-associated symptoms and viral clearance in hematology-oncology patients.

References

1. Grant W. Observations on the late influenza the febris catarrhalis epidemica of Hippocrates: as it appeared at London in 1775 and 1782. *Published by the author* in **1782**. Free e-book available from Universidad Complutense de Madrid, 2010. https://books.google.nl/books?id=-gpkv_f8u8YC&dq=Observations+on+the+late+influenza+the+febris+catarrhalis+epidemica&hl=nl&source=gbs_navlinks_s , accessed April 24th in 2015.

2. Thompson, Theophilus. Annals of influenza or epidemic catarrhal fever in Great Britain from 1510 to 1837. *The New Sydenham Society, London*, **1852**. Free e-book available from Yale University, Cushing/Whitney Medical Library, 2011. https://archive.org/details/annalsofinfluenz00unse, accessed April 24th in 2015.

3. Editorial. Etymologia: influenza. *Emerg Infect Dis* **2006**; 12: 179.

4. Shope RE. Influenza: history, epidemiology, and speculation. *Public Health Rep* **1958**; 73: 165–78.

5. Kempińska-Mirosławska B, Woźniak-Kosek A. The influenza epidemic of 1889-90 in selected European cities—a picture based on the reports of two Poznań daily newspapers from the second half of the nineteenth century. *Med Sci Monit* **2013**; 19: 1131–41.

6. Mathers G. Etiology of the epidemic acute respiratory infections commonly called influenza. *JAMA* **1917**; LXVIII: 678–80.

7. MacCallum WG. Pathology of the pneumonia following influenza. *JAMA* **1919**; 72: 720–23.

8. Patterson SW. The pathology of influenza in France. *MJA* **1920**; 1: 207–10.

9. Armstrong GL, Conn LA, Pinner RW. Trends in infectious disease mortality in the United States during the 20th century. *JAMA* **1999**; 281: 61–6.

10. Taubenberger JK. The Origin and Virulence of the 1918 "Spanish" Influenza Virus. *Proc Am Philos Soc* **2006**; 150: 86–112.

11. Editorial. The influenza outbreak. *JAMA* **1918**; 71: 1138.

12. Köhler M, Köhler W. Zentralblatt für bakteriologie - 100 years ago an outbreak of fowl plague in Tyrol in 1901. *Int J Med Microbiol* **2001**; 291: 319–21.

13. Selter, H. *Deutsch med Woch* **1918**; 44: 932.

14. Nicolle C, Lebailly C. Quelques notions expérimentales sur le virus de la grippe. Comptes rendus de l'Académie des Sciences **1918**; 167: 607–10.

15. Shope RE. Swine influenza: III. Filtration experiments and etiology. *J Exper Med* **1931**; 54: 373–85.

16. Smith W, Andrewes CH, Laidlaw PP. A virus obtained from influenza patients. *Lancet* **1933**; 222: 66–8.

17. Smith W. Cultivation of the Virus of Influenza. *Br J Exp Pathol* **1935**; 16: 508–12.

18. Dochez AR, Mills KC, Kneeland Y. Studies on the virus of influenza. *J Exp Med* **1936**; 63: 581–98.

19. Hirst GK. The agglutination of red cells by allantoic fluid of chick embryos infected with influenza virus. *Science* **1941**; 94: 22–3.

20. Source: www.berkefeld.com/en/company/history/.

21. Ghedin E, Sengamalay NA, Shumway M, *et al.* Large-scale sequencing of human influenza reveals the dynamic nature of viral genome evolution. *Nature* **2005**; 437: 1162–6.

22. Jagger BW, Wise HM, Kash JC, *et al.* An overlapping protein-coding region in influenza A virus segment 3 modulates the host response. *Science* **2012**; 337: 199–204.

23. Wise HM, Hutchinson EC, Jagger BW, *et al.* Identification of a novel splice variant form of the influenza A virus M2 ion channel with an antigenically distinct ectodomain. *PLoS Pathog* **2012**; 8: e1002998.

24. Shaw ML, Palese P. *Orthomyxoviridae.* In: Knipe DM, Howley PM. Editors-In-Chief. *Fields Virology*, 5th edn. Lippincott Williams & Wilkins, **2007**; pp. 1649.

25. Templeton KE, Scheltinga SA, Beersma MF, Kroes AC, Claas EC. Rapid and sensitive method using multiplex real-time PCR for diagnosis of infections by influenza a and influenza B viruses, respiratory syncytial virus, and parainfluenza viruses 1, 2, 3, and 4. *J Clin Microbiol* **2004**; 42: 1564–9.

26. van der Vries E, Jonges M, Herfst S, *et al.* Evaluation of a rapid molecular algorithm for detection of pandemic influenza A (H1N1) 2009 virus and screening for a key oseltamivir resistance (H275Y) substitution in neuraminidase. *J Clin Virol* **2010**; 47: 34–7.

27. Dulyachai W, Makkoch J, Rianthavorn P, *et al.* Perinatal pandemic (H1N1) 2009 infection, Thailand. *Emerg Infect Dis* **2010**; 16: 343–4.

28. van Riel D, den Bakker MA, Leijten LM, *et al.* Seasonal and pandemic human influenza viruses attach better to human upper respiratory tract epithelium than avian influenza viruses. *Am J Pathol* **2010**; 176: 1614–8.

29. Montalto NJ. An office-based approach to influenza: clinical diagnosis and laboratory testing. *Am Fam Physician* **2003**; 67: 111–8.

30. Cowling BJ, Chan KH, Fang VJ, *et al.* Comparative epidemiology of pandemic and seasonal influenza A in households. *N Engl J Med* **2010**; 362: 2175–84.

31. Loeb M, Singh PK, Fox J, *et al.* Longitudinal study of influenza molecular viral shedding in Hutterite communities. *J Infect Dis* **2012**; 206: 1078–84.

32. Suess T, Remschmidt C, Schink SB, *et al.* Comparison of shedding characteristics of seasonal influenza virus (sub)types and influenza A(H1N1)pdm09; Germany, 2007–2011. *PLoS One* **2012**; 7: e51653.

33. Kaiser L, Fritz RS, Straus SE, Gubareva L, Hayden FG. Symptom pathogenesis during acute influenza: interleukin-6 and other cytokine responses. *J Med Virol* **2001**; 64: 262–8.

34. Maxwell ES, Ward TG, Van Metre TE Jr. The relation of influenza virus and bacteria in the etiology of pneumonia. *J Clin Invest* **1949**; 28: 307–18.

35. Louria DB, Blumenfeld HL, Ellis JT, Kilbourne ED, Rogers DE. Studies on influenza in the pandemic of 1957-1958. II. Pulmonary complications of influenza. *J Clin Invest* **1959**; 38: 213–65.

36. Hers JF, Masurel N, Mulder J. Bacteriology and histopathology of the respiratory tract and lungs in fatal Asian influenza. *Lancet* **1958**; 270: 1141–3.

37. Abbott JD, Fernando HV, Gurling K, Meade BW. Pulmonary aspergillosis following post-influenzal bronchopneumonia treated with antibiotics. *Br Med J* **1952**; 1: 523–5.

38. Dawood FS, Chaves SS, Pérez A, *et al*; Emerging Infections Program Network. Complications and associated bacterial coinfections among children hospitalized with seasonal or pandemic influenza, United States, 2003–2010. *J Infect Dis* **2014**; 209: 686–94.

39. Rothberg MB, Haessler SD. Complications of seasonal and pandemic influenza. *Crit Care Med* **2010**; 38: e91–7.

40. Kilbourne ED. Influenza pandemics of the 20th century. *Emerg Infect Dis* **2006**; 12: 9–14.

41. Masurel N. Relation between Hong Kong virus and former human A2 isolates and the A-EQU12 virus in human sera collected before 1957. *Lancet* **1969**; 293: 907–910.

42. Worobey M, Han GZ, Rambaut A. Genesis and pathogenesis of the 1918 pandemic H1N1 influenza A virus. *Proc Natl Acad Sci U S A* **2014**; 111: 8107–12.

43. Hampson AW, Mackenzie JS. The influenza viruses. *Med J Aust* **2006**; 185: S39–43.

44. Armstrong GL, Conn LA, Pinner RW. Trends in infectious disease mortality in the United States during the 20th century. *JAMA* **1999**; 281: 61–6.

45. Tumpey TM, Basler CF, Aguilar PV, *et al*. Characterization of the reconstructed 1918 Spanish influenza pandemic virus. *Science* **2005**; 310: 77–80.

46. Charu V, Chowell G, Palacio Mejia LS, *et al*. Mortality burden of the A/H1N1 pandemic in Mexico: a comparison of deaths and years of life lost to seasonal influenza. *Clin Infect Dis* **2011**; 53: 985–93.

47. Fleming DM, van der Velden J, Paget WJ. The evolution of influenza surveillance in Europe and prospects for the next 10 years. *Vaccine* **2003**; 21: 1749–53.

48. www.nivel.nl/sites/default/files/NIVELsurveillance_2015_wk22b.pdf

49. WHO Global Epidemiological Surveillance Standards for Influenza. Geneva: World Health Organization; **2013**.

50. Bolotin S, Pebody R, White PJ, *et al*; UK Severe Influenza Surveillance System (USISS) Steering Group. A new sentinel surveillance system for severe influenza in England shows a shift in age distribution of hospitalised cases in the post-pandemic period. *PLoS One* **2012**; 7: e30279.

51. Chaves SS, Aragon D, Bennett N, *et al*. Patients hospitalized with laboratory-confirmed influenza during the 2010-2011 influenza season: exploring disease severity by virus type and subtype. *J Infect Dis* **2013**; 208: 1305–14.

52. Ferguson NM, Anderson RM. Predicting evolutionary change in the influenza A virus. *Nat Med* **2002**; 8: 562–3.

53. Jonges M, van der Lubben IM, Dijkstra F, Verhoef L, Koopmans M, Meijer A. Dynamics of antiviral-resistant influenza viruses in the Netherlands, 2005–2008. *Antiviral Res* **2009**; 83: 290–7.

54. Gubareva LV, Kaiser L, Hayden FG. Influenza virus neuraminidase inhibitors. *Lancet* **2000**; 355: 827–35.

55. Moscona A. Neuraminidase inhibitors for influenza. *N Engl J Med* **2005**; 353: 1363–73.

56. Furuta Y, Gowen BB, Takahashi K, Shiraki K, Smee DF, Barnard DL. Favipiravir (T-705), a novel viral RNA polymerase inhibitor. *Antiviral Res* **2013**; 100: 446–54.

57. Park S, Kim JI, Lee I, *et al*. Combination effects of peramivir and favipiravir against oseltamivir-resistant 2009 pandemic influenza A(H1N1) infection in mice. *PLoS One* **2014**; 9: e101325.

58. Aoki FY, Macleod MD, Paggiaro P, *et al*; IMPACT Study Group. Early administration of oral oseltamivir increases the benefits of influenza treatment. *J Antimicrob Chemother* **2003**; 51: 123–9.

59. Jefferson T, Jones MA, Doshi P, *et al*. Neuraminidase inhibitors for preventing and treating influenza in healthy adults and children. *Cochrane Database Syst Rev* **2014**; 4: CD008965.

60. Dobson J, Whitley RJ, Pocock S, Monto AS. Oseltamivir treatment for influenza in adults: a meta-analysis of randomised controlled trials. *Lancet* **2015**. pii: S0140–6736(14)62449–1.

61. McGeer A, Green KA, Plevneshi A, *et al*; Toronto Invasive Bacterial Diseases Network. Antiviral therapy and outcomes of influenza requiring hospitalization in Ontario, Canada. *Clin Infect Dis* **2007**; 45: 1568–75.

62. Lee N, Choi KW, Chan PK, *et al*. Outcomes of adults hospitalised with severe influenza. *Thorax* **2010**; 65: 510–5.

63. Muthuri SG, Venkatesan S, Myles PR, *et al*; PRIDE Consortium Investigators, Nguyen-Van-Tam JS. Effectiveness of neuraminidase inhibitors in reducing mortality in patients admitted to hospital with influenza A H1N1pdm09 virus infection: a meta-analysis of individual participant data. *Lancet Respir Med* **2014**; 2: 395–404.

64. Chemaly RF, Ghosh S, Bodey GP, *et al*. Respiratory viral infections in adults with hematologic malignancies and human stem cell transplantation recipients: a retrospective study at a major cancer center. *Medicine (Baltimore)*. **2006**; 85: 278–87.

65. Ljungman P, de la Camara R, Perez-Bercoff L, *et al*; Infectious Diseases Working Party, European Group for Blood and Marrow Transplantation; Infectious Complications Subcommittee, Spanish Group of Haematopoietic Stem-cell Transplantation. Outcome of pandemic H1N1 infections in hematopoietic stem cell transplant recipients. *Haematologica* **2011**; 96: 1231–5.

66. Choi SM, Boudreault AA, Xie H, Englund JA, Corey L, Boeckh M. Differences in clinical outcomes after 2009 influenza A/H1N1 and seasonal influenza among hematopoietic cell transplant recipients. *Blood* **2011**; 117: 5050–6.

67. Lee N, Leo YS, Cao B, *et al*. Neuraminidase inhibitors, superinfection and corticosteroids affect survival of influenza patients. *Eur Respir J* **2015**; 45: 1642–52.

68. Chaves SS, Pérez A, Miller L, *et al*. Impact of Prompt Influenza Antiviral Treatment on Extended Care Needs After Influenza Hospitalization Among Community-Dwelling Older Adults. *Clin Infect Dis* **2015**. pii: civ733.

69. Lee N, Chan PK, Wong CK, *et al*. Viral clearance and inflammatory response patterns in adults hospitalized for pandemic 2009 influenza A(H1N1) virus pneumonia. *Antivir Ther* **2011**; 16: 237–47.

70. Gubareva LV, Kaiser L, Matrosovich MN, Soo-Hoo Y, Hayden FG. Selection of influenza virus mutants in experimentally infected volunteers treated with oseltamivir. *J Infect Dis* **2001**; 183: 523–31.

71. Samson M, Pizzorno A, Abed Y, Boivin G. Influenza virus resistance to neuraminidase inhibitors. *Antiviral Res* **2013**; 98: 174–85.

72. Potier M, Mameli L, Bélisle M, Dallaire L, Melancon SB. Fluorometric assay of neuraminidase with a sodium (4-methylumbelliferyl-alpha-D-N acetylneuraminate) substrate. *Anal Biochem* **1979**; 94: 287–96.

73. Weinstock DM, Zuccotti G. The evolution of influenza resistance and treatment. *JAMA* **2009**; 301: 1066–9.

74. Bloom JD, Gong LI, Baltimore D. Permissive secondary mutations enable the evolution of influenza oseltamivir resistance. *Science* **2010**; 328: 1272–5.

75. Collins PJ, Haire LF, Lin YP, *et al*. Structural basis for oseltamivir resistance of influenza viruses. *Vaccine* **2009**; 27: 6317–23.

76. Holmes EC. Virology. Helping the resistance. *Science* **2010**; 328: 1243–4.

77. Mertz D, Kim TH, Johnstone J, *et al*. Populations at risk for severe or complicated influenza illness: systematic review and meta-analysis. *BMJ* **2013**; 347: f5061.

78. Pebody RG, McLean E, Zhao H, *et al*. Pandemic Influenza A (H1N1) 2009 and mortality in the United Kingdom: risk factors for death, April 2009 to March 2010. *Euro Surveill* **2010**; 15. pii: 19571.

79. Campbell A, Rodin R, Kropp R, *et al*. Risk of severe outcomes among patients admitted to hospital with pandemic (H1N1) influenza. *CMAJ* **2010**; 182: 349–55.

80. Louie JK, Acosta M, Samuel MC, *et al*; California Pandemic (H1N1) Working Group. A novel risk factor for a novel virus: obesity and 2009 pandemic influenza A (H1N1). *Clin Infect Dis* **2011**; 52: 301–12.

81. Thompson MG, Shay DK, Zhou H, *et al*; Influenza Div, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention (CDC). Estimates of Deaths Associated with Seasonal Influenza — United States, 1976-2007. *MMWR Morb Mortal Wkly Rep* **2010**; 59; 1057–1062.

82. Hayward AC, Harling R, Wetten S, *et al*. Effectiveness of an influenza vaccine programme for care home staff to prevent death, morbidity, and health service use among residents: cluster randomised controlled trial. *BMJ* **2006**; 333: 1241.

83. Bridges CB, Harper S. The full-court press for influenza prevention in elderly persons. *Clin Infect Dis* **2004**; 39: 465–7.

84. Ferguson NM, Galvani AP, Bush RM. Ecological and immunological determinants of influenza evolution. *Nature* **2003**; 422: 428–33.

85. Beck CR, McKenzie BC, Hashim AB, *et al*. Influenza vaccination for immunocompromised patients: systematic review and meta-analysis from a public health policy perspective. *PLoS One* **2011**; 6: e29249.

86. Udell JA, Zawi R, Bhatt DL, *et al*. Association between influenza vaccination and cardiovascular outcomes in high-risk patients: a meta-analysis. *JAMA* **2013**; 310: 1711–20.

87. Nichol KL, Nordin JD, Nelson DB, Mullooly JP, Hak E. Effectiveness of influenza vaccine in the community-dwelling elderly. *N Engl J Med* **2007**; 357: 1373–81.

88. Darvishian M, Bijlsma MJ, Hak E, van den Heuvel ER. Effectiveness of seasonal influenza vaccine in community-dwelling elderly people: a meta-analysis of test-negative design case-control studies. *Lancet Infect Dis* **2014**; 14: 1228–39.

89. Govaert TM, Thijs CT, Masurel N, Sprenger MJ, Dinant GJ, Knottnerus JA. The efficacy of influenza vaccination in elderly individuals. A randomized double-blind placebo-controlled trial. *JAMA* **1994**; 272: 1661–5.

90. Chan TC, Hung IF, Luk JK, *et al*. Efficacy of dual vaccination of pandemic H1N1 2009 influenza and seasonal influenza on institutionalized elderly: a one-year prospective cohort study. *Vaccine* **2011**; 29: 7773–8.

91. Hobson D, Curry RL, Beare AS, Ward-Gardner A. The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses. *J Hyg (Lond)* **1972**; 70: 767–77.

92. Potter CW, Oxford JS. Determinants of immunity to influenza infection in man. *Br Med Bull* **1979**; 35: 69–75.

93. van de Sandt CE, Kreijtz JH, Rimmelzwaan GF. Evasion of influenza A viruses from innate and adaptive immune responses. *Viruses* **2012**; 4: 1438–76.

94. Subbarao K, Murphy BR, Fauci AS. Development of effective vaccines against pandemic influenza. *Immunity* **2006**; 24: 5–9.

95. Iwasaki A, Pillai PS. Innate immunity to influenza virus infection. *Nat Rev Immunol* **2014**; 14: 315–28.

96. Kim TS, Sun J, Braciale TJ. T cell responses during influenza infection: getting and keeping control. *Trends Immunol* **2011**; 32: 225–31.

97. Schnell D, Mayaux J, de Bazelaire C, *et al*. Risk factors for pneumonia in immunocompromised patients with influenza. *Respir Med* **2010**; 104: 1050–6.

98. Nichols WG, Guthrie KA, Corey L, Boeckh M. Influenza infections after hematopoietic stem cell transplantation: risk factors, mortality, and the effect of antiviral therapy. *Clin Infect Dis* **2004**; 39: 1300–6.

99. Khanna N, Steffen I, Studt JD, *et al*. Outcome of influenza infections in outpatients after allogeneic hematopoietic stem cell transplantation. *Transpl Infect Dis* **2009**; 11: 100–5.

100. Gubareva LV, Matrosovich MN, Brenner MK, Bethell RC, Webster RG. Evidence for zanamivir resistance in an immunocompromised child infected with influenza B virus. *J Infect Dis* **1998**; 178: 1257–62.

101. Weinstock DM, Gubareva LV, Zuccotti G. Prolonged shedding of multidrug-resistant influenza A virus in an immunocompromised patient. *N Engl J Med* **2003**; 348: 867–8.

102. Baz M, Abed Y, McDonald J, Boivin G. Characterization of multidrug-resistant influenza A/H3N2 viruses shed during 1 year by an immunocompromised child. *Clin Infect Dis* **2006**; 43: 1555–61.

103. Ison MG, Gubareva LV, Atmar RL, Treanor J, Hayden FG. Recovery of drug-resistant influenza virus from immunocompromised patients: a case series. *J Infect Dis* **2006**; 193: 760–4.

104. Redelman-Sidi G, Sepkowitz KA, Huang CK, *et al*. 2009 H1N1 influenza infection in cancer patients and hematopoietic stem cell transplant recipients. *J Infect* **2010**; 60: 257–63.

105. Tramontana AR, George B, Hurt AC, *et al*. Oseltamivir resistance in adult oncology and hematology patients infected with pandemic (H1N1) 2009 virus, Australia. *Emerg Infect Dis* **2010**; 16: 1068–75.

106. Passweg JR, Baldomero H, Bader P, *et al*. Hematopoietic SCT in Europe 2013: recent trends in the use of alternative donors showing more haploidentical donors but fewer cord blood transplants. *Bone Marrow Transplant* **2015**; 50: 476–82.

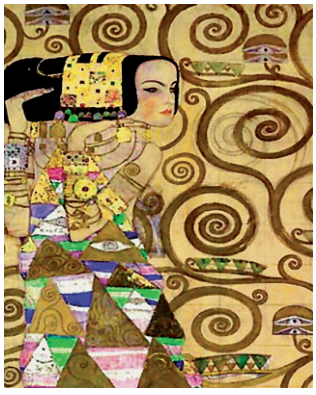
107. Gratwohl A, Baldomero H. Trends of hematopoietic stem cell transplantation in the third millennium. *Curr Opin Hematol* **2009**; 16: 420–6.

Chapter 2

Clinical evaluation of viral acute respiratory tract infections in children presenting to the emergency department of a tertiary referral hospital in the Netherlands

Gooskens J [1], van der Ploeg V [1], Sukhai RN [2],
Vossen ACTM [1], Claas ECJ [1], Kroes ACM [1]

BMC Pediatrics **2014**; *14: 297*



1 Department of Medical Microbiology
Leiden University Medical Center, Leiden, the Netherlands
2 Department of Pediatrics
Leiden University Medical Center, Leiden, the Netherlands

Abstract

Background: The relative incidence and clinical impact of individual respiratory viruses remains unclear among children presenting to the hospital emergency department with acute respiratory tract infection (ARTI).

Methods: During two winter periods, respiratory virus real-time multiplex PCR results were evaluated from children (<18 years) presenting to the emergency department of a tertiary referral hospital with ARTI that had been sampled within 48 hours of hospital presentation. In an attempt to identify virus-specific distinguishing clinical features, single virus infections were correlated with presenting signs and symptoms, clinical findings and outcomes using multivariate logistic regression.

Results: In total, 274 children with ARTI were evaluated and most were aged <3 years (236/274, 86%). PCR detected respiratory viruses in 224/274 (81.8%) children and included 162 (59%) single and 62 (23%) mixed virus infections. Respiratory syncytial virus (RSV) and human rhinovirus (HRV) single virus infections were common among children aged <3 years, but proportional differences compared to older children were only significant for RSV (95% CI 1.3–15). Clinical differentiation between viral ARTIs was not possible due to common shared presenting signs and symptoms and the high frequency of mixed viral infections. We observed virus-associated outcome differences among children aged <3 years. Oxygen treatment was associated with RSV (OR 3.6) and inversely correlated with FLU (OR 0.05). Treatment with steroids (OR 3.4) or bronchodilators (OR 3.4) was associated with HRV. Severe respiratory complications were associated with HRV (OR 3.5) and inversely correlated with RSV (OR 0.24).

Conclusions: Respiratory viruses are frequently detected in young children presenting to the hospital emergency department with ARTI and require PCR diagnosis since presenting signs and symptoms are not discriminant for a type of virus. RSV and HRV bear a high burden of morbidity in the pediatric clinical setting.

Background

Children suffer through multiple episodes of viral acute respiratory tract infection (ARTI) annually and symptoms range from common mild upper respiratory illness to lower respiratory tract infection (LRTI) [1, 2]. Large-scale studies during the past decades were unable to provide accurate virus-specific clinical correlations due to the low sensitivity of viral culture and immunofluorescence techniques and inability of these diagnostic methods to detect non-culturable or mixed viral infections [1, 3]. The routine implementation of multiplex PCR in recent years has allowed for sensitive and accurate identification of single and mixed viral infections [4, 5]. The burden of individual respiratory viruses remains unclear among different pediatric age-groups in the clinical setting. Recent molecular population-based studies show that viral ARTIs exceed 21% of pediatric emergency department visits during the seasonal influenza winter season and annual hospitalization rates exceed 1.5 per 100 children aged <3 years [6-8]. These findings suggest a high burden of viral ARTI in young children. The burden of viral respiratory pathogens in children is underestimated since human rhinovirus (HRV) infections were not evaluated in these studies. Recent clinical studies have included HRV molecular diagnostics, but interpretation of virus-associated correlations is hampered by age- and symptom-related enrollment differences [9, 10] or by sampling during the 2009 influenza pandemic [11, 12]. This study assessed the relative incidence of respiratory virus infections in children presenting to the emergency department of a tertiary hospital with ARTI during two winter seasons and evaluated virus-specific clinical correlations in young children.

Methods

Study design and patient populations. This retrospective cohort study included all children aged <18 years presenting to the emergency department of Leiden University Medical Center (LUMC) during the 2006 and 2007 winter seasons (November–April) with ARTI who were sampled within 48 hours of hospital presentation. The LUMC is a tertiary referral hospital for the south-western part of the Netherlands covering a population of approximately 2 million inhabitants. Clinical diagnosis of ARTI was made by the attending physician. Routine diagnostic specimens were prospectively analyzed by respiratory virus multiplex PCR to evaluate the relative incidence of respiratory viruses among different pediatric age-groups (children aged <3 years and children aged 3–17

years). Structured medical records of children aged <3 years with single virus infections (RSV, HRV, FLU or Other) were evaluated for baseline characteristics, presenting signs and symptoms and clinical outcomes.

Respiratory specimens and molecular diagnostics. Qualified medical personnel obtained diagnostic respiratory specimens and a single specimen was evaluated for each child sampled within 48 hours of hospital presentation. Respiratory specimens included nasopharyngeal washes, throat swabs, nasal swabs, sputum or tracheal aspirates. Nucleic acid was extracted by using a MagnaPure LC total nucleic acid kit (Roche Diagnostics, Mannheim, Germany) as described [4]. A fixed amount of equine arteritis virus served as an RNA internal control and phocid herpesvirus was used as a DNA internal control. Respiratory virus multiplex PCR detected respiratory syncytial virus (RSV), human rhinovirus (HRV), influenzavirus (FLU) A/B, parainfluenzavirus (PIV) 1/2/3/4, human metapneumovirus (HMPV), human coronavirus (HCoV) 229E/NL63/OC43, and adenovirus (HAdV) in multiple tubes as described [4]. Primers, probes and amplification methods of the multiplex PCR used in this study are described in reference 4 for laboratory 2. In a pilot run during the 2007 winter season, we performed additional real-time PCR analysis of HCoV HKU1, *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* [4]. During this pilot run, we performed additional analysis of human bocavirus (HBoV) by real-time PCR amplification of a 138-bp fragment of the NS1 gene as described by others [13].

Ethics. The study was conducted in accordance with ethical principles expressed in the World Medical Association Declaration of Helsinki. The study procedures complied with legal and regulatory standards and clinical data was obtained by following professional codes of conduct. All necessary precautions were taken to prevent identification of any child included in the study. The Medical Ethics Committee (MEC) of Leiden University Medical Center reviewed the study protocol (C14.128) and final version of the manuscript and confirms that the study is based on clinical data collected in the context of routine clinical practice. For this retrospective analysis of routine clinical data the committee declares that no formal ethical approval and written informed consent is needed.

Study procedure - viral distribution. Routine diagnostic PCR results from all eligible children were analyzed. Distribution of RSV, HRV, FLU and Other single virus infections was depicted by age-groups (young children aged <3 years and children aged 3–17 years). PIV, HMPV, HAdV and HCoV were aggregated into a distinct group (Other) due to small numbers and to enable more accurate comparative virus-associated correlations.

Study procedure – presenting signs and symptoms. The attending physician recorded signs and symptoms of young children aged <3 years using structured medical records. Presenting signs and symptoms manifesting within 48 hours of hospital presentation of children with RSV, HRV, FLU or Other single virus infections were evaluated. Virus-associated symptoms were compared and included fever >38.5 °C, cough, rhinitis, pharyngitis, wheezing, crepitation, dyspnea or tachypnea. The clinical presence of chest wall retractions, nasal flaring, moaning or labored breathing were used to define dyspnea World Health Organization clinical diagnostic criteria defined tachypnea among children aged <2 months (≥60 breaths per minute, bpm), aged 2 to 12 months (≥50 bpm) and aged ≥12 months (≥40 bpm).

Study procedure – outcomes. The attending physician recorded outcomes of children aged <3 years with RSV, HRV, FLU and Other single virus infections. We evaluated laboratory and pulmonary imaging findings that were obtained within 96 hours of hospital presentation and adverse outcome manifestations (hospital admission, severe respiratory complications, mortality) that were recorded within ≤7 days of hospital presentation. Laboratory findings included C-reactive protein (CRP) and white blood cell count (WBC). We evaluated cut-offs that would indicate absence of serious bacterial infections (CRP <35 mg/l; WBC <15 x10^9/L) [14, 15]. We compared bacteriology results that were obtained by PCR (atypical bacteria) or from routine cultures from non-sterile (urine, sputum) and sterile sites (blood, cerebrospinal fluid). Pulmonary imaging findings were assessed for signs of LRTI (radiologic presence of alveolar or peribronchial infiltrates, interstitial opacities, and hyperinflation). Supplemental treatments included bronchodilators, steroids, oxygen supplementation and antibiotics. Oxygen supplementation was provided during sustained oxygen saturation ≤92% or during dyspnea with abnormal PCO2 levels. Adverse outcomes included hospital admission, development of severe respiratory complications and all-cause mortality ≤7 days of hospital presentation. Severe respiratory complications were defined as apnea, respiratory intubation and Apparent Life Threatening Events (ALTE). ALTE was defined as an acute change in an infant's breathing behavior perceived as possibly life threatening by the child's caretaker.

Statistical analysis. Statistical analyses were performed using SPSS software version 20.0 (SPSS, Chicago, IL). Continuous variables were presented as mean or median (with range) and categorical variables as frequencies (with percentages). Mann Whitney U-test and Kruskal Wallis test were used as non-parametric tests to compare age (months) between 2 or more groups, because of non-normally distributed data. Chi square was

performed to compare categorical variables between 2 groups. Logistic regression analysis compared categorical variables between virus groups including presenting signs and symptoms, diagnostic, treatment and outcome findings. Multivariate analyses adjusted for age, gender and relevant history or chronic underlying disorders (stepwise). A 2-sided value of $P < 0.05$ was considered significant.

Results

Patient enrollment and viral etiology. During two winter seasons, we enrolled 274 children presenting to the emergency department with ARTI and sampled within 48 hours of hospital presentation. The majority of children were aged <3 years (236/274, 86%) compared to older children aged 3–17 years (38/274, 14%). Multiplex PCR detected respiratory viruses in 82% (224/274) of all children and included single virus infections (162 of 224, 72%) and mixed viral infections (62 of 224, 28%) **(Figure 1)**. Single virus infections were caused by RSV (69 of 224, 31%), HRV (53 of 224, 24%), FLU (16 of 224, 7%) and Other (24 of 224, 11%). In a pilot run among a total 131 children during the 2007 winter season, PCR yielded HBoV (7%) and HCoV HKU1 (1%), but no *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae*. HBoV- and HCoV HKU1-associated clinical findings were not evaluated due to a rare occurrence of single virus infections (n = 4 HBoV; n = 0 HCoV HKU1) and due to incomplete data (single season).

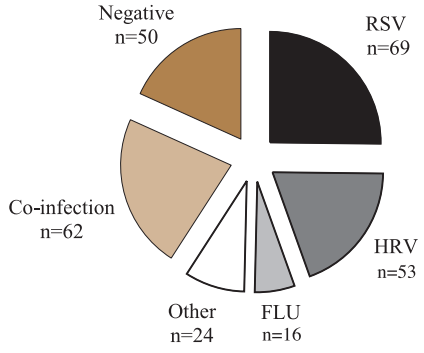


Figure 1. Viral etiologies of 274 children with ARTI presenting to the emergency department of a tertiary referral hospital.

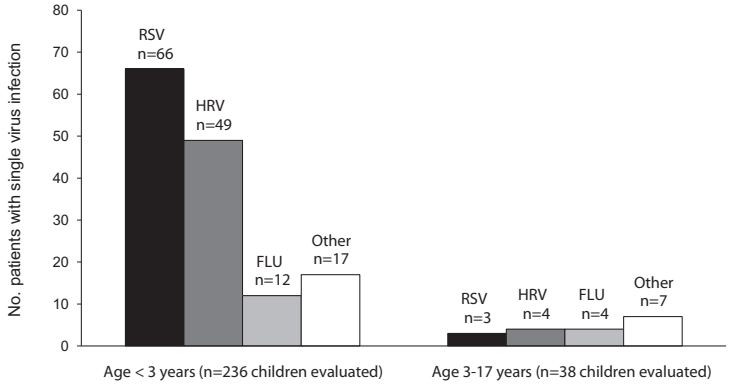


Figure 2. Distribution of single respiratory virus infections among different pediatric age groups.

Respiratory virus distribution. The distribution of single virus infections among different age groups was evaluated in an attempt to establish virus-associated clinical correlations. Mixed viral infections were not analyzed due to the small sample size and the difficulty to establish clinical relevance. Single virus infections were common among young children aged <3 years (144 of 236, 61%) compared to older children (18 of 38, 47%) but these proportional differences were not statistically significant **(Figure 2)**. RSV and HRV were more common among children aged <3 years compared to older children, but proportional differences were only significant for RSV (28% vs 7.9%; 95% CI 1.3–15). In contrast, young children were less common infected with Other single viruses (including PIV, HMPV, HAdV or HCoV) compared to older children (7.2% vs 18%; 95% CI 0.13–0.90). FLU single virus infections were uncommon in both age groups with a similar low prevalence (range, 5 to 11%).

Virus-specific clinical correlations - baseline characteristics. Evaluation of baseline characteristics was limited to children aged <3 years with single virus infections (RSV, HRV, FLU, Other) with clinical data available. We included demographics (sex, age) and relevant history encompassing prematurity at birth (gestational age <37 weeks), bronchial hyperreactivity (bronchoconstriction in response to stimuli) and underlying pulmonary, cardiovascular and immunodeficiency disorders. Base-line characteristics were equally distributed among virus groups, except for a male predominance **(Table 1)** among children with HRV vs FLU (73% vs 36%; 95% CI 1.2–19). Proportional differences at baseline underscore the need for statistical adjustment for possible confounders during comparative analyses.

Table 1. Baseline characteristics of children aged <3 years with single respiratory virus infections.

	Total N (%)	RSV N (%)	HRV N (%)	FLU N (%)	Other N (%)
Children <3 y with single virus ARTI					
Total included	144	66	49	12	17
Analysis of signs and symptoms (data available)	138	64	46	11	17
Analysis of clinical outcomes (data available)	123	53	44	11	15
Demographics (analysis of clinical outcomes)					
Male	76 (62)	30 (57)	32 (73) $	4 (36) $	10 (67)
Mean age, months [range]	8 [0 – 35]	7 [0 – 35]	7 [0 – 24]	10 [1 – 26]	7 [0 – 35]
Median age, months [range]	4 [0 – 35]	3 [0 – 35]	4 [0 – 24]	9 [1 – 26]	8 [0 – 33]
Clinical history (analysis of clinical outcomes)					
Prematurity at birth	11 (9)	6 (11)	3 (7)	1 (9)	1 (7)
History of BHR	12 (9)	5 (9)	5 (11)	1 (9)	1 (7)
Chronic pulmonary disorder	2 (2)	0	2 (5)	0	0
Chronic cardiovascular disorder	12 (10)	3 (6)	6 (14)	1 (9)	2 (13)
Chronic immunodeficiency disorder	5 (4)	0	2 (5)	1 (9)	2 (13)

$ Baseline characteristics are equally distributed, except for a male predominance with HRV vs FLU (95%CI 1.2-19).

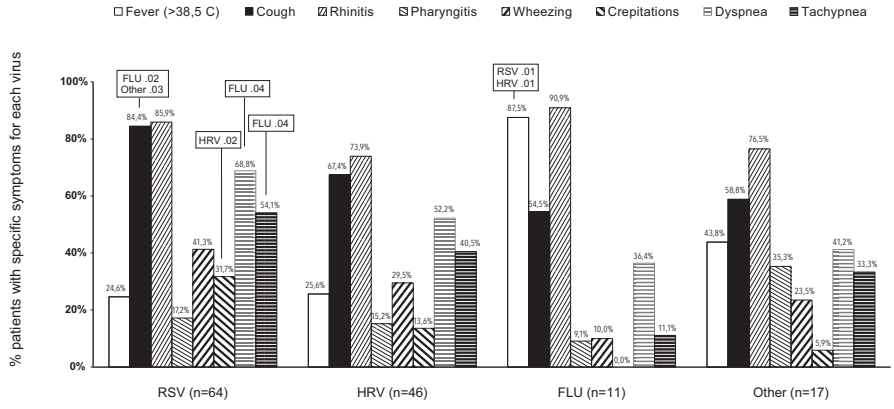


Figure 3. Signs and symptoms of 138 children aged <3 years presenting to the emergency department of a tertiary referral hospital with single virus ARTI.

The proportions of presenting signs and symptoms were compared between virus groups using logistic regression analysis and stepwise multivariate adjustment for demographics and relevant clinical history. Significant proportional differences (P <0.05) between virus groups are depicted and include fever (FLU vs RSV, P = 0.01; FLU vs HRV, P = 0.01) and other findings (cough, crepitation, dyspnea and tachypnea).

Virus-specific clinical correlations – signs and symptoms. Presenting signs and symptoms were compared among 138 children aged <3 years with single virus infections and clinical data available **(Table 1, Figure 3)**. Six children were previously excluded from comparative analyses due to incomplete documentation. Children aged 3–17 years were not evaluated due to small numbers and age-related confounding differences. Multivariate analyses adjusted for possible confounders including age and sex, and stepwise for relevant underlying disorders. Significant virus-associated presenting signs and symptoms **(Figure 3)** included fever (FLU vs RSV, $P = 0.01$; FLU vs HRV, $P = 0.01$), cough (RSV vs FLU, $P = 0.02$; RSV vs Other, $P = 0.03$), crepitation (RSV vs HRV, $P = 0.02$), dyspnea and tachypnea (RSV vs FLU, $P = 0.04$).

Virus-specific clinical correlations – clinical outcomes. Clinical outcomes were compared among 123 children aged <3 years with single virus infections and clinical data available **(Table 1, Table 2)**. A total of 21 children were previously excluded from comparative analyses due to incomplete documentation or hospital transfer without follow-up data. Excluded cases were equally distributed among virus groups **(Table 1)**. High levels of CRP (≥35 mg/l) or WBC (≥15 x 10^9/L) and antibiotic treatment were equally distributed among virus groups and suggested no differences in potential serious bacterial infections. Bacterial cultures and PCRs were often negative and were equally distributed among individual virus infections. This supports the assumption that there were no differences in potential serious bacterial infections among virus groups. Hospital admission (~80%) was equally distributed and fortunately there was no mortality. Overall and individual comparisons were made among children with RSV, HRV, FLU and Other single virus infections **(Table 2)** following multivariate adjustment for possible confounders.

RSV. Overall, RSV infection was associated with supplemental oxygen requirement (OR 3.6) and inversely correlated with severe respiratory complications (OR 0.24). Individual comparisons revealed that RSV was associated with supplemental oxygen requirement compared to FLU (OR 26) and Other (OR 4.2).

Table 2. Outcomes associated with single respiratory virus infections in children <3 years.

	Single virus ARTI					Statistical analysis		
	Total N (%)	RSV N (%)	HRV N (%)	FLU N (%)	Other N (%)	vs	Multivariate[$] OR (95%CI)	*P* value
Diagnostics								
Chemistry/hematology								
CRP ≥ 35 mg/L	28/81 (35)	11/32 (34)	11/29 (38)	2/9 (22)	4/11 (36)			NS
WBC ≥ 15 x10^9/L	27/80 (34)	11/32 (34)	11/28 (39)	2/9 (22)	3/11 (27)			NS
Bacteriology								NS
PCR atypical bacteria	0/10 (0)	0/2 (0)	0/4 (0)	0/1 (0)	0/3 (0)			NS
Culture, sterile site	2/45 (4)	0/15 (0)	1/17 (6)	0/7 (0)	1/6 (17)			NS
Culture, non-sterile site	1/19 (5)	0/7 (0)	0/8 (0)	0/1 (0)	1/3 (33)			NS
Pulmonary imaging								
LRTI[#]	31/63 (37)	15/24 (63)	10/25 (40)	3/6 (50)	3/8 (38)			NS
Treatment								
Antibiotics	50/122 (41)	24/53 (45)	17/43 (40)	3/11 (27)	6/15 (40)			NS
Bronchodilators	31/113 (27)	12/48 (25)	15/39 (38)	2/11 (18)	2/15 (13)	HRV vs Other	7.0 (1.2-42)	.03
						HRV overall	3.0 (1.2-7.8)	.02
Steroids	26/117 (22)	8/52 (15)	13/39 (33)	2/11 (18)	3/15 (20)	HRV vs RSV	3.5 (1.2-11)	.03
						HRV overall	3.4 (1.2-9.3)	.02
Oxygen therapy	67/118 (57)	38/52 (73)	22/41 (54)	1/10 (10)	6/15 (40)	RSV vs FLU	26 (3.0-225)	< .01
						RSV vs Other	4.2 (1.3-14)	.02
						HRV vs FLU	11 (1.2-97)	.03
						RSV overall	3.6 (1.6-8.0)	< .01
						FLU overall	.05 (.01-.40)	< .01
Adverse outcome								
Hospital admission	99/123 (80)	46/53 (87)	33/44 (75)	8/11 (73)	12/15 (80)			NS
Severe respiratory complication	15/123 (12)	3/53 (6)	9/44 (20)	1/11 (9)	2/15 (13)	HRV vs RSV	5.0 (1.2-21)	.03
						HRV overall	3.5 (1.0-11)	.04
						RSV overall	.24 (.06-.92)	.04
Apnea	5/123 (4)	1/53 (2)	4/44 (9)	0/11 (0)	0/15 (0)			NS
Intubation	7/123 (6)	1/53 (2)	5/44 (11)	0/11 (0)	1/15 (7)			NS
ALTE	6/123 (5)	1/53 (2)	3/44 (7)	1/11 (9)	1/15 (7)			NS
Mortality ≤ 7 days	0/123 (0)	0/53 (0)	0/44 (0)	0/11 (0)	0/15 (0)			NS

ARTI, Acute Respiratory Tract Infection, CRP, C-reactive protein; WBC, White Blood Cell count; LRTI, lower respiratory tract infection; ALTE, Apparent Life Threatening Events; NS, not significant.

LRTI was defined as radiologic presence of alveolar infiltrates, interstitial opacities, peribronchial infiltrates or hyperinflation.

$ Multivariate correction for sex, age and underlying disease (forward stepwise adjustment) using logistic regression analysis.

HRV. Overall, HRV infection was associated with bronchodilator therapy (OR 3.0), steroid treatment (OR 3.4) and severe respiratory complications (OR 3.5). Individual comparisons revealed that HRV was associated with bronchodilator therapy compared to Other (OR 7.0), steroid treatment compared to RSV (OR 3.5), supplemental oxygen requirement compared to FLU (OR 11) and severe respiratory complications (apnea, respiratory intubation or ALTE) compared to RSV (OR 5.0). HRV subtyping was not performed and therefore specific subtypes could not be associated with clinical outcome.

FLU. Overall, Flu infection was inversely correlated with supplemental oxygen requirement (OR 0.05). Individual comparisons revealed no FLU specific outcome associations.

Discussion

This study confirmed a frequent viral etiology among 82% (224 of 274) of children aged <18 years presenting to the emergency department of a tertiary hospital with ARTI. These findings add to a growing body of literature on the epidemiology and virus-associated clinical features in the clinical setting [6-12].

The high detection rate of respiratory viruses using multiplex PCR was similar to previously published rates exceeding 80% in the pediatric clinical setting [10, 11]. Much lower detection rates (range 58% – 67%) in a few other studies are likely due to age- and symptom-related enrollment differences or due to sampling during non-winter seasons [9, 12]. A high rate of mixed viral infections in this study (23%) is similar to findings in other studies (range 14% – 30%) [9-11].

Children presenting to the hospital emergency department with ARTI and sampled within 48 hours were predominantly aged <3 years (236 of 274, 86%). HRV and RSV single virus infections were common among children aged <3 years, but proportional differences compared to older children were only significant for RSV (95% CI 1.3–15). Previous studies report a similar predominance of RSV among young children in the clinical setting [7, 8, 10] but reports on HRV age-distribution are mixed [10, 16]. FLU cases were often young children (n = 12) compared to older children (n = 4) but the small numbers and the lack of a population-based design restrict firm epidemiologic conclusions.

Viral ARTI signs and symptoms are widely presumed to be aspecific but confirmation of this assumption is lacking. This study confirmed that viral ARTI presenting signs and symptoms are aspecific by comparative statistical analysis among children aged <3 years with single virus infections. Fever was often associated with influenza (FLU vs RSV, $P = 0.01$; FLU vs HRV, $P = 0.01$) and cough was often associated with RSV (RSV vs FLU, $P = 0.02$; RSV vs Other, $P = 0.03$). Unfortunately, these and other findings were insufficient to differentiate between individual viral ARTIs due to common shared signs and symptoms among viruses and the high frequency of mixed viral infections [9]. A febrile disease with ARTI symptoms was observed among ≥25% of total viruses infections, therefore influenza-like illness required PCR confirmation to establish FLU diagnosis [17, 18].

Virus-specific comparative outcome analysis among children aged <3 years with single virus infections unveiled clinical outcome similarities **(Table 2)**. Laboratory infection parameters (CRP, WBC), pulmonary imaging LRTI findings and antibiotic use were similar among virus groups. The findings contrast with a previous study which reported high CRP levels, elevated leukocyte counts and frequent antibiotic use during HRV [10]. In this study, children with RSV often received antibiotics (45%). Previous studies show that rapid confirmation of RSV can limit antibiotic use [19, 20], but antibiotic stewardship guided by respiratory virus PCR results may be difficult to implement [21].

Relevant virus-specific outcome differences include supplemental oxygen treatment requirement (RSV), steroid and bronchodilator treatment (HRV) and development of severe respiratory complications (HRV). Supplemental treatments with corticosteroids and bronchodilators were remarkably associated with HRV. There is insufficient evidence on the benefit of corticosteroids or bronchodilators during viral LRTI and most guidelines do not recommend the routine use for cases of RSV LRTI [22]. However, corticosteroids may improve HRV-induced wheezing and bronchodilators may be effective for individual children experiencing LRTI with underlying reactive airway disease. This may explain why corticosteroids and bronchodilators were more commonly provided to children with HRV. Young children presenting to the hospital emergency department with the combined symptoms of cough, pulmonary crepitation and oxygen-dependent viral LRTI were more likely to be infected with RSV than with other viruses as reported by others [12, 23]. With the knowledge that RSV is more often associated with oxygen-dependent viral LRTI, it would seem counter-intuitive that HRV (and not RSV) is associated with more severe respiratory complications **(Table 2)**. HRV often causes mild symptoms, but the findings of this and other studies suggest that the occurrence of

severe HRV respiratory complications may be underestimated [24, 25]. In this study, FLU infection was inversely correlated with supplemental oxygen treatment compared to other infections as described by others [26]. We emphasize that the low number of FLU cases does not allow for any firm conclusions on this matter. Previous studies suggest that FLU manifestations may be relatively mild among young children presenting to the hospital during seasonal influenza, but rare life-threatening events and severe cases of concomitant bacterial pneumonia do occur [12, 27].

Limitations. Children were evaluated at a tertiary care setting and this possibly limits the validity of virus-specific clinical correlations in other settings. The retrospective design of the study and evaluation of the patients by different attending physicians may have introduced a reporting bias. The inclusion of children sampled within 48 hours of hospital presentation may have introduced a selection bias towards younger patients due to a more cautious clinical and diagnostic approach of parents and pediatricians in that age group [28]. This could explain why the majority of children included in this study were aged <3 years (236/274, 86%). The respiratory specimens were of different types (including nasopharyngeal washes, throat swabs, nasal swabs, sputum or tracheal aspirates) which could have bearing on the sensitivity of the PCR for the various viruses and thus their relative frequency. The number of children with viral ARTI is an underestimation since our multiplex PCR did not detect HBoV, HCoV HKU1, human influenza C virus and enteroviruses that may cause viral respiratory infections. Molecular differentiation of HRV and enteroviruses is difficult and the HRV assay used in this study cross-reacts with a few enteroviruses that are associated with respiratory infections. This variation in HRV types may explain severe HRV infections and emphasizes that future studies should elucidate the specific role of HRV and enteroviruses using molecular subtyping. In this study, multiple comparisons were performed which can lead to more type I errors (more false positives) since 5% of the comparisons have uncorrected *P* values <0.05. Statistical adjustment of confidence intervals (e.g. Bonferroni corrections) can be applied to reduce incorrect rejection of true null hypotheses and to lower type I errors, but these corrections can increase the type II error (more false negatives) and lead to interpretation errors [29]. In this observational study, no adjustments were made for multiple comparisons in an attempt to find novel virus-specific clinical correlations. This approach is intended for hypothesis generation and not for hypothesis testing. Future prospective studies using improved standardized study protocols are therefore awaited to confirm virus-associated clinical findings and for hypothesis testing.

Conclusions. This study confirmed that a viral agent is frequently found in young children with ARTI presenting to the pediatric emergency department of a tertiary referral hospital. Molecular diagnostics are required to confirm respiratory virus infections since presenting symptoms could not discriminate between the viruses. RSV and HRV infections bear the highest burden of morbidity in the pediatric clinical setting.

Competing interests

The authors declare that they have no competing interests. There was no financial support.

Acknowledgements

Ron Wolterbeek (Department of Medical Statistics and Bioinformatics) provided valuable statistical advice. We thank Pulak Goswami for language editing support.

References

1. Monto AS. Studies of the community and family: acute respiratory illness and infection. *Epidemiol Rev* **1994**; 16: 351–73.

2. Yorita KL, Holman RC, Sejvar JJ, Steiner CA, Schonberger LB. Infectious disease hospitalizations among infants in the United States. *Pediatrics* **2008**; 121: 244–52.

3. Jacobs JW, Peacock DB, Corner BD, Caul EO, Clarke SK. Respiratory syncytial and other viruses associated with respiratory disease in infants. *Lancet* **1971**; 1: 871–6.

4. Loens K, van Loon AM, Coenjaerts F, *et al*. GRACE Study Group. Performance of different mono- and multiplex nucleic acid amplification tests on a multi-pathogen external quality assessment panel. *J Clin Microbiol* **2012**; 50: 977–87.

5. Freymuth F, Vabret A, Cuvillon-Nimal D, *et al*. Comparison of multiplex PCR assays and conventional techniques for the diagnostic of respiratory virus infections in children admitted to hospital with an acute respiratory illness. *J Med Virol* **2006**; 78: 1498–504.

6. Forster J, Ihorst G, Rieger CH, *et al*. Prospective population-based study of viral lower respiratory tract infections in children under 3 years of age (the PRI.DE study). *Eur J Pediatr* **2004**; 163: 709–16.

7. Iwane MK, Edwards KM, Szilagyi PG, *et al*. New Vaccine Surveillance Network. Population-based surveillance for hospitalizations associated with respiratory syncytial virus, influenza virus, and parainfluenza viruses among young children. *Pediatrics* **2004**; 113: 1758–64.

8. Bourgeois FT, Valim C, Wei JC, McAdam AJ, Mandl KD. Influenza and other respiratory virus-related emergency department visits among young children. *Pediatrics* **2006**; 118: e1–8.

9. Cilla G, Onate E, Perez-Yarza EG, Montes M, Vicente D, Perez T. Viruses in community-acquired pneumonia in children aged less than 3 years old: high rate of viral co-infection. *J Med Virol* **2008**; 80: 1843–9.

10. Garcia-Garcia ML, Calvo C, Pozo F, Villadangos PA, Perez-Brena P, Casas I. Spectrum of respiratory viruses in children with community-acquired pneumonia. *Pediatr Infect Dis J* **2012**; 31: 808–13.

11. Laurent C, Dugue AE, Brouard J, *et al*. Viral epidemiology and severity of respiratory infections in infants in 2009: a prospective study. *Pediatr Infect Dis J* **2012**; 31: 827–31.

12. Pierangeli A, Scagnolari C, Selvaggi C, *et al*. Virological and clinical characterization of respiratory infections in children attending an emergency department during the first autumn-winter circulation of pandemic A (H1N1) 2009 influenza virus. *Clin Microbiol Infect* **2012**; 18: 366–73.

13. Allander T, Tammi MT, Eriksson M, Bjerkner A, Tiveljung-Lindell A, Andersson B. Cloning of a human parvovirus by molecular screening of respiratory tract samples. *Proc Natl Acad Sci U S A* **2005**; 102: 12891–6.

14. Pulliam PN, Attia MW, Cronan KM. C-reactive protein in febrile children 1 to 36 months of age with clinically undetectable serious bacterial infection. *Pediatrics* **2001**; 108: 1275–9.

15. Yo CH, Hsieh PS, Lee SH, *et al*. Comparison of the test characteristics of procalcitonin to C-reactive protein and leukocytosis for the detection of serious bacterial infections in children presenting with fever without source: a systematic review and meta-analysis. *Ann Emerg Med* **2012**; 60: 591–600.

16. Miller EK, Lu X, Erdman DD, *et al*. New Vaccine Surveillance Network. Rhinovirus-associated hospitalizations in young children. *J Infect Dis* **2007**; 195: 773–81.

17. Wallace LA, Collins TC, Douglas JD, McIntyre S, Millar J, Carman WF. Virological surveillance of influenza-like illness in the community using PCR and serology. *J Clin Virol* **2004**; 31: 40–5.

18. Schnepf N, Resche-Rigon M, Chaillon A, *et al*. High burden of non-influenza viruses in influenza-like illness in the early weeks of H1N1v epidemic in France. *PLoS One* **2011**; 6: e23514.

19. Woo PC, Chiu SS, Seto WH, Peiris M. Cost-effectiveness of rapid diagnosis of viral respiratory tract infections in pediatric patients. *J Clin Microbiol* **1997**; 35: 1579–81.

20. Purcell K, Fergie J. Concurrent serious bacterial infections in 2396 infants and children hospitalized with respiratory syncytial virus lower respiratory tract infections. *Arch Pediatr Adolesc Med* **2002**; 156: 322–4.

21. Wishaupt JO, Russcher A, Smeets LC, Versteegh FG, Hartwig NG. Clinical impact of RT-PCR for pediatric acute respiratory infections: a controlled clinical trial. *Pediatrics* **2011**; 128: e1113–20.

22. Tregoning JS, Schwarze J. Respiratory viral infections in infants: causes, clinical symptoms, virology, and immunology. *Clin Microbiol Rev* **2010**; 23: 74–98.

23. Hall CB, Weinberg GA, Iwane MK, *et al*. The burden of respiratory syncytial virus infection in young children. *N Engl J Med* **2009**; 360: 588–98.

24. Iwane MK, Prill MM, Lu X, *et al*. Human rhinovirus species associated with hospitalizations for acute respiratory illness in young US children. *J Infect Dis* **2011**; 204: 1702–10.

25. Asner SA, Petrich A, Hamid JS, Mertz D, Richardson SE, Smieja M. Clinical severity of rhinovirus/enterovirus compared to other respiratory viruses in children. *Influenza Other Respir Viruses* **2014**; 8: 436–42.

26. Hite LK, Glezen WP, Demmler GJ, Munoz FM. Medically attended paediatric influenza during the resurgence of the Victoria lineage of influenza B virus. *Int J Infect Dis* **2007**; 11: 40–7.

27. Ruf BR, Knuf M. The burden of seasonal and pandemic influenza in infants and children. *Eur J Pediatr* **2014**; 173: 265–76.

28. Gasparini R, Durando P, Ansaldi F, *et al*. Influenza and respiratory syncytial virus in infants and children: relationship with attendance at a paediatric emergency unit and characteristics of the circulating strains. *Eur J Clin Microbiol Infect Dis* **2007**; 26: 619–28.

29. Rothman KJ. No adjustments are needed for multiple comparisons. *Epidemiology* **1990**; 1: 43–6.

Chapter 3

Severe influenza resembling hemorrhagic shock and encephalopathy syndrome

Gooskens J [1], Kuiken T [4], Claas ECJ [1], Harinck HI [2],
Thijssen JCP [1], Baelde HJ [3], Kroes ACM [1]

Journal of Clinical Virology **2007**; *39: 136–40*

1 Department of Medical Microbiology
Leiden University Medical Center, Leiden, the Netherlands
2 Department of Intensive Care
Leiden University Medical Center, Leiden, the Netherlands
3 Department of Pathology
Leiden University Medical Center, Leiden, the Netherlands
4 Department of Virology
Erasmus University Medical Center, Rotterdam, the Netherlands

Abstract

Influenza-associated encephalopathy is a clinically diverse syndrome and severe cases are not well documented outside Japan. Clinical, pathological and molecular aspects are described of two fatal cases presenting during 2004 and 2005 winter seasons in the Netherlands. Results showed that severe influenza can resemble hemorrhagic shock and encephalopathy syndrome, and proper testing for influenza virus should be considered in similar cases. The failure to detect viral replication in non-pulmonary organs including the brain would support the pathogenesis of this syndrome is based on proinflammatory cytokine responses.

Introduction

Neurological impairment during acute human influenza is defined as influenza-associated encephalopathy (IAE). Patients with IAE have rarely been documented in Europe and North America after the 1957 pandemic [2, 5, 9, 20] and recent clinical presentations are variable [12, 26]. In contrast, cases have frequently been described among Japanese children, characterized by rapidly progressive coma (often <24 h) and high mortality rates (50–100 annually from 1995 to 2000) [19, 21]. Viral RNA detection in cerebrospinal fluid has been reported [6], but is considered a rare finding [7, 20]. Neuroinvasion is therefore disputed as a cause of neurological symptoms, whereas an indirect role for proinflammatory cytokines is suggested as a more likely explanation [15, 26]. Neuroinvasion of avian influenza virus (A/H5N1) has been confirmed in animal models and is suggested for human cases [4]. Distinct clinical forms of encephalopathy associated with acute human influenza include Reye's syndrome, encephalitis, acute necrotizing encephalopathy [19] and the hemorrhagic shock and encephalopathy syndrome (HSE) [22]. We report two patients with IAE and unusual symptoms of hemorrhage, multiple organ failure and shock, admitted to our hospital during the 2003–2004 and 2004–2005 winter seasons. Pathological and molecular studies were performed on tissue specimens following autopsy in one case.

Case reports

Patient 1. A previously healthy 9-year-old girl was found somnolent in the morning with evidence of recent haematemesis. She had a 3-day history of sore throat, cough and 38.5 °C fever, with nausea, vomiting and fever exceeding 40 °C during the previous night. There was no influenza vaccination or acetylsalicylic acid ingestion. During medical transportation she developed coma and required intubation. On admission, the Glasgow Coma Scale was 3 with signs of respiratory distress, endotracheal hemorrhage, epistaxis and diarrhea. Physical examination revealed a temperature of 37.4 °C and blood pressure of 100/40 mmHg. There was no nuchal rigidity, papilledema, pathological reflexes, petechiae, rash or mucous membrane abnormalities. Laboratory examination revealed hypoxia (85% O2 during mechanical ventilation), combined respiratory and metabolic acidosis (pH 7.18, lactate 2.6 mmol/l), low white blood cell count (WBC 3200 mm^{-3} , 59% lymphocytes), renal failure (creatinine 197 µmol/l, urea 9.5 mmol/l) and elevated LDH (752 U/l) and CPK (1455 U/l). She developed anemia (haemoglobin 9.0 to 4.4 mmol/l), leucopenia (WBC 600 mm^{-3}), thrombocytopenia (31×10^{9} l^{-1}), coagulopathy (PTT 31.5 s, aPTT 88.6 s, fibrinogen 1.2 g/l and D-dimer >5000 µg/l), elevated liver enzymes (ASAT 193 U/l, ALAT 39 U/l), glucose (11.8 mmol/l) and amylase (2045 U/l). Chest radiograph showed bilateral infiltrate formation and computed tomography (CT) of the brain appeared normal. Cerebrospinal fluid was not obtained due to coagulopathy. Rapid influenza antigen testing (nasal wash), influenza serology (complement fixation), and viral throat cultures were negative. PCR of throat and nasal wash specimens was positive for influenza A virus and negative for influenza B virus, human respiratory syncytial virus, parainfluenza virus 1–4, human metapneumovirus, rhinovirus, *Mycoplasma sp.* and *Chlamydophila sp.* [17, 23, 24]. Viral culture from nasal wash revealed an influenza virus, characterized by duplicate haemagglutination inhibition tests as influenza A/H3N2/Fujian/411/02-like virus according to standard methods [13, 16] using turkey erythrocytes and four haemagglutinating units of virus. Multiple blood and other bacterial cultures were negative. Her condition rapidly deteriorated despite broad-spectrum antibiotic treatment, dexamethasone and drotrecogin-alfa. She died within 24 hours due to shock, multiple organ failure and disseminated intravascular coagulation (DIC). No autopsy was performed. This case occurred in the midst of the community influenza season in December 2003.

Patient 2. A 17-year-old male adolescent with a stable ventricular septal defect became progressively somnolent following a 1-day history of flu-like symptoms, diarrhea and

neck pain. There was no influenza vaccination or acetylsalicylic acid ingestion. During admission, he progressed into coma (Glasgow Coma Scale 10 to 3) without respiratory symptoms. Profuse hemorrhagic secretions were retrieved during intubation. Physical examination revealed a temperature of 33.7 °C, blood pressure of 90/50 mmHg and skin mottling. There was no nuchal rigidity, papilledema, pathological reflexes, petechiae, rash or mucous membrane abnormalities. Laboratory results revealed 100% oxygen saturation (breathing 5 l oxygen), severe lactic acidosis (pH 6.78, lactate 15.7 mmol/l), leukocytosis 31,200 mm^{-3}, haemoglobin 9.5 mmol/l, progressive coagulopathy (thrombocyte count 88×10^{9} l^{-1}, PTT 19.0 s, aPTT 98.6 s, fibrinogen 1.3 g/l), renal failure (creatinine 288 µmol/l, urea 12.6 mmol/l), and elevated liver enzymes (ASAT 108 U/l, ALAT 25 U/l), glucose (22.4 mmol/l), thyroid-stimulating hormone (14.630 mU/l), LDH (1210 U/l) and CPK (1782 U/l). Toxicology screening was negative. Chest radiograph and electrocardiography appeared normal. CT of the brain was normal, but revealed sphenoid and maxillary sinusitis. Cerebrospinal fluid was not obtained due to coagulopathy. Multiple blood and other bacterial cultures were negative. His condition rapidly deteriorated despite broad-spectrum antibiotic treatment, dexamethasone and drotrecogin-alfa. He died within 24 hours due to progressive multiple organ failure, DIC and shock. Autopsy was performed following parental permission. PCR detected influenza A virus [23] from fresh pulmonary and sinus tissue specimens, but not from pleural and pericardial fluid obtained during autopsy. PCR was negative for other respiratory viruses. Influenza virus cultured from sinus tissue was antigenically characterized as influenza A/H3N2/Wyoming/003/03-like virus. This case occurred in the midst of the community influenza season in February 2005.

Pathological and molecular analysis

Pathology. Autopsy (patient 2) and tissue preparation was performed according to standard procedures, taking precautions to minimize RNA degradation (rapid tissue preparation and fixation to minimize autolysis) and to prevent contamination (separate autopsy of the brain; individual tissue processing in separate cassettes; solitary tissue cutting by sterile microtome and disposable blades). Macroscopic findings included edematous lungs with hemorrhagic pleural fluid. Microscopic examination did not detect encephalitis, necrosis or other abnormalities in the pons, striatum, mesencephalon, hippocampus, cerebellum or four ventricle biopsy specimens. Pulmonary tissue revealed multifocal hemorrhage, bronchitis and early diffuse alveolar damage.

The liver contained no fatty changes and other organs had no major abnormalities. *Streptococcus pneumoniae, Neisseria meningitidis*, herpes simplex virus, varicella-zoster virus and enterovirus were excluded by PCR or polysaccharide antigen testing on post-mortem cerebrospinal fluid. Fresh specimens were not available for further molecular testing.

Immunohistochemistry. Formalin-fixed paraffin-embedded (FFPE) tissue sections of 4 µm were deparaffinized, hydrated and treated with pronase for 10 min at 37 °C. The monoclonal mouse-anti-Influenza A nucleoprotein (IgG2a, Hb65, ATCC) was diluted in PBS/0.1% BSA and incubated with the section for 1 h at room temperature, and bound antibody was detected with an alkaline phosphatase labeled (AP) goat-anti-mouse IgG2a. AP was revealed with fast red chromogen substrate resulting in pink/red precipitate. Focal specific nuclear staining was mainly observed in pulmonary bronchial epithelium cells and in very few cells in the alveoli (type undetermined), but not in other organs **(Table 1)**.

Molecular analysis of viral expression. RNA was extracted from alternate clinical and negative control FFPE tissue sections. Ten 4 µm sections were deparaffinized (xylene), rehydrated and digested overnight at 50 °C in 200 µl lysis buffer (500 µg/ml proteinase K (Roche, Mannheim, Germany) in 20mM Tris (pH 7.4), 1mM EDTA (pH 8.0), 2% SDS). RNA was isolated (TRIzol reagent, Invitrogen, Carlsbad, CA, USA), precipitated (2-propanol, 3 µl of 2 mg/ml glycogen), washed (500 µl of 75% ethanol), dried and re-suspended (60 µl). Purity and quantity of extracted RNA was measured (NanoDrop Technologies, Wilmington, USA). OD 260/280 ratios were ≥1.8 for most sections indicating high purity [3] except for brain tissues with OD ratios <1.8. In addition, the RNA concentration extracted from brain specimens was somewhat lower. Influenza PCR was performed using previously published primers and probe with an equine arteritis virus internal control [23, 17]. Influenza A RNA was detected in all organs **(Table 1)**. A molecular assay was developed for detection of influenza mRNA indicating viral replication. The primers 851FLUA-M2s 5'-GAGYCTTCTAACCGAGGTC and 852FLUA-M2as 5'-CAACAACAAGCGGGTCAC amplified an 83 bp spliced fragment of M2 mRNA as confirmed by sequence analysis **(Figure 1)**, and a 771 bp unspliced fragment of M1 mRNA or viral RNA. The optimized assay was performed using the Qiagen OneStep RT-PCR kit (Qiagen, Hilden, Germany) with 0.2mM dNTP's, 3.5mM MgCl2, 30 pmol each primer and 10 µl extracted RNA. Cycling conditions were 30 min at 50 °C (cDNA synthesis) and 45 cycles (95 °C, 30 s; 51 °C, 30 s; 30 s at 72 °C). PCR product was

analyzed by gel electrophoresis. Spliced M2 mRNA was detected in pulmonary tissue, but not in other organs (Table 1). The sensitivity of the PCR to detect influenza mRNA is unknown and especially difficult to determine in fixed specimens. Spliced M2 mRNA was consistently detected from fixed pulmonary tissue. In addition, spliced mRNA was consistently detected down to a 1:3125 dilution in controls of influenza infected MRC-5 cells, with the knowledge that mRNA is a low abundant messenger.

Table 1. Molecular and immunohistochemical detection of influenza A RNA, antigen and spliced mRNA in paraffin-embedded tissue specimens.

Paraffin-embedded specimens	Influenza A PCR (Ct value)	Influenza B PCR (Ct value)	Influenza A expression		Multiplex EAV spike (Ct value) [g]	Concentration of total extracted RNA (ng/µl)	RNA/protein OD ratio [h] (260/280)
			Antigen	M2 mRNA			
nControl (fixed lung) [a]	–	–	–		27.2	81	1.8
Heart	+ (36.9)	–	–	–	28.0	83	1.8
nControl (fixed lung)	–	–	–		27.3	92	1.8
Liver	+ (37.5)	–	–	–	27.6	289	1.8
nControl (fixed lung)	–	–	–		27.5	90	1.8
Kidney	+ (35.6)	–	–	–	26.2	118	1.8
nControl (fixed lung)	–	–	–		27.1	74	1.8
Mesencephalon	–	–	–	–	27.2	8.9	1.6
nControl (fixed lung)	–	–	–		27.2	69	1.8
Pons	+ (37.6)	–	–	–	26.6	18	1.7
nControl (fixed lung)	–	–	–		28.1	66	1.9
Lung	+ (24.8)	–	+	+	30.1	545	1.8
nControl (fixed tonsil) [a]	–	–	–	–	26.8	114	1.8
nControl (isotype/omission) [b]			–				
Influenza B control (fixed) [c]	–	+ (33.3)	–		26.6	51	1.7
Influenza A control (fixed) [d]			+				
Influenza A control (culture) [e]				+			
Influenza A control (culture) [f]	+ (33.1)	–					

Ct, cycle threshold; M2, matrix gene segment encoding influenza M2 protein; EAV, equine arteritis virus.

a Fixed negative controls, paraffin-embedded pulmonary (cynomolgus macaques) and tonsil (human) tissues.

b Fixed negative controls, isotype- and omission controls.

c Fixed influenza B control, post-mortem paraffin-embedded lung tissue from a patient with influenza B pneumonia.

d Fixed influenza A antigen positive control, paraffin-embedded pulmonary tissue of an experimentally infected cat with influenza A (04-7992-2).

e mRNA positive control, RNA extracted from MRC-5 human lung cells infected by influenza A virus (field isolate).

f Influenza A RNA positive control, RNA extracted from supernatant of LLC-MK2 cells infected by influenza A virus (field isolate).

g Co-amplification with EAV spike as internal control revealed no evidence for inhibition.

h High OD 260/280 ratios (≥1.8) indicate purity of the RNA (Chung et al., 2006).

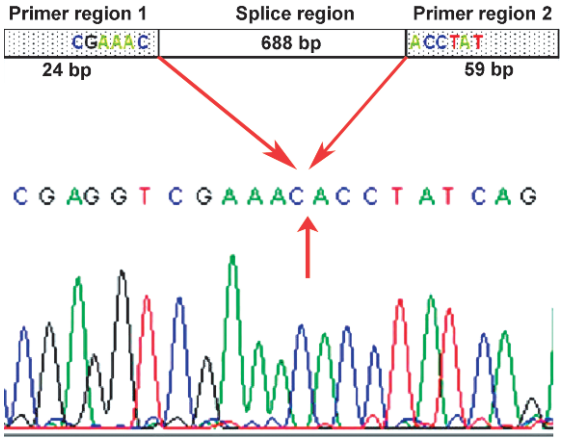


Figure 1. Sequence analysis of the 83 bp M2 mRNA PCR product amplified from RNA extracted from MRC-5 human lung cells infected with influenza A virus (field isolate).
M2 mRNA was confirmed by detection of spliced influenza matrix gene RNA. The splice site (arrow) corresponds to position C51-A740 (A/New York/206/2005; Gen-Bank Accession number CY006132).

Discussion

Two patients with IAE are described, who had rapidly progressive coma during acute influenza infection. The clinical findings and outcome differed substantially from recent Dutch cases with IAE [18, 26]. Although fatal cases of IAE occurred in Japan, fatal cases of IAE have not been documented in the Netherlands after the 1957 pandemic, when 68 patients (mainly children) died during a 3-month period [25].

Clinical similarities to recent Japanese cases include sudden high fever, vomiting or diarrhea, early onset and rapid progression of coma, multiple organ failure and haemodynamic shock [15]. Comparable laboratory results include thrombocytopenia, prolonged coagulation tests and elevated levels of transaminases, LDH and CPK. Differences in our cases include a higher age (most Japanese cases <5 years old), lack of documented convulsions and absence of convincing brain CT abnormalities. Acute necrotizing encephalopathy was excluded in both patients by brain CT [14]. In addition, influenza A RNA was isolated from brain tissue of patient 2. Influenza RNA has not been reported in brain tissue or cerebrospinal fluid of patients with acute necrotizing encephalopathy. Reye's syndrome is considered unlikely as the patients received no

acetylsalicylic acid, were hyperglycemic, and had only mildly elevated liver enzyme levels. The absence of hepatic fatty changes and neural inflammatory cell infiltration also excludes Reye's syndrome and encephalitis as the cause of coma in patient 2.

Importantly, clinical symptoms of both patients strongly resembled HSE using the described criteria [1, 11]. Criteria for HSE for patient 1 were not met due to unavailability of cerebrospinal fluid and the solitary elevated hepatocellular enzyme levels without clear evidence for hepatic dysfunction. Criteria for HSE for patient 2 were not met because of the modest reduction of haemoglobin levels and the solitary elevated hepatocellular enzyme levels without clear evidence for -hepatic dysfunction. Hepatocellular enzyme profiles reflected rhabdomyolysis rather than hepatic dysfunction in both patients. HSE is a severe syndrome with unknown aetiology [10], and associations with acute influenza are confined to Japan. Similar to Japanese findings [22], we detected influenza RNA in multiple organs including the brain taken at autopsy from patient 2. In contrast to Japanese findings, we found no evidence of viral replication in nonpulmonary tissues containing influenza RNA, which could be explained by residual deposition of disseminated influenza RNA from replication elsewhere (lungs) or by the presence of undetectable low-level influenza antigen and mRNA in these non-pulmonary tissues. This would further implicate proinflammatory cytokine responses in the pathogenesis of this syndrome. The rapid clinical deterioration in both patients suggests that antiviral therapy will have little chance of success in hospitalized patients presenting with advanced stage of IAE and HSE-like symptoms. Antiviral intervention during early stages of influenza-like illness might have altered the outcome of the patients described. Additional supportive and immunomodulating therapeutic measures during hospital admission have been suggested [8]. The protective role of influenza vaccination during early stages of childhood, to prevent rare IAE and HSE-like complications during later stages of childhood or adolescence, remains to be determined.

In conclusion, severe influenza can resemble HSE syndrome, emphasizing the need for appropriate diagnostic efforts to demonstrate influenza virus infections in similar cases. Molecular detection of influenza mRNA can be used to detect local influenza replication in archival and clinical specimens.

Conflict of interest

There was no financial support for the study. None of the authors have associations that might pose a conflict of interest.

Acknowledgements

Influenza immunostaining was kindly performed by D. van Riel and influenza antigen characterization by haemagglutination inhibition testing was performed by Dr. G.F. Rimmelzwaan, Department of Virology, Erasmus Medical Center, WHO National Influenza Center. Brain tissue specimens were reviewed for neuropathological characteristics by Dr. A.C. Jobsis and Dr. S.G. van Duinen, Department of Pathology, Leiden University Medical Center. We thank Prof. W.J. Spaan and Dr. H.C. van Leeuwen, Department of Medical Microbiology, Leiden University Medical Center, for helpful suggestions and advice.

References

1. Bacon CJ, Hall SM. Haemorrhagic shock encephalopathy syndrome in the British Isles. *Arch Dis Child* **1992**; 67: 985–93.

2. Bhat N, Wright JG, Broder KR, *et al*. Influenza Special Investigations Team. Influenza-associated deaths among children in the United States, 2003-2004. *N Engl J Med* **2005**; 353: 2559–67.

3. Chung JY, Braunschweig T, Hewitt SM. Optimization of recovery of RNA from formalin-fixed, paraffin-embedded tissue. *Diagn Mol Pathol* **2006**; 15: 229–36.

4. de Jong MD, Bach VC, Phan TQ, *et al*. Fatal avian influenza A (H5N1) in a child presenting with diarrhea followed by coma. *N Engl J Med* **2005**; 352: 686–91.

5. Flewett TH, Hoult JG. Influenzal encephalopathy and postinfluenzal encephalitis. *Lancet* **1958**; 2: 11–5.

6. Fujimoto S, Kobayashi M, Uemura O, *et al*. PCR on cerebrospinal fluid to show influenza-associated acute encephalopathy or encephalitis. Lancet **1998**; 352: 873–5.

7. Ito Y, Ichiyama T, Kimura H, *et al*. Detection of influenza virus RNA by reverse transcription-PCR and proinflammatory cytokines in influenza-virus-associated encephalopathy. *J Med Virol* **1999**; 58: 420–5.

8. Kawashima H, Togashi T, Yamanaka G, *et al*. Efficacy of plasma exchange and methylprednisolone pulse therapy on influenza-associated encephalopathy. *J Infect* **2005**; 51: E53–6.

9. Kirton A, Busche K, Ross C, Wirrell E. Acute necrotizing encephalopathy in Caucasian children: two cases and review of the literature. *J Child Neurol* **2005**; 20: 527–32.

10. Levin M, Hjelm M, Kay JD, *et al*. Haemorrhagic shock and encephalopathy: a new syndrome with a high mortality in young children. *Lancet* **1983**; 2: 64–7.

11. Levin M, Pincott JR, Hjelm M, *et al*. Hemorrhagic shock and encephalopathy: clinical, pathologic, and biochemical features. *J Pediatr* **1989**; 114: 194–203.

12. Maricich SM, Neul JL, Lotze TE, *et al*. Neurologic complications associated with influenza A in children during the 2003-2004 influenza season in Houston, Texas. *Pediatrics* **2004**; 114: e626–33.

13. Masurel N, Ophof P, de Jong P. Antibody response to immunization with influenza A/USSR/77 (H1N1) virus in young individuals primed or unprimed for A/New Jersey/76 (H1N1) virus. *J Hyg (Lond)* **1981**; 87: 201–9.

14. Mizuguchi M, Abe J, Mikkaichi K, *et al*. Acute necrotising encephalopathy of childhood: a new syndrome presenting with multifocal, symmetric brain lesions. *J Neurol Neurosurg Psychiatry* **1995**; 58: 555–61.

15. Morishima T, Togashi T, Yokota S, *et al*; Collaborative Study Group on Influenza-Associated Encephalopathy in Japan. Encephalitis and encephalopathy associated with an influenza epidemic in Japan. *Clin Infect Dis* **2002**; 35: 512–7.

16. Palmer DF, Dowdle WR, Coleman MT, Schild GC. Haemagglutination inhibition test. In: *Anonymous: Advanced laboratory techniques for influenza diagnosis. Procedural guide. Atlanta: US Department of Health, Education and Welfare* **1975**; 25–62.

17. Scheltinga SA, Templeton KE, Beersma MF, Claas EC. Diagnosis of human metapneumovirus and rhinovirus in patients with respiratory tract infections by an internally controlled multiplex real-time RNA PCR. *J Clin Virol* **2005**; 33: 306–11.

18. Smidt MH, Stroink H, Bruinenberg JF, Peeters M. Encephalopathy associated with influenza A. *Eur J Paediatr Neurol* **2004**; 8: 257–60.

19. Sugaya N. Influenza-associated encephalopathy in Japan. *Semin Pediatr Infect Dis* **2002**; 13: 79–84.

20. Steininger C, Popow-Kraupp T, Laferl H, *et al*. Acute encephalopathy associated with influenza A virus infection. *Clin Infect Dis* **2003**; 36: 567–74.

21. Sugaya N, Takeuchi Y. Mass vaccination of schoolchildren against influenza and its impact on the influenza-associated mortality rate among children in Japan. *Clin Infect Dis* **2005**; 41: 939–47.

22. Takahashi M, Yamada T, Nakashita Y, *et al*. Influenza virus-induced encephalopathy: clinicopathologic study of an autopsied case. *Pediatr Int* **2000**; 42: 204–14.

23. Templeton KE, Scheltinga SA, Beersma MF, Kroes AC, Claas EC. Rapid and sensitive method using multiplex real-time PCR for diagnosis of infections by influenza a and influenza B viruses, respiratory syncytial virus, and parainfluenza viruses 1, 2, 3, and 4. *J Clin Microbiol* **2004**; 42: 1564–9.

24. Templeton KE, Scheltinga SA, Graffelman AW, *et al*. Comparison and evaluation of real-time PCR, real-time nucleic acid sequence-based amplification, conventional PCR, and serology for diagnosis of Mycoplasma pneumoniae. *J Clin Microbiol* **2003**; 41: 4366–71.

25. van Geer I, Mulder J. Asian influenza mortality in 1957. *Ned Tijdschr Geneeskd* **1958**; 102: 1208–11.

26. van Zeijl JH, Bakkers J, Wilbrink B, Melchers WJ, Mullaart RA, Galama JM. Influenza-associated encephalopathy: no evidence for neuroinvasion by influenza virus nor for reactivation of human herpesvirus 6 or 7. *Clin Infect Dis* **2005**; 40: 483–5.

Chapter 4

Rapid molecular detection of influenza outbreaks in nursing homes

Gooskens J [1], Swaan CM [2], Claas ECJ [1], Kroes ACM [1]

Journal of Clinical Virology **2008**; *41: 7–12*

1 Department of Medical Microbiology
Leiden University Medical Center, Leiden, the Netherlands
2 Public Health Services
GGD Hollands Midden, Leiden, the Netherlands

Abstract

Background: Nursing home influenza outbreaks occur in spite of established vaccination programs, and require rapid and sensitive laboratory confirmation for timely intervention.

Objectives: To evaluate diagnostic approaches for rapid confirmation of nursing home influenza outbreaks.

Study design: Influenza virus real-time PCR and Directigen Flu A+B enzyme immunoassay were performed on nasopharyngeal swabs, nasopharyngeal washes and throat swabs collected from residents with clinical suspicion of influenza during seven probable nursing home outbreaks in 2004–2005 and 2005–2006. The efficacy of specimen sampling and transport management by Public Health Service outbreak team was evaluated.

Results: PCR detected influenza RNA in 80% (68/85) of specimens from 81% (38/47) residents, confirming six suspected outbreaks. Immunoassay sensitivity was highest on nasopharyngeal swabs (38%; 11/29) with a positive predictive value of 100% compared to PCR. Nasopharyngeal swabs were equally sensitive to nasopharyngeal washes by PCR. Nasopharyngeal wash sampling appeared unpractical due to common underlying disability of residents. Outbreak team support was associated with a shorter time to PCR diagnosis compared to outbreaks with no logistical support (mean, 28.2 h vs. 84 h; $P = 0.05$).

Conclusions: Influenza real-time PCR on nasopharyngeal swabs, obtained by Public Health Service outbreak teams, enabled rapid and sensitive confirmation of nursing home influenza outbreaks.

Introduction

Influenza outbreaks in nursing homes are associated with increased morbidity, hospitalization and mortality among residents [8]. Influenza vaccination effectively reduces the incidence of influenza-like illness (ILI) and associated complications [17], but outbreaks continue to occur with a high burden of disease [6, 12, 16, 19, 26]. The low protection rate among vaccinated elderly is related to the aging immune system, underlying disease and in some cases immunosuppressive medication. Influenza outbreaks are further determined by local vaccination rates of residents and staff [12], vaccine match to circulating strains and waning vaccine-induced immunity due to late seasonal occurrence. New nursing home influenza guidelines therefore recommend additional hygiene and cohorting measures during suspect outbreaks [4, 26], extending to re-offering of influenza vaccination and oseltamivir 'postexposure prophylaxis' during confirmed outbreaks [4, 7, 21]. These measures invariably require rapid and sensitive diagnostic confirmation, a paradigm that is challenged by the use of slow and insensitive conventional laboratory techniques and lack of established logistical support regarding specimen transport and communication strategy [14]. Nursing home influenza outbreak-control would likely benefit from the support of Public Health Services (PHS) to raise influenza awareness, facilitate diagnostic efforts and implement control measures [5, 11]. For diagnostic efforts, nasopharyngeal washes are considered most sensitive [18], however this has not yet been specifically evaluated among elderly. Recent studies demonstrate the importance of detection of other respiratory viruses associated with outbreaks [1, 2, 8, 13]. The present study evaluated rapid influenza immunoassay and PCR detection on nursing homes specimens, and the efficacy of PHS outbreak team support.

Methods

Public Health Service outbreak teams. PHS and nursing home health care workers from the region 'Hollands Midden' in the Netherlands received training in early recognition of clinical influenza, respiratory tract sampling techniques and application of new outbreak-control guidelines. Regional nursing homes were invited by mail to report probable influenza outbreaks during the 2004–2005 and 2005–2006 winter seasons to the PHS department. PHS outbreak teams (physicians and nurses) were responsible for specimen and data collection, transport, advice in outbreak management, and commu-

nication with laboratory staff. A microbiological laboratory performed influenza immunoassay daily during and outside office hours and daily PCR during office hours.

Outbreak procedures. In seven nursing homes with a probable outbreak, clinical specimens were simultaneously obtained from residents with clinical suspicion for influenza. A probable influenza outbreak was defined as two or more residents from the same ward developing four of the following symptoms within 48 h of each other: headache, myalgia, throat pain, fever of sudden onset, cold chills, dry cough and malaise. Nursing homes requested PHS support in five of the seven probable outbreaks. The two remaining outbreaks were sampled and managed by local nursing home health care workers. Temperature and respiratory symptoms were recorded and data obtained by questionnaire included demographics (age, sex), influenza vaccination status, antibiotic use, acute onset (≤48 h) and symptom duration. Clinical data from confirmed cases were compared to ILI criteria defined as a fever (≥38 ˚C), plus a new onset or acute worsening of one or more respiratory symptoms. Nasopharyngeal wash (5 ml PBS each nostril) volume recovery was variable (2–8 ml). Nasopharyngeal swabs (flexible wire, rayon) and throat swabs (woodstick, cotton) were stored in 3ml VTM. Directigen Flu A+B enzyme immunoassay (Becton Dickinson, MD, USA) was performed following the manufacturer's instructions. Multiplex real-time PCR detection of influenza and other respiratory viruses were performed as described previously [20, 25]. PCR cycle threshold (Ct) levels were determined as a surrogate quantitative marker of viral genome equivalents. Viral culture was performed for antigenic typing of circulating viruses from selected specimens.

Influenza typing. Antigenic typing was performed on cultured influenza strains by duplicate haemagglutination inhibition (HI) testing as described previously [9]. For outbreaks with antigenic heterogeneous strains, the two antigenic most deviant viruses were compared by sequence analysis of 1015 bp of the hemagglutinin HA_1 gene fragment (including globular antigenic domains) using primers 983FLUH3s 5'-ATCATTGCTTTGAGCTACATT, 984FLUH3as 5'-AACGTGACTATGCCAAAC, 985FLUH3as 5'-CCCAAATGTACAATTTGTCAA and 986FLUH3as 5'-CTTCCCAACCATTTTCTATG.

Statistical analysis. SPSS software version 11.0.1 (SPSS, Chicago, Illinois, USA) was used to calculate differences in categorical measurements (Pearson chi-square) and continuous variables (unpaired and paired Student's *t*-test). Comparative evaluation of diagnostic delay (hours) with and without outbreak team support was performed using Mann–

Whitney *U*-test. Linear regression analysis was used to correlate PCR results from nasopharyngeal swabs to paired nasopharyngeal washes and throat swabs.

Results

Laboratory detection of influenza and other respiratory viruses. PCR detected influenza RNA in 68/85 (80%) specimens among 38/47 (81%) residents (Table 1A). PCR-based typing revealed 36 (95%) residents with influenza A, 2 (5%) residents with influenza B and no residents with mixed influenza infections. Sensitivity of viral culture (54%; 14/26) and immunoassay (22%; 14/65) were low compared to PCR. Some outbreaks were missed by immunoassay on individual specimens (Table 1A). The immunoassay sensitivity was highest on nasopharyngeal swabs (38%; 11/29) with 100% positive predictive value compared to PCR. Rhinovirus (n = 2), human metapneumovirus (n = 2) and parainfluenza virus 4 (n = 1) were only detected from patients with influenza and not associated with outbreaks.

Characteristics of residents with confirmed influenza. We enrolled 47 residents with 38 confirmed cases of influenza (26 female, median age 85 years) (Table 2). Main symptoms were fever (83%), cough (63%) and malaise (60%). Absence of fever among symptomatic elderly with confirmed influenza (n = 6) was the most important cause of a low sensitivity using ILI criteria (69%). Confirmed cases were frequently vaccinated (82%) and often treated with antibiotics during infection (57%). Physical (aging, underlying or current disease) or mental (dementia) disability frequently limited the collection of clinical data (Table 2) and nasopharyngeal wash sampling (Table 1A) among elderly residents.

Influenza outbreaks, diagnostic intervals and typing results. Influenza was confirmed in 6 of 7 probable outbreaks (5 by influenza A/H3N2; 1 by both influenza A and B strains not further typed), occurring during normal influenza seasons (Table 1B). Time from sampling to report of immunoassay results with outbreak team support was 10.8 h (mean, range 4–19 h). PCR diagnostic intervals with the use of outbreak team support (mean 28.2 h, range 20–42 h) were significantly shorter than without support (mean 84 h, range 72–96 h) (Mann–Whitney *U*-test, $P = 0.05$). Logistical problems included delays in specimen transport and handling, laboratory testing and communication of laboratory results outside normal office hours. Cultured influenza viruses were typed A/H3N2 by HI testing (Table 1B). Viruses from outbreak A, and occasionally

outbreak B and D were closely related to the vaccine strains. Influenza A/H3N2 viruses from outbreaks B and D revealed intrinsic HI titer variability (range 160–960), suggesting co-circulation of multiple H3N2 strains. Sequence analysis of two most antigenic deviant viruses for each outbreak (Outbreak B 05014614, 05014606; Outbreak D 06017519, 06017544), demonstrated identical HA_1 gene sequences within each outbreak. Strains from both outbreaks differed in HA_1 gene sequence indicating seasonal drift.

Table 1A. Molecular and antigen detection of influenza virus and other respiratory virus.

Nursing home	Influenza PCR+		Immunoassay+/PCR+			Multiplex PCR+
	Residents	Specimens	NPW	NPS	TS	Residents with other respiratory viruses
2005						
A	8/9	13/14	1/5	4/8	NA	0
B	8/10	8/10	NA	NA	1/8	0
C	8/8	14/14	0/6 [a]	3/8	NA	1 hMPV
2006						
D	7/8	19/22	0/5 [a]	2/7	0/7 [a]	2 hRV
E	2/4	2/4	NA	0/2 [a]	NA	1 PIV4
F	0/2	0/6	NA	NA	NA	0
G	5/6	12/15	1/3	2/4	0/2 [a]	1 MPV
Total	**38/47**	**68/85**	**2/19**	**11/29**	**1/17**	

NA, not applicable; NPW, nasopharyngeal wash; NPS, nasopharyngeal swab; TS, throat swab; hMPV, human metapneumovirus; hRV, human rhinovirus; PIV4, parainfluenza virus 4.

a Influenza outbreak not detected by rapid antigen test on current specimens.

Table 1B. Influenza outbreak management and typing results.

Nursing home residents	Outbreak date	Outbreak management			Influenza typing		
		Team	PCR (h)	Antigen test (h)	PCR typing	HI typing	Antigenic matching
2005							
A (9)	February 2005	+	22	5	A	A/H3N2	≥960
B (10)	February 2005	–	96 [a]	NA	A	A/H3N2	r 160-960 [b]
C (8)	March 2005	+	42	19	A	A/H3N2	NA
2006							
D (8)	March 2006	+	20	15	A	A/H3N2	r 160-960 [b]
E (4)	March 2006	–	72 [a]	NA	A	A/H3N2	160
F (2)	March 2006	+	29	NA	NA	NA	NA
G (6)	March 2006	+	28	4	A+B	Culture–	Culture–

NA, not applicable; HI, haemagglutination inhibition.

a PCR diagnostic interval without outbreak team support.

b Post-infection ferret antisera to H3N2 strains A/Wyoming/003/03 (home B) and A/California/007/04 (home D).

Table 2. Demographic and clinical characteristics of residents with confirmed influenza by PCR.

Characteristics	Influenza PCR+	
Patients		
Total	38/47	(81%)
Female	26/38	(68%)
Median age (range years)	85	(61–97)
Influenza vaccination	23/28	(82%)
Antibiotic therapy	12/21	(57%)
Symptoms		
Fever (≥38 °C)	29/35	(83%)
Cough	22/35	(63%)
Malaise	12/20	(60%)
URTI (throat pain/runny nose)	16/29	(55%)
Muscle pain	13/28	(46%)
Acute onset (≤48 h)	15/35	(43%)
Headache	6/20	(30%)
Vomiting	6/27	(22%)
Diarrhea	4/27	(15%)
ILI criteria+	24/35	(69%)
Prolonged duration (≥7 days)	9/12	(75%)

Evaluation of influenza PCR cycle threshold values. Immunoassay positive nasopharyngeal specimens had significantly lower PCR Ct values (mean 22.9, range 17.6–28.7) compared to immunoassay negative specimens (mean 31.5, range 18.9–43.3; $P < 0.0001$) **(Figure 1)**. Total nasopharyngeal swabs seemed more sensitive than nasopharyngeal washes by immunoassay (11/29 vs. 2/19) and PCR (mean Ct values 28.1 vs. 30.6; $P = 0.18$). However, paired nasopharyngeal swabs and nasopharyngeal washes (n = 18) revealed more comparable immunoassay results (4/18 vs. 2/18) and equally sensitive and correlated PCR results ($r^2 = 0.55$, $P < 0.0001$; mean Ct difference −0.1, $P = 0.94$) **(Figure 2A)**. Nasopharyngeal swabs were significantly more sensitive than paired throat swabs by immunoassay test (4/11 vs. 0/11) and PCR (–4.7, $P = 0.005$) with significant correlation ($r^2 = 0.61$, $P = 0.005$) **(Figure 2B)**.

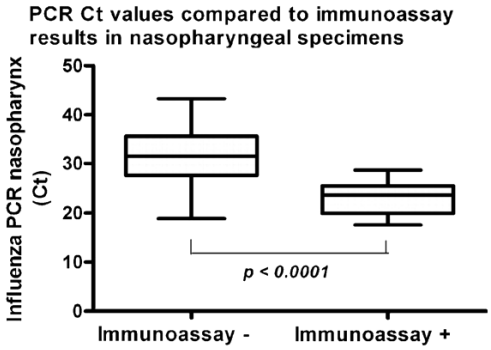


Figure 1. PCR Ct values compared to immunoassay results in nasopharyngeal specimens.

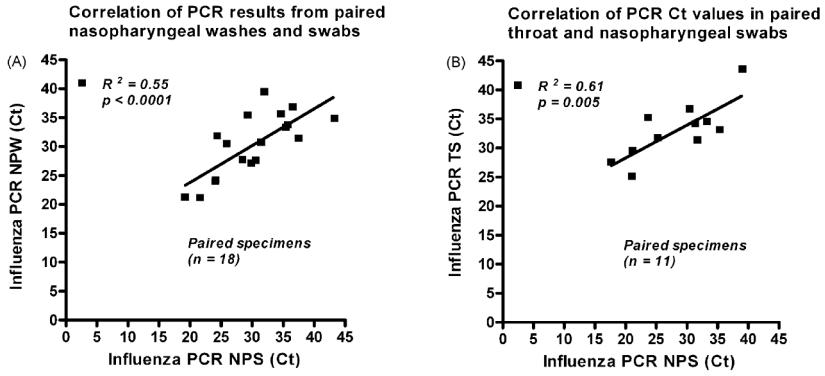


Figure 2. Correlation of PCR Ct values from paired nasopharyngeal washes and swabs (A) and from paired throat and nasopharyngeal swabs (B).

Discussion

Early intervention during nursing home influenza outbreak leads to improved control [3, 7, 21], but depends on rapid and sensitive laboratory confirmation that proves difficult to implement [5, 14]. This study evaluates diagnostic approaches for rapid confirmation of nursing home influenza outbreaks, with support of the PHS department.

Evaluation of the adequacy of sampling revealed important practical and diagnostic insights. Nasopharyngeal washes are considered most sensitive specimens for detection

of respiratory viruses in adults [18], however wash sampling proved to be unpractical among frail elderly with common mental or physical disability. Nasopharyngeal swabs revealed a high concordance to paired nasopharyngeal wash specimens by PCR **(Figure 2A)**, not significantly affected by VTM dilution. Nasopharyngeal swab PCR Ct values were continuously 4.7 cycles lower than in paired throat swabs indicating a 10-100-fold higher sensitivity. Outbreak team support was associated with shorter PCR diagnostic intervals (mean, 28.2 h vs. 84 h; $P = 0.05$), indicating that outbreak team measures directly can accelerate nursing home influenza outbreak diagnosis. We conclude that PCR on nasopharyngeal swabs obtained by PHS outbreak teams provide most practical and sensitive confirmation of nursing home influenza outbreaks.

PCR sensitivity was clearly superior to culture and immunoassay. Compared to PCR, immunoassay sensitivity on total specimens was low (22%; 14/65) similar to other studies [10, 23], but higher on nasopharyngeal swabs (38%). Immunoassays are notoriously unreliable to detect outbreaks, illustrated in this study on individual specimens **(Table 1A)**. A similar performance could be expected from other immunoassay tests [15, 22]. Alternative sampling techniques could be studied to increase immunoassay sensitivity, including the use of flocked swabs. Immunoassays remain attractive for their convenience, speed and high positive predictive value (100%), however negative results should always be confirmed by more sensitive tests.

All influenza outbreaks presented in the midst of influenza seasons. Outbreaks among residents with clinical suspicion for influenza were not associated to other respiratory viruses capable of causing outbreaks [1, 2, 8, 13]. Influenza outbreaks are mainly associated with low vaccination coverage among residents or poor vaccine match. Relevant findings were that residents with confirmed influenza were often vaccinated (82%) and that all cultured influenza viruses matched the vaccine strains. These results are in agreement to recent 2004–2005 cross-sectional surveys in the Netherlands [26] describing nursing homes with high vaccine coverage frequently experiencing clinical (49%) and laboratory confirmed (29%) influenza outbreaks. Similarly, Canadian surveys demonstrated influenza outbreaks in more than one third of highly immunized nursing homes [24]. High attack rates (≥50%) and case-fatality rates are reported during influenza outbreaks in nursing homes with high vaccine coverage [6, 14], underlining the importance of rapid diagnostics and outbreak prevention and control. In this study, influenza viruses isolated from three outbreaks (A, B and D) were antigenically highly related to the vaccine strains. This could be explained by waning immunity since the outbreaks

presented 5–6 months after vaccination, or insufficient vaccine protective immunity against the circulating strains as documented previously for 2004–2005 influenza outbreaks [16]. Influenza viruses with variable HI titers (range 160–960) isolated during Outbreak B and D had identical HA_1 sequences. Alternative explanations for antigenic variability of these strains are posttranslational modification of the HA_1, mutations in other antigenic domains, difference in avidity due to conformational changes or intrinsic haemagglutination inhibition test variability. The clinical significance of these findings remains to be determined.

In conclusion, this study offers further insight to the feasibility of practical and rapid confirmation of nursing home influenza outbreaks by PCR with support of trained PHS outbreak teams. Data on logistical aspects of rapid confirmation of outbreaks are much awaited with the increasing concerns about epidemiological aspects of influenza.

Conflict of interest

There was no financial support for the study. None of the authors have associations that might pose a conflict of interest.

Acknowledgements

We thank Dr. G.F. Rimmelzwaan and R. van Beek of the National Influenza Center for influenza antigen characterization of circulating strains by haemagglutination inhibition testing. In addition, we thank the staff and nurses of the participating nursing homes and the Public Health Service department for their commitment to implement new outbreak-control guidelines and collect specimens and clinical data.

References

1. Birch CJ, Clothier HJ, Seccull A, *et al*. Human coronavirus OC43 causes influenza-like illness in residents and staff of aged-care facilities in Melbourne, Australia. *Epidemiol Infect* **2005**; 133: 273–7.

2. Boivin G, De Serres G, Hamelin ME, *et al*. An outbreak of severe respiratory tract infection due to human metapneumovirus in a long-term care facility. *Clin Infect Dis* **2007**; 44: 1152–8.

3. Bowles SK, Lee W, Simor AE, *et al*; Oseltamivir Compassionate Use Program Group. Use of oseltamivir during influenza outbreaks in Ontario nursing homes, 1999-2000. *J Am Geriatr Soc* **2002**; 50: 608–16.

4. Bridges CB, Fukuda K, Uyeki TM, Cox NJ, Singleton JA. Prevention and control of influenza. Recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Recomm Rep* **2002**; 51: 1–31.

5. Bridges CB, Harper S. The full-court press for influenza prevention in elderly persons. *Clin Infect Dis* **2004**; 39: 465–7.

6. Coles FB, Balzano GJ, Morse DL. An outbreak of influenza A (H3N2) in a well immunized nursing home population. *J Am Geriatr Soc* **1992**; 40: 589–92.

7. Drinka PJ, Krause P, Nest L, Gravenstein S, Goodman B, Shult P. Delays in the application of outbreak control prophylaxis for influenza A in a nursing home. *Infect Control Hosp Epidemiol* **2002**; 23: 600–3.

8. Ellis SE, Coffey CS, Mitchel EF, Dittus RS, Griffin MR. Influenza- and respiratory syncytial virus-associated morbidity and mortality in the nursing home population. *J Am Geriatr Soc* **2003**; 51: 761–7.

9. Gooskens J, Kuiken T, Claas EC, *et al*. Severe influenza resembling hemorrhagic shock and encephalopathy syndrome. *J Clin Virol* **2007**; 39: 136–40.

10. Grondahl B, Puppe W, Weigl J, Schmitt HJ. Comparison of the BD Directigen Flu A+B Kit and the Abbott TestPack RSV with a multiplex RT-PCR ELISA for rapid detection of influenza viruses and respiratory syncytial virus. *Clin Microbiol Infect* **2005**; 11: 848–50.

11. Gupta RK, Zhao H, Cooke M, *et al*. Public health responses to influenza in care homes: a questionnaire-based study of local Health Protection Units. *J Public Health (Oxf)* **2007**; 29: 88–90.

12. Hayward AC, Harling R, Wetten S, *et al*. Effectiveness of an influenza vaccine programme for care home staff to prevent death, morbidity, and health service use among residents: cluster randomised controlled trial. *BMJ* **2006**; 333: 1241–4.

13. Hicks LA, Shepard CW, Britz PH, *et al*. Two outbreaks of severe respiratory disease in nursing homes associated with rhinovirus. *J Am Geriatr Soc* **2006**; 54: 284–9.

14. Hill T, Platzer A, Reyes C. Influenza deaths in spite of immunization and prophylaxis. *Clin Infect Dis* **2005**; 40: 492–3.

15. Hurt AC, Alexander R, Hibbert J, Deed N, Barr IG. Performance of six influenza rapid tests in detecting human influenza in clinical specimens. *J Clin Virol* **2007**; 39: 132–5.

16. Iorio AM, Neri M, Lepri E, *et al*. An influenza A/H3 outbreak during the 2004/2005 winter in elderly vaccinated people living in a nursing home. *Vaccine* **2006**; 24: 6615–9.

17. Jefferson T, Rivetti D, Rivetti A, Rudin M, Di Pietrantonj C, Demicheli V. Efficacy and effectiveness of influenza vaccines in elderly people: a systematic review. *Lancet* **2005**; 366: 1165–74.

18. Kaiser L, Briones MS, Hayden FG. Performance of virus isolation and Directigen Flu A to detect influenza A virus in experimental human infection. *J Clin Virol* **1999**; 14: 191–7.

19. Monto AS, Rotthoff J, Teich E, *et al*. Detection and control of influenza outbreaks in well-vaccinated nursing home populations. *Clin Infect Dis* **2004**; 39: 459–64.

20. Scheltinga SA, Templeton KE, Beersma MF, Claas EC. Diagnosis of human metapneumovirus and rhinovirus in patients with respiratory tract infections by an internally controlled multiplex real-time RNA PCR. *J Clin Virol* **2005**; 33: 306–11.

21. Shijubo N, Yamada G, Takahashi M, Tokunoh T, Suzuki T, Abe S. Experience with oseltamivir in the control of nursing home influenza A outbreak. *Intern Med* **2002**; 41: 366–70.

22. Smit M, Beynon KA, Murdoch DR, Jennings LC. Comparison of the NOW Influenza A & B, NOW Flu A, NOW Flu B, and Directigen Flu A+B assays, and immunofluorescence with viral culture for the detection of influenza A and B viruses. *Diagn Microbiol Infect Dis* **2007**; 57: 67–70.

23. Stein J, Louie J, Flanders S, *et al*. Performance characteristics of clinical diagnosis, a clinical decision rule, and a rapid influenza test in the detection of influenza infection in a community sample of adults. *Ann Emerg Med* **2005**; 46: 412–9.

24. Stevenson CG, McArthur MA, Naus M, Abraham E, McGeer AJ. Prevention of influenza and pneumococcal pneumonia in Canadian long-term care facilities: how are we doing? *CMAJ* **2001**; 164: 1413–9.

25. Templeton KE, Scheltinga SA, Beersma MF, Kroes AC, Claas EC. Rapid and sensitive method using multiplex real-time PCR for diagnosis of infections by influenza a and influenza B viruses, respiratory syncytial virus and parainfluenza viruses 1 2 3 and 4. *J Clin Microbiol* **2004**; 42: 1564–9.

26. van der Sande MA, Ruijs WL, Meijer A, Cools HJ, van der Plas SM. Use of oseltamivir in Dutch nursing homes during the 2004–2005 influenza season. *Vaccine* **2006**; 24: 6664–9.

Chapter 5

Prolonged influenza virus infection during lymphocytopenia and frequent detection of drug-resistant viruses

Gooskens J [1], Jonges M [2], Claas ECJ [1], Meijer A [2], Kroes ACM [1]

The Journal of Infectious Diseases **2009**; *199: 1435–41*

1 Department of Medical Microbiology
Leiden University Medical Center, Leiden, the Netherlands
2 Virology section
National Institute of Public Health and the Environment,
Bilthoven, the Netherlands

Abstract

The factors that cause prolonged human influenza virus respiratory tract infection and determine its clinical impact and the development of drug-resistant viruses are unclear. During a 3-year period, symptomatic influenza virus excretion for ≥2 weeks was observed among 8 immunocompromised patients and found to be associated with lymphocytopenia at onset (8 of 8 patients) more often than with granulocytopenia (2 of 8 patients) or monocytopenia (2 of 8 patients). Six (75%) of 8 patients developed influenza lower respiratory tract infection (10 episodes), and receipt of oseltamivir treatment was significantly associated with clinical improvement (8 of 8 episodes vs. 0 of 2 untreated episodes; $P = .02$). Complete viral clearance was strongly correlated with lymphocyte reconstitution ($P = .04$) but was never observed during the first 2 weeks after oseltamivir treatment. Neuraminidase inhibitor-resistant influenza viruses emerged in 2 (67%) of 3 patients eligible for resistance analysis. In conclusion, prolonged influenza virus infection was associated with lymphocytopenia, influenza lower respiratory tract infection, and frequent development of drug resistance during antiviral therapy. Clinical improvement in influenza lower respiratory tract infection is observed during oseltamivir treatment, but complete viral clearance is dependent on lymphocyte reconstitution, irrespective of receipt of antiviral medication.

Introduction

Human influenza viruses cause frequent morbidity and mortality, particularly in high-risk populations [1]. Antiviral drugs are important for treatment of infection and control of transmission during seasonal and pandemic influenza [2]. The recent and unexplained widespread emergence of circulating human influenza viruses with reduced sensitivity to adamantanes and neuraminidase (NA) inhibitors (NAIs) is therefore of major concern [2-5]. The potential modes by which NAI-resistant influenza viruses emerge merit further analysis. Particularly among children and immunocompromised hosts, prolonged viral excretion is associated with a higher incidence of drug resistance during antiviral therapy [6-13]. The frequency with which such prolonged influenza viral excretion occurs, the clinical consequences of this condition, the development of antiviral resistance, and its correlation with impaired cell-mediated immunity are unclear, however [1, 14]. During a 3-year period, these questions were addressed through careful analysis of findings in 8 patients who presented with prolonged influenza virus respiratory tract infection.

Methods

Patients and specimens. The study included patients with prolonged influenza virus respiratory tract infection (defined below) observed at Leiden University Medical Center from March 2005 through April 2008. Respiratory specimens were obtained during episodes of symptomatic respiratory tract disease. Routine follow-up sampling was not performed during asymptomatic episodes that occurred in patients with established prolonged influenza virus infection, except for patient 1 and on single occasions for patients 3–6 to document complete viral clearance. Influenza virus type A or B RNA was detected in respiratory specimens by reverse-transcriptase polymerase chain reaction (PCR) [15]. Medical records were reviewed for underlying immunodeficiency and clinical characteristics during presentation, follow-up, and outcome, including upper or lower respiratory tract infection symptoms, intubation, antiviral therapy, viral clearance, leukocyte counts, hematopoietic stem cell transplantation (HSCT), and survival. Minimum cell count percentages for granulocytes, monocytes, and lymphocytes determined within 1 week before or after initial detection of viral RNA (i.e., onset) were correlated with percentages determined within 2 weeks after final influenza virus RNA detection or within 1 week before fatal influenza pneumonia.

Definitions. Prolonged influenza virus respiratory tract infection was defined as PCR-positive status combined with symptomatic respiratory tract disease for ≥2 weeks after primary laboratory confirmation of influenza virus infection. This definition is based on documented maximum durations of excretion and approximate median duration of symptoms among immunocompetent children (duration of excretion, <14 days; duration of symptoms, 7.1 days) [16, 17] and adults (duration of excretion, 5.5 days; duration of symptoms, 6 days) [18, 19] and mean durations of excretion among immunocompromised autologous HSCT recipients (6.7 days) and allogeneic HSCT recipients (11.1 days) [20]. Influenza lower respiratory tract infection was diagnosed clinically (as dyspnea) and by PCR, and fatal influenza pneumonia was diagnosed by histology and PCR of pulmonary tissue specimens. Minimum reference cell counts for granulocytes (1800 cells/mm^3), monocytes (135 cells/mm^3), and lymphocytes (900 cells/mm^3) were used for adults, and pediatric values were age dependent. These thresholds were used to determine granulocytopenia, monocytopenia, and lymphocytopenia. Precursor virus was defined as influenza virus detected in a patient before antiviral therapy was started. Seasonal viruses were defined as community-acquired influenza viruses collected from patients with influenza-like illness or acute respiratory infection who had consulted a general practitioner involved in the sentinel surveillance system in The Netherlands. Baseline NAI susceptibility for seasonal viruses was defined as the IC_{50} value (i.e., the mean concentration of NAI needed to inhibit the NA enzyme activity by 50%), after removal of outlier IC_{50} values. NAI drug resistance was defined as a viral IC_{50} value ≥8-fold higher than that of the corresponding sensitive precursor virus [21] and detection of specific NA gene mutations known to confer NAI drug resistance. In the absence of a precursor virus isolate, the IC_{50} of a presumed drug-resistant isolate was compared with baseline NAI susceptibility to calculate the *n*-fold change in drug susceptibility.

Influenza virus characterization and drug susceptibility testing. Influenza virus-eswere cultured in Madin-Darby canine kidney cells from specimens stored at -80°C. Antigens were characterized by hemagglutination inhibition testing, as described elsewhere [22]. Phenotypic susceptibility (IC_{50}) for oseltamivir carboxylate (GS4071; Roche Diagnostics) and zanamivir (GG167; GlaxoSmithKline) was determined as described elsewhere [23]. Emergence of NAI-resistant viruses was confirmed by phenotypic and sequence analysis of precursor viruses. Influenza virus RNA was extracted from culture supernatant or clinical specimens using the QIAamp Viral RNA kit (Qiagen). Specific primers (available on request) were used for transcription (ThermoScript reverse transcriptase; Invitrogen) and cDNA amplification (Phusion High-Fidelity

Taq polymerase; Finnzymes) of influenza NA, hemagglutinin (HA) and matrix 2 (M2) genes. DNA sequences were analyzed using Bionumerics software (version 5.1; Applied Maths).

Data analysis. Patients treated with oseltamivir and untreated patients were compared with respect to clinical recovery from lower respiratory tract infection, by use of Pearson's chi-square tests and 2-sided Fisher's exact tests (SPSS software, version 14.0.2; SPSS). The percentages of the granulocyte, monocyte, and lymphocyte minimum cell counts at onset were compared with the percentages of these counts during viral clearance, by use of the Wilcoxon matched-pairs signed rank test.

Results

Patients with prolonged influenza virus respiratory tract infection. Eight immunocompromised patients (median age, 52 years [range, 0–66 years]) were identified who had prolonged influenza virus excretion (median duration of excretion, 29.5 days [range, 14–275 days]) and symptomatic respiratory tract disease (Table 1). These patients' underlying diseases included hematological malignancy (n = 7) and severe combined immunodeficiency (n = 1). The duration of symptoms observed by healthcare workers before the first detection of influenza virus was 2 days for patient 5 (coryza and fever), 2 days for patient 8 (dyspnea), 4 days for patients 2 and 3 (dyspnea and fever), 14 days for patient 6 (cough), and 20 days for patient 7 (cough). For patient 1, the duration of symptoms (cough) was 6 weeks before the first viral isolation, according to the history provided by a parent. Symptom duration could not reliably be determined for patient 4, who had chronic underlying pulmonary disease. Lymphocytopenia was more common at the onset of prolonged influenza virus infection (8 of 8 patients) than were granulocytopenia (2 of 8 patients) or monocytopenia (2 of 8 patients).

Influenza virus characterization. Viruses cultured from 4 patients were characterized as influenza A/Wyoming/003/03 (H3N2)–like (patient 1), A/California/007/04 (H3N2)–like (patient 2), A/Wisconsin/67/05 (H3N2)–like (patient 5) matching corresponding vaccine strains, and A/Solomon Islands/03/06 (H1N1)–like with a poor vaccine match (patient 8) (i.e., ~16-fold difference by duplicate hemagglutination inhibition titers). The remaining 4 influenza viruses A (H3N2) (patient 3), A (H1N1) (patient 6), and B (patients 4 and 7) could not be cultured or remained uncharacterized.

Table 1. Patients with prolonged influenza virus excretion and sequential susceptibility results.

Patient							Relevant genetic amino acid substitutions (GenBank accession no.)			NA inhibition assay, IC_{50}, nmol/L	
No.	Sex	Age, years	Underlying disease (duration)	Flu typing (duration of excretion) [a]	Infection date	Antiviral therapy	NA gene [b]	HA_1 gene [c]	M2 gene [d]	Oseltamivir	Zanamivir
8	M	66	Acute myelogenous leukemia (46 months), allogeneic stem cell transplantation (34 months), donor lymphocyte infusions (25 months)	A H1N1 (23 days)	Day 1	None	H274Y (AB462370)	NP	NP	471	0.96
7	F	58	Acute myelogenous leukemia (5 months), allogeneic stem cell transplantation (3 months)	B (14 days)	Day 0	None	None (AB462368)	NP	. . .	9.9	4.4
6	M	60	Non-Hodgkin lymphoma (7 years), allogeneic stem cell transplantation (8 months)	A H1N1 (27 days)	Day 0	None	H274Y (AB465342)	NP	NP	ND	ND
5	M	13	Non-Hodgkin lymphoma (34 months)	A H3N2 (32 days)	Day 4	None	None (AB462351)	None (AB462373)	None (AB462350)	0.32	0.57
4	M	19	Anaplastic large cell lymphoma (10 years), allogeneic stem cell transplantation (33 months), donor lymphocyte infusions (12 months)	B (14 days)	Day 0	None	ND	NP	. . .	ND	ND
3	M	57	Non-Hodgkin lymphoma (2 months)	A H3N2) (55 days)	Day 1	None	None (AB462363)	None (AB462362)	S31N (AB462364)	ND	ND
					Day 22	None	None (AB462366)	L111L/I (AB462365)	S31N (AB462367)	ND	ND
					Day 55	None	ND	ND	ND	ND	ND

Table 1. *(continued)* **Patients with prolonged influenza virus excretion and sequential susceptibility results.**

Patient							Relevant genetic amino acid substitutions (GenBank accession no.)			NA inhibition assay, IC_{50}, nmol/L	
No.	**Sex**	**Age, years**	**Underlying disease (duration)**	**Flu typing (duration of excretion)** [a]	**Infection date**	**Antiviral therapy**	**NA gene** [b]	**HA_1 gene** [c]	**M2 gene** [d]	**Oseltamivir**	**Zanamivir**
2	M	47	Non-Hodgkin lymphoma (10 years), allogeneic stem cell transplantation (16 months), donor lymphocyte infusions (11 months)	A H3N2 (64 days)	Day 0	None	None (AB462355)	None (AB462354)	S31N (AB462356)	ND	ND
					Day 7	Oseltamivir	E119V, R292K (AB462358)	A138S (AB462357)	S31N (AB462359)	94	3.3
					Day 38	None	None (AB462360)	ND	S31N (AB462361)	ND	ND
					Day 64	None	ND	ND	ND	ND	ND
1	F	0	Severe combined immunodeficiency (3 months)	A H3N2 (275 days)	Day 0	None	None (AB462526)	135T/A, 189N/K (AB462525)	None (AB462527)	0.56	1.2
					Day 78	Oseltamivir	R292K (AB462529)	H56Y, T135A, N189K (AB462528)	A30S (AB462530)	4382	26
					Days 85 and 89 [d]	Oseltamivir	E119V (AB462532) (AB462535)	T135T/A, D188D/N N189N/K (AB462531) (AB462534)	None (AB462533) (AB462536)	Range, 44–62	Range, 1.0–1.4
					Days 152, 173, and 244 [d]	None	E119V (AB462538) (AB462541) (AB462543)	H56H/Y, Q57Q/R, Y94Y/H, T128T/A, T135T/A, A138A/S, D188D/N, N189N/K (AB462537) (AB462540) (AB462542)	None (AB462539) (AB462544)	Range, 46–50	Range, 0.96–1.0
					Day 260	Oseltamivir	E119V (AB462546)	Y94Y/H, T128T/A, D188D/N (AB462545)	None (AB462547)	53	1.1

NOTE. HA_1, hemagglutinin 1; M2, matrix 2; NA, neuraminidase;
ND, no data obtained because of primer mismatch, poor viral growth, or insufficient NA activity; NP, test not performed.

a Duration of continuous influenza virus excretion demonstrated by polymerase chain reaction.
b NA and M2 mutations associated with antiviral resistance.
c HA_1 mutations were compared with the precursor virus.
d Combined sequence and antiviral resistance results for multiple viruses are depicted.

Detection of antiviral-resistant viruses. Five of 8 patients were treated with oseltamivir (Figure 1). Two of the 5 (patients 4 and 8) were excluded from resistance analysis because of low viral levels and no viral growth or because of NAI-resistant precursor influenza virus. Among the 3 patients for whom resistance analysis was performed (patients 1–3), NAI-resistant viruses emerged in patients 1 and 2. In patient 1, an influenza A (H3N2) NA gene R292K escape mutant emerged during therapy, with a >7000-fold increase in resistance to oseltamivir and a >20-fold increase in resistance to zanamivir, compared with precursor virus (Table 1). This virus reverted to an NA gene E119V mutant virus that had ~100-fold reduced susceptibility to oseltamivir compared with precursor virus but was sensitive to zanamivir (Table 1). Continuous E119V virus excretion was confirmed for 6 weeks during oseltamivir therapy and for >4 months after cessation of antiviral therapy. Sequence analysis during follow-up revealed transient HA gene T/A135T/A, A138S, D188D/N, and N/K189N/K substitutions at or near the receptor binding site compared with precursor virus. In patient 2, a mixed population of influenza A (H3N2) NA gene R292K (10%) and NA gene E119V (90%) escape mutants with HA gene A138S substitution at the receptor binding site emerged during therapy (Table 1). Because precursor virus isolation in cell culture was unsuccessful, possibly owing to low viral load, it was not possible to generate precursor IC_{50} values. Nevertheless, HA and NA sequences derived from the precursor clinical specimen were free of known resistance markers and were very similar to those of seasonal A (H3N2) viruses. Therefore, the IC_{50} of the antiviral-resistant A (H3N2) virus isolate that emerged could validly be compared with its baseline NAI susceptibility and was ~300-fold for oseltamivir and ~6-fold for zanamivir. The mixed population of NAI-resistant viruses subsequently reverted to sensitive wild-type virus 3 weeks after discontinuation of oseltamivir. Influenza A M2 gene mutations conferring adamantane resistance were detected in samples from 3 of 6 patients (patients 1–3). The influenza A (H3N2) M2 gene S31N mutations observed in samples from patients 2 and 3 were pre-existent and genetically stable, in contrast to the newly acquired A30S mutation that subsequently reverted to adamantane-sensitive virus in patient 1.

Clinical outcome and viral clearance. Six (75%) of 8 patients had 10 episodes of influenza lower respiratory tract infection (Figure 1), and 4 patients required mechanical ventilation. Oseltamivir treatment was associated with clinical improvement of lower respiratory tract infection (8 of 8 episodes; patients 1–4 and patient 8), compared with untreated episodes in patients who died of influenza pneumonia (improvement in 0 of 2 episodes; patients 7 and 8) ($P = .02$, by Fisher's exact test). Influenza lower respiratory tract infection reappeared in 4 patients after antiviral therapy and clinical

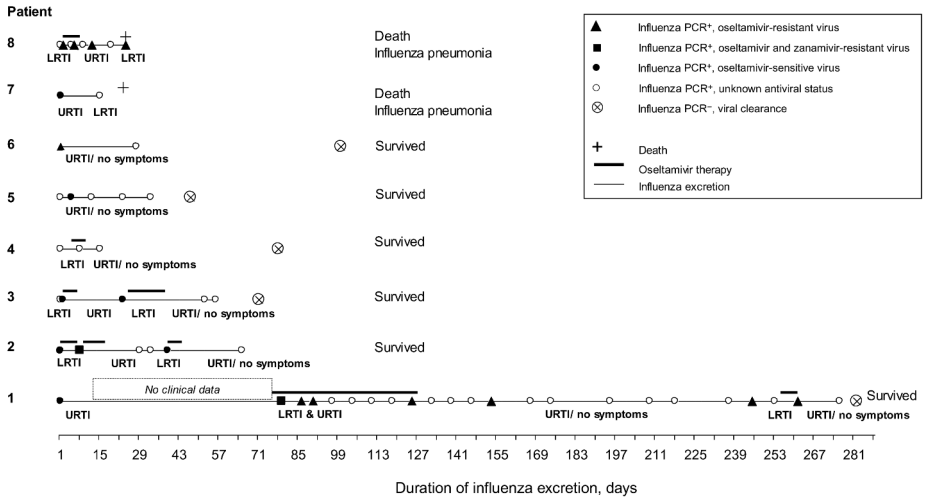


Figure 1. Timeline for patients with prolonged excretion of influenza virus.

Patient numbers correlate with numbers depicted in Table 1. Day 0 correlates with the day influenza virus was initially detected by culture or PCR. Data on antiviral susceptibility or resistance were lacking for many time points when no viral isolate or RNA nucleotide sequence could be obtained because of low viral levels and no viral growth. LRTI, lower respiratory tract infection; URTI, upper respiratory tract infection.

improvement **(Figure 1)**. Renewed clinical deterioration was likely caused by the same influenza A (H3N2) virus in each of 3 patients, because the NA gene sequences were identical (≤0.1% mutation frequency) to those of the precursor virus. NA sequences differed significantly among the 3 patients (>0.5% mutation frequency). Confirmed viral clearance in 5 patients correlated with lymphocyte reconstitution ($P = .04$) **(Figure 2)** and was attributed to successful HSCT (patient 1 at day 247 and patient 5 at day 22) or to spontaneous lymphocyte reconstitution (patients 3, 4, and 6). In contrast, decreasing lymphocyte counts were observed in 2 patients with fatal influenza pneumonia **(Figure 2)**. Viral clearance was never observed during the first 2 weeks after 8 courses of oseltamivir administered to 5 patients (5 regular courses of 5–6 days; 3 prolonged courses of 13–37 days) **(Figure 1)** or after unsuccessful HSCT (day 110, patient 1). Three patients never received oseltamivir treatment **(Figure 1)**; 1 died of unrecognized fatal influenza pneumonia (patient 7), and 2 rapidly cleared the virus after lymphocyte reconstitution that followed successful HSCT (patient 5) or spontaneous lymphocyte reconstitution that followed "mild" lymphocytopenia (i.e., 55% of reference minimum cell count) at onset (patient 6).

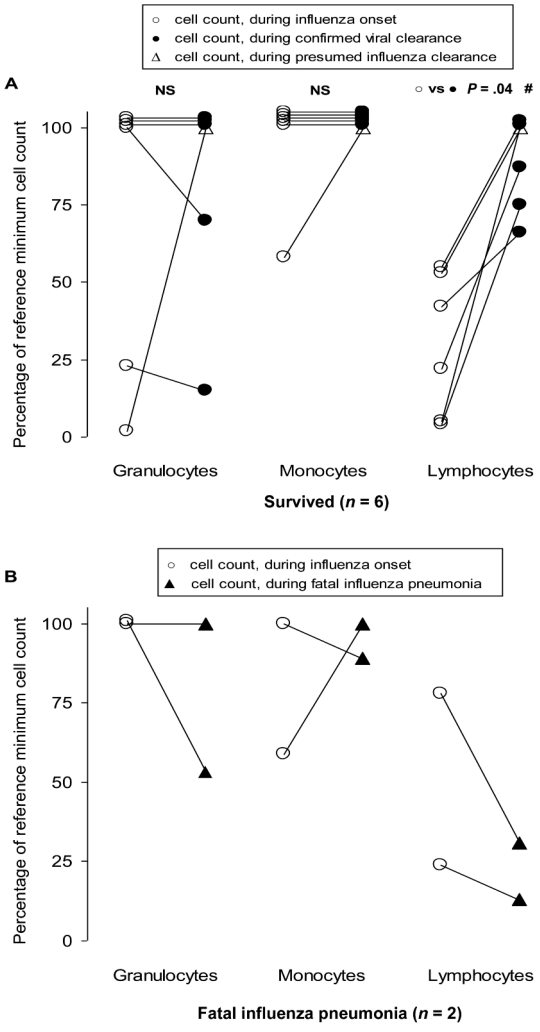


Figure 2. Cellular immune response (cell counts) during prolonged influenza excretion among survivors (2A) and patients with fatal influenza pneumonia (2B).

Cell count during influenza onset was obtained ≤1 week before or after first positive polymerase chain reaction (PCR) result for influenza; cell count during confirmed viral clearance or presumed influenza was obtained ≤2 weeks after final positive PCR result; cell count during fatal influenza pneumonia was obtained ≤1 week before fatal influenza pneumonia. #Wilcoxon matched pairs signed rank test revealed significant lymphocyte reconstitution during confirmed viral clearance. NS, not significant.

Discussion

The occurrence and clinical manifestations of prolonged influenza virus respiratory tract infection and the associated risk of developing antiviral resistance are poorly documented. This study included 8 immunocompromised patients who had symptomatic prolonged viral excretion for ≥2 weeks, with development of influenza lower respiratory tract infection in 6 patients (10 episodes) and frequent detection of drug resistance after therapy.

The occurrence of prolonged influenza virus infection among 8 patients during a 3-year period in a hospital setting appeared to be more common than would have been expected from the few cases documented in the literature [8-12]. All 8 patients in the current study who had prolonged influenza virus infection were immunocompromised with lymphocytopenia, but a few presented with either granulocytopenia (2 of 8 patients) or monocytopenia (2 of 8 patients). This suggests that unimpaired cell-mediated immunity is important for the timely elimination of influenza viruses, a result that agrees with earlier observations [14]. In this study, complete viral clearance was associated with lymphocyte reconstitution ($P = .04$) **(Figure 2)** and was never observed within the first 2 weeks after oseltamivir therapy **(Figure 1)**. This provides new evidence that complete influenza virus clearance depends on lymphocyte reconstitution, irrespective of receipt of oseltamivir therapy. Variable outcomes among patients who never received antiviral therapy indicate that the level and duration of lymphocytopenia could determine later outcome during prolonged influenza virus infection.

NAI-resistant influenza viruses emerged in 2 (67%) of 3 patients eligible for resistance analysis. This rate is much higher than incidences of oseltamivir resistance reported for immunocompetent adults or adolescents (0.33%–2%) and pediatric patients (4%–18%) during clinical trials [6, 7, 13]. Emergence of NAI-resistant influenza viruses was confirmed in patients 1 and 2. In patient 1, an influenza A (H3N2) virus with NA gene R292K substitution, which conferred high-level oseltamivir resistance (>7000-fold increase, compared with precursor virus) and zanamivir resistance (>20-fold increase, compared with precursor virus) **(Table 1)**, emerged from wild-type NAI-sensitive precursor virus during oseltamivir therapy during the summer months. There was no molecular variability that would suggest the presence of a mixed population. This was followed by the rapid emergence of influenza virus with NA gene E119V framework mutation, which conferred reduced oseltamivir susceptibility (~100-fold decrease),

compared with precursor virus **(Table 1)**. Viral growth seemed unaffected, as observed elsewhere [24], because the E119V mutant virus was cultured from the patient's samples for 6 weeks during oseltamivir therapy and for >4 months after cessation of antiviral therapy **(Figure 1)**.

Although amino acid substitutions and subpopulations were detected in HA, NA, and M2 genes, sequence comparison of the viruses 244 and 260 days after infection with the precursor virus revealed only 1 fixed nucleotide substitution in both HA and NA genes (NA gene E119V). In addition, 260 days after the onset of infection in patient 1, the Dutch 2005–2006 influenza epidemic had started, caused by a significantly deviated influenza A (H3N2) strain. Reinfection with a new influenza A (H3N2) strain is therefore unlikely, a conclusion further supported by the fact that a single virus sequence was obtained during the summer, when there was no circulation of influenza virus in the community. The E119V mutant virus likely retained its pathogenicity during continuous influenza excretion, illustrated by renewed clinical deterioration to viral lower respiratory tract infection after a 4-month period of upper respiratory tract infection or no symptoms. Transient T/A135T/A, A138S, D188N, and N/K189N/K amino acid changes at or near the HA receptor binding site, compared with precursor virus, may have contributed to persistence of the E119V variant, partially compensating for potential reduced NA activity by a decreased HA affinity [25]. HA gene receptor binding site alterations may emerge and persist more easily among severely immunocompromised patients, with continuous high-level viral replication and decreased immunogenic selection control among viruses with aberrant HA antigens. R292K variants appear impaired, as described elsewhere [24], because this mutation did not persist in the patient and was possibly selected out by overgrowth of the E119V variant.

In patient 2, a mixed population of NA gene R292K (10%) and NA gene E119V (90%) mutants with HA gene A138S substitution at the receptor binding site and reduced NAI susceptibility emerged during oseltamivir therapy and reverted back to wild-type NAI-sensitive virus after antiviral treatment was discontinued. The emergence of R292K substitution in viruses from patients 1 and 2, which confers high-level oseltamivir resistance and reduced zanamivir susceptibility, is of concern because these mutants hamper therapy with NAIs. Apparently, with selective pressure and lack of immunological containment, NAI-resistant minor variant viruses can emerge that retain their replicative ability and evade antiviral therapy. The cessation of oseltamivir therapy allowed reversion to sensitive wild-type influenza virus A (H3N2) in patient 2, a result

consistent with prior findings [8, 9] and in contrast to the result observed for patient 1, in whom the E119V mutation remained fixed after cessation of oseltamivir therapy and until viral clearance.

None of the patients received adamantane therapy; it was considered unreliable because of the rapid development of resistance [2, 3]. The M2 protein S31N substitution in viruses from patients 2 and 3 was not unexpected, because most 2006 national sentinel influenza A (H3N2) viruses also displayed this amino acid substitution (data not shown). The detected M2 protein A30S substitution in patient 1 was apparently acquired by spontaneous mutation and therefore unrelated to exposure, as has been proposed earlier for the S31N mutation [5]. A30S mutation has been observed in vivo in avian influenza virus A(H7N2) field isolates [26]. M2 protein mutations at position 30, conferring amantadine resistance are typically A30V or A30T in humans [27]; we are not aware of documented A30S mutation in human influenza viruses.

Lymphocytopenia at the time of infection has been documented as an important risk factor for developing influenza virus pneumonia [20]. In this study, the occurrence of influenza lower respiratory tract infection in 6 (75%) of 8 patients and the subsequent fatal outcome in 2 (33%) of 6 patients was similar to the rates of infection and death (80% and 33%, respectively) reported among severely immunocompromised patients elsewhere [28]. Clinical improvement of influenza lower respiratory tract infection, in which it became upper respiratory tract infection or symptoms ceased was significantly associated with oseltamivir treatment (8 of 8 episodes) compared with untreated episodes (0 of 2 episodes) ($P = .02$). During continuous influenza virus excretion, the remarkable reappearance of specific viral lower respiratory tract infection symptoms was observed in 4 patients (patients 1, 2, 3, and 8). This affirms the importance of timely recognition and treatment of influenza lower respiratory tract infection among severely immunocompromised hosts.

We conclude that prolonged influenza virus respiratory tract infection is observed among immunocompromised patients with lymphocytopenia and is associated with frequent development of influenza lower respiratory tract infection and antiviral resistance during therapy. PCR-based monitoring for this category of patients should enable the early detection of influenza viruses and could help prompt the initiation of timely therapeutic and preventive measures. Lymphocyte reconstitution is associated with viral clearance, irrespective of receipt of antiviral therapy, a result that warrants

further exploration of therapeutic approaches that aim to improve specific immune recovery during prolonged influenza infection. These strategies are important for the improvement of individual outcomes and for helping to prevent the emergence and continuous excretion of drug-resistant viruses.

Conflict of interest

There was no financial support for the study. M. Jonges reports that he was funded by the Impulse Veterinary Avian Influenza Research in the Netherlands program of the Economic Structure Enhancement Fund (FES) (contact: Marion.Koopmans@rivm.nl). The other authors declare not to have any disclosures of financial or potential conflicts of interest.

Acknowledgements

This study relied on the availability of routine diagnostic specimens sent to our laboratory by numerous medical specialists from the departments of hematology, pediatrics, and intensive care at Leiden University Medical Center. We thank Dr. Marcel F. Peeters from the Laboratory for Medical Microbiology and Immunology at St. Elisabeth Hospital in Tilburg, The Netherlands, for providing early diagnostic specimens and viral isolates obtained from patient 1. We gratefully acknowledge the support.

References

1. Whitley RJ, Monto AS. Prevention and treatment of influenza in high-risk groups: children, pregnant women, immunocompromised hosts, and nursing home residents. *J Infect Dis* **2006**; 194: S133–8.

2. Moscona A. Neuraminidase inhibitors for influenza. *N Engl J Med* **2005**; 353: 1363–73.

3. Bright RA, Shay DK, Shu B, Cox NJ, Klimov AI. Adamantane resistance among influenza A viruses isolated early during the 2005–2006 influenza season in the United States. *JAMA* **2006**; 295: 891–4.

4. Lackenby A, Hungnes O, Dudman SG, *et al*. Emergence of resistance to oseltamivir among influenza A(H1N1) viruses in Europe. *Euro Surveill* **2008**; 13. pii: 8026.

5. Simonsen L, Viboud C, Grenfell BT, *et al*. The genesis and spread of reassortment human influenza A/H3N2 viruses conferring adamantane resistance. *Mol Biol Evol* **2007**; 24: 1811–20.

6. Ward P, Small I, Smith J, Suter P, Dutkowski R. Oseltamivir (Tamiflu) and its potential for use in the event of an influenza pandemic. *J Antimicrob Chemother* **2005**; 55(Suppl 1): i5–i21.

7. Reece PA. Neuraminidase inhibitor resistance in influenza viruses. *J Med Virol* **2007**; 79: 1577–86.

8. Baz M, Abed Y, McDonald J, Boivin G. Characterization of multidrug-resistant influenza A/H3N2 viruses shed during 1 year by an immunocompromised child. *Clin Infect Dis* **2006**; 43: 1555–61.

9. Ison MG, Gubareva LV, Atmar RL, Treanor J, Hayden FG. Recovery of drug-resistant influenza virus from immunocompromised patients: a case series. *J Infect Dis* **2006**; 193: 760–4.

10. Weinstock DM, Gubareva LV, Zuccotti G. Prolonged shedding of multidrug-resistant influenza A virus in an immunocompromised patient. *N Engl J Med* **2003**; 348: 867–8.

11. Englund JA, Champlin RE, Wyde PR, *et al*. Common emergence of amantadine- and rimantadine-resistant influenza A viruses in symptomatic immunocompromised adults. *Clin Infect Dis* **1998**; 26: 1418–24.

12. Gubareva LV, Matrosovich MN, Brenner MK, Bethell RC, Webster RG. Evidence for zanamivir resistance in an immunocompromised child infected with influenza B virus. *J Infect Dis* **1998**; 178: 1257–62.

13. Kiso M, Mitamura K, Sakai-Tagawa Y, *et al*. Resistant influenza A viruses in children treated with oseltamivir: descriptive study. *Lancet* **2004**; 364: 759–65.

14. Hayden FG. Prevention and treatment of influenza in immunocompromised patients. *Am J Med* **1997**; 102: 55–60; discussion 75–6.

15. Templeton KE, Scheltinga SA, Beersma MF, Kroes AC, Claas EC. Rapid and sensitive method using multiplex real-time PCR for diagnosis of infections by influenza A and influenza B viruses, respiratory syncytial virus and parainfluenza viruses 1 2 3 and 4. *J Clin Microbiol* **2004**; 42: 1564–9.

16. Frank AL, Taber LH, Wells CR, Wells JM, Glezen WP, Paredes A. Patterns of shedding of myxoviruses and paramyxoviruses in children. *J Infect Dis* **1981**; 144: 433–41.

17. Whitley RJ, Hayden FG, Reisinger KS, *et al*. Oral oseltamivir treatment of influenza in children. *Pediatr Infect Dis J* **2001**; 20: 127–33.

18. Hayden FG, Treanor JJ, Fritz RS, *et al*. Use of the oral neuraminidase inhibitor oseltamivir in experimental human influenza: randomized controlled trials for prevention and treatment. *JAMA* **1999**; 282: 1240–6.

19. Nicholson KG, Aoki FY, Osterhaus AD, *et al*. Efficacy and safety of oseltamivir in treatment of acute influenza: a randomised controlled trial. Neuraminidase Inhibitor Flu Treatment Investigator Group. *Lancet* **2000**; 355: 1845–50.

20. Nichols WG, Guthrie KA, Corey L, Boeckh M. Influenza infections after hematopoietic stem cell transplantation: risk factors, mortality, and the effect of antiviral therapy. *Clin Infect Dis* **2004**; 39: 1300–6.

21. Mishin VP, Hayden FG, Gubareva LV. Susceptibilities of antiviral-resistant influenza viruses to novel neuraminidase inhibitors. *Antimicrob Agents Chemother* **2005**; 49: 4515–20.

22. Gooskens J, Kuiken T, Claas EC, *et al*. Severe influenza resembling hemorrhagic shock and encephalopathy syndrome. *J Clin Virol* **2007**; 39: 136–40.

23. Potier M, Mameli L, Bélisle M, Dallaire L, Melancon SB. Fluorometric assay of neuraminidase with a sodium (4-methylumbelliferyl-alpha- D-N acetylneuraminate) substrate. *Anal Biochem* **1979**; 94: 287–96.

24. Yen HL, Herlocher LM, Hoffmann E, *et al*. Neuraminidase inhibitor-resistant influenza viruses may differ substantially in fitness and transmissibility. *Antimicrob Agents Chemother* **2005**; 49: 4075–84.

25. Wagner R, Matrosovich M, Klenk HD. Functional balance between haemagglutinin and neuraminidase in influenza virus infections. *Rev Med Virol* **2002**; 12: 159–66.

26. Ilyushina NA, Govorkova EA, Webster RG. Detection of amantadine-resistant variants among avian influenza viruses isolated in North America and Asia. *Virology* **2005**; 341: 102–6.

27. Suzuki H, Saito R, Masuda H, Oshitani H, Sato M, Sato I. Emergence of amantadine-resistant influenza A viruses: epidemiological study. *J Infect Chemother* **2003**; 9: 195–200.

28. Yousuf HM, Englund J, Couch R, *et al*. Influenza among hospitalized adults with leukemia. *Clin Infect Dis* **1997**; 24: 1095–9.

Chapter 6

Morbidity and mortality associated with nosocomial transmission of oseltamivir-resistant influenza A(H1N1) virus

Gooskens J [1], Jonges M [3], Claas ECJ [1],
Meijer A [3], van den Broek PJ [2], Kroes ACM [1]

JAMA **2009**; *301: 1042–6*

1 Department of Medical Microbiology
Leiden University Medical Center, Leiden, the Netherlands
2 Department of Infectious Diseases
Leiden University Medical Center, Leiden, the Netherlands
3 Virology section
National Institute of Public Health and the Environment,
Bilthoven, the Netherlands

Abstract

Context: The sudden emergence and rapid spread of oseltamivir-resistant influenza A(H1N1) viruses with neuraminidase (NA) gene H274Y amino acid substitution is the hallmark of global seasonal influenza since January 2008. Viruses carrying this mutation are widely presumed to exhibit attenuated pathogenicity, compromised transmission, and reduced lethality.

Objective: To investigate nosocomial viral transmission in a cluster of patients with influenza A(H1N1) virus infection.

Design, Setting, and Patients: Descriptive outbreak investigation of 2 hematopoietic stem cell transplant recipients and an elderly patient who developed hospital-acquired influenza A virus infection following exposure to an index patient with community-acquired H274Y-mutated influenza A(H1N1) virus infection in a medical ward at a Dutch university hospital in February 2008. The investigation included a review of the medical records, influenza virus polymerase chain reaction and culture, phenotypic oseltamivir and zanamivir susceptibility determination, and hemagglutinin chain 1 (HA_1) gene and NA gene sequence analysis.

Main Outcome Measure: Phylogenetic relationship of patient cluster influenza A(H1N1) viruses and other 2007-2008 seasonal influenza A(H1N1) viruses.

Results: Viral HA_1 and NA gene sequence analysis from the 4 patients revealed indistinguishable nucleotide sequences and phylogenetic clustering of H274Y-mutated, oseltamivir-resistant influenza A(H1N1) virus, confirming nosocomial transmission. Influenza virus pneumonia (3 patients) and attributable mortality (2 patients) during active infection was observed in patients with lymphocytopenia at onset.

Conclusion: Seasonal oseltamivir-resistant influenza A(H1N1) viruses with NA gene H274Y mutation are transmitted and retain significant pathogenicity and lethality in high-risk patients.

Introduction

A global emergence and rapid spread of oseltamivir-resistant influenza A(H1N1) viruses carrying a neuraminidase (NA) gene with an H274Y (N2 numbering; H275Y in N1 numbering) amino acid substitution has been observed since January 2008 [1-3]. Viruses carrying this mutation are presumed to exhibit attenuated pathogenicity [4], compromised transmission [5], and reduced lethality [6]. However, current widespread circulation of oseltamivir-resistant influenza A(H1N1) viruses associated with typical influenza illnesses and viral pneumonia suggest that these viruses retain significant transmissibility and pathogenicity [2, 3, 7, 8]. While these resistant variants may cause significant mortality and retain efficient transmission, these properties have not yet been firmly established.

Methods

In February 2008, an outbreak of influenza A(H1N1) virus occurred in a medical ward at a Dutch university hospital. Clinical specimens from symptomatic contact patients of the presumed index patient were tested by influenza polymerase chain reaction (PCR) and sequences were further analyzed. Medical records of contact patients with related influenza virus infection were reviewed for underlying disease, clinical findings, and outcome. Screening specimens were obtained from contacts of the last outbreak patient (patient 4) to rule out further spread of the virus.

Influenza Virus Diagnostics. Influenza virus detection was performed on clinical respiratory specimens using rapid antigen testing and PCR as described previously [9], along with viral culture. Antigenic characterization (hemagglutination inhibition testing) and phenotypic oseltamivir and zanamivir susceptibility (IC_{50}, concentration of drug needed to inhibit enzyme activity by 50%) were determined as described [10, 11]. Viral RNA extracted from clinical specimens was further transcribed and amplified. Hemagglutinin chain 1 (HA_1) and neuraminidase gene sequences from patients with confirmed influenza virus infection were analyzed and phylogenetically related to other 2007-2008 seasonal influenza viruses obtained at the hospital or sentinel surveillance isolates collected nationwide using Bionumerics version 5.1 (Applied Maths, Sint-Martens-Latem, Belgium).

Table 1. Clinical characteristics of 4 hospitalized patients with oseltamivir-resistant influenza A (H1N1) virus infection.

	Patient 1 (Index)	Patient 2	Patient 3	Patient 4
Sex	Male	Female	Male	Male
Age, y	47	57	89	66
Medical history	Systemic lupus erythematosus, sarcoidosis, diabetes	Multiple myeloma, allogeneic stem cell transplantation following reduced-intensity conditioning, engraftment	Diabetes, cardiac ischemia	Acute myelogenous leukemia, allogeneic stem cell transplantation following myeloablative conditioning, chronic graft-vs-host disease
Immunosuppressive therapy	Prednisolone	None	None	Mycophenolate mofetil, prednisone, recent cyclosporine
Reason for admission	Fever, dyspnea	Fever, mild cough	Fever	Fever
Confirmed diagnosis	Influenza lower respiratory tract infection	Bacteremia, source unknown	Bacteremia, urosepsis	Bacteremia, abdominal sepsis, active graft-vs-host disease
First positive test result for influenza A [a]	January 29, 2008	February 11, 2008	February 15, 2008	February 14, 2008
No. of days following admission in hospital	0	16	17	17
Antiviral therapy	Oseltamivir	Oseltamivir	Oseltamivir	Oseltamivir
Absolute lymphocyte count within 48 h of symptom onset, cells/μL	699 (lymphocytopenia)	3195 (normal count)	726 (lymphocytopenia)	217 (lymphocytopenia)
Respiratory symptoms	Dyspnea, oxygen dependent	Cough	Dyspnea, oxygen dependent	Dyspnea, oxygen dependent
Chest radiograph result	Left lower-lobe consolidation	No pulmonary abnormalities	Bilateral lower-lobe consolidations	Perihilar consolidations
Absolute lymphocyte count during follow-up, cells /μL [b]	1134 (normal count)	4739 (normal count)	Not available	129 (lymphocytopenia)
Influenza outcome	Viral clearance	Viral clearance	Fatal influenza pneumonia [c]	Fatal influenza pneumonia [c,d]

a Determined by polymerase chain reaction (PCR).
b Lymphocyte count determined 2 to 6 weeks after influenza confirmation.
c Clinical diagnosis (dyspnea, PCR detection of influenza A in respiratory specimens).
d Pathological diagnosis (histological features, PCR detection of influenza A in pulmonary tissue).

Ethical Considerations. The investigation of this outbreak did not involve any planned activity that could have been reviewed prospectively by an institutional review board or ethics committee. Nevertheless, all necessary precautions were taken to prevent identification of the patients and health care workers involved. The physicians involved all gave signed permission to use clinical data and were informed on the outcome of the investigation. All of them agreed with the intention to publication. Details including the age and role of the health care workers were omitted or described in a nonspecific way, while we also took care to preserve all clinically meaningful details. The chairman of the ethics committee at the Leiden University Medical Center was consulted retrospectively and agreed to the approach as described for reporting the clinical information obtained during the investigation and included herein.

Results

Nosocomial Influenza A(H1N1) Virus Outbreak. The clinical characteristics and timeline of the outbreak of influenza A(H1N1) virus are shown in the **Table** and in **Figure 1**, respectively. Community-acquired oseltamivir-resistant influenza A(H1N1) virus with NA gene H274Y mutation, isolated from the presumed index case, was detected in 3 additional patients (mean oseltamivir IC_{50}, 484 nM; mean zanamivir IC_{50}, 1.1 nM). The presumed index case (patient 1), who was vaccinated for 2007-2008 seasonal influenza and received high-dose (cumulative) prednisolone therapy for systemic lupus erythematosus, was admitted to the hospital on January 29, 2008, with fever, cough, dyspnea, and lymphocytopenia. Mechanical ventilation and broad-spectrum empirical antibacterial treatment were initiated for acute respiratory failure and apparent pulmonary consolidations by chest radiography. Oseltamivir administration was initiated following influenza A virus detection using rapid antigen testing and PCR along with contact and droplet isolation. No other viral and bacterial respiratory pathogens were detected and blood cultures remained negative. The patient was transferred to a medical ward following clinical improvement on February 1 and isolation precautions were continued for the duration of symptoms until February 3. Viral clearance was confirmed by PCR upon lymphocyte reconstitution in an outpatient setting on March 4, 2008. Two hematopoietic stem cell transplant recipients (patient 2 and patient 4) and an 89-year-old elderly patient (patient 3) developed hospital-acquired influenza A virus infection **(Table)**. These patients were present at the same time as the index patient at the medical ward without isolation procedures **(Figure 1)**, but the 4

patients never shared rooms. Patient 2 developed mild influenza symptoms with rapid viral clearance, whereas both patients 3 and 4 developed pulmonary consolidations and fatal respiratory failure with viral excretion under broad-spectrum antibacterial therapy. The influenza vaccination status of these patients is not known. The attribution of mortality to influenza was supported by detection of influenza A(H1N1) viral RNA from post-mortem pulmonary tissue and histopathological pulmonary findings consistent with viral pneumonia in patient 4, with the exclusion of other pathogens. Further nosocomial spread to other contacts within the wards was excluded by PCR.

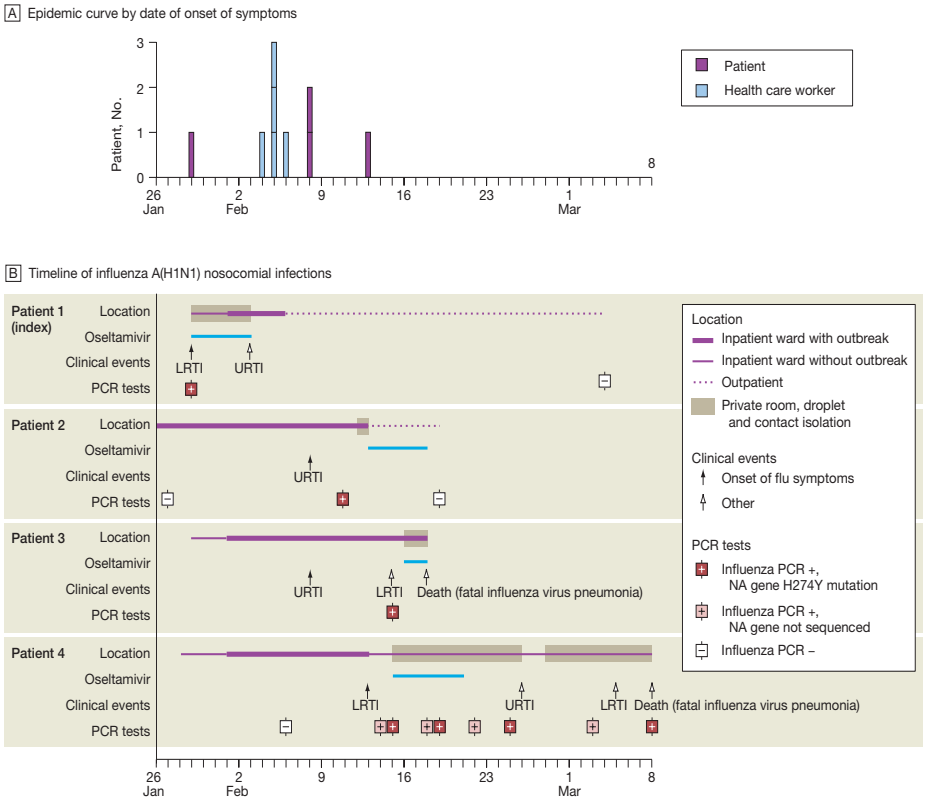


Figure 1. Epidemic curve by date of onset of symptoms and timeline of influenza A(H1N1) nosocomial infections.

During influenza A(H1N1) virus outbreak, all 4 infected patients were admitted in the same department and never shared rooms. LRTI indicates lower respiratory tract infection; NA, neuraminidase; and URTI, upper respiratory tract infection.

Phylogenetic Relationship. HA_1 gene and NA gene sequence analysis of viruses from the 4 outbreak patients revealed indistinguishable nucleotide sequences and phylogenetic clustering **(Figure 2)**. In addition to NA gene H274Y substitution, a rare T284A substitution was sequenced (NA gene sequence GenBank accession numbers AB476754, AB476755, AB476756, AB462370) and could therefore be recognized as a marker for transmission. An unlinked national surveillance isolate (A/NL/088/08) with identical HA_1 gene nucleotide sequence revealed no NA gene phylogenetic clustering and lacked the specific T284A substitution. The NA gene T284A mutation was not observed in other

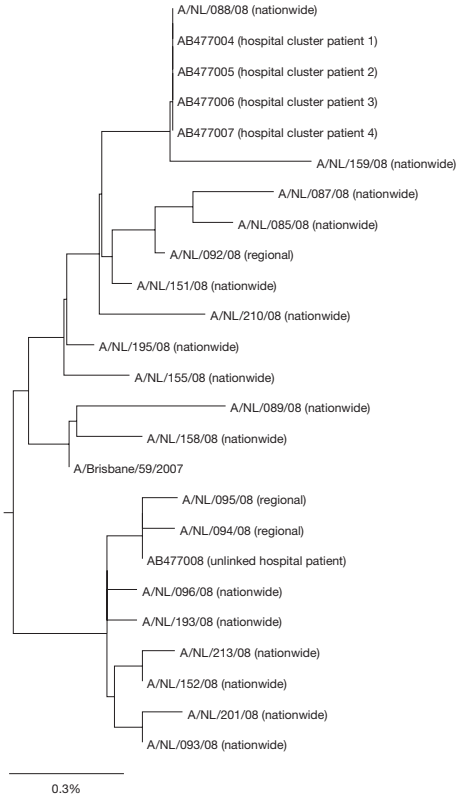


Figure 2. Phylogenetic relationship of nosocomial patient cluster influenza A(H1N1) viruses and other 2007-2008 seasonal influenza A viruses.

Influenza A(H1N1) virus HA_1 gene sequences obtained from the patient cluster (n=4, marked as hospitalized cluster patient 1, 2, 3, 4) were related to available unlinked 2007-2008 seasonal influenza A(H1N1) viral sequences obtained at the hospital (n=1, marked as unlinked hospitalized patient), surveillance isolates collected within a 10-km regional zone from the hospital (n=3, marked as regional), nationwide collected surveillance isolates (n=17, marked as nationwide), and vaccine strain A/Brisbane/59/2007. The HA_1 gene (nucleotides 1-1071) neighbor-joining tree was rooted on vaccine strain A/Solomon Islands/3/2006. Viral sequence Genbank accession numbers are depicted for hospitalized patients.

2007-2008 seasonal influenza A(H1N1) viruses or in worldwide sequences submitted to public databases, reinforcing the unique genetic relatedness of this influenza A(H1N1) virus patient cluster.

Health Care Workers. Five health care workers developed influenza-like illness (onset February 4–6, 2008) during admission of the presumed index patient. One health care worker, vaccinated for the 2007-2008 seasonal influenza, developed influenza-like illness following established contact with the index patient and continued working. Four other health care workers took sick leave within 24 to 48 hours of symptom onset. However, no samples for influenza testing were obtained from any of these 5 health care workers. Thus, their role in possibly contributing to this apparent nosocomial spread of influenza could not be confirmed. Viruses cultured from the patient cluster revealed a poor antigenic match with the 2007-2008 vaccine reference strain A/Solomon Islands/3/06 (approximately 16-fold difference by duplicate hemagglutination inhibition testing). This may in part explain the sustained susceptibility and infectivity of the vaccinated index patient and suspected health care worker resulting in the hospital outbreak.

Comment

This outbreak provided evidence that circulating oseltamivir-resistant influenza A(H1N1) viruses with NA gene H274Y mutation are transmitted between humans. Limitations of this observational study include the small number of patients, therefore the findings require careful interpretation and do not allow conclusions on the frequency of this complication in hospital settings. The vaccination status of secondarily infected cases (patients 2, 3, and 4) remained unclarified. Information on the mechanism of spread was limited by the circumstances in this study. Data obtained from clinical specimens suggest different routes of transmission; however, this could not be further explored because the sampling of symptomatic health care workers and testing of fomites are not routinely performed. However, analysis of data obtained from clinical specimens provided some insight to different routes of transmission and suggested a limited viral spread.

Early identification and prolonged isolation precautions appear prudent in the care for infected immunocompromised patients to prevent nosocomial influenza virus outbreaks. This study confirmed that circulating H274Y-mutated A(H1N1) viruses can retain

significant pathogenicity and lethality, as shown in these elderly or immunocompromised patients with lymphocytopenia, underlining the urgency for the introduction of new effective antiviral agents and therapeutic strategies [12].

Conflict of interest

M. Jonges reports that he was funded by the Impulse Veterinary Avian Influenza Research in the Netherlands program of the Economic Structure Enhancement Fund (FES) (contact: Marion.Koopmans@rivm.nl). The Economic Structure Enhancement Fund and the Dutch GP surveillance network were not involved in the design and conduct of the study; collection, management, analysis, and interpretation of the data; or in the preparation, review, or approval of the manuscript. The other authors declare not to have any disclosures of financial or potential conflicts of interest.

Acknowledgements

The Dutch GP surveillance network provided respiratory specimens for virological surveillance of influenza-like illness from which viral sequences were included in this analysis for comparison. We thank Gé A. Donker, PhD, NIVEL Netherlands Institute for Health Services Research, who is the coordinator of the Dutch GP sentinel surveillance network. Dr Donker was not financially compensated for providing the surveillance isolates.

References

1. Lackenby A, Hungnes O, Dudman SG, *et al*. Emergence of resistance to oseltamivir among influenza A(H1N1) viruses in Europe. *Euro Surveill* **2008**; 13. pii: 8026.

2. World Health Organization. Epidemic and pandemic alert response: influenza A(H1N1) virus resistance to oseltamivir (December 30, **2008**). http://www.who.int/csr/disease/influenza/h1n1_table/en/index.html; accessibility verified February 10th, 2009.

3. Centers for Disease Control and Prevention. CDC issues interim recommendations for the use of influenza antiviral medications in the setting of oseltamivir resistance among circulating influenza A (H1N1) viruses, 2008-09 influenza season (December 19, **2008**). http://www2a.cdc.gov/HAN/ArchiveSys/ViewMsgV.asp?AlertNum=00279; accessibility verified February 10th, 2009.

4. Ives JA, Carr JA, Mendel DB, *et al*. The H274Y mutation in the influenza A/H1N1 neuraminidase active site following oseltamivir phosphate treatment leave virus severely compromised both in vitro and in vivo. *Antiviral Res* **2002**; 55: 307–17.

5. Herlocher ML, Truscon R, Elias S, *et al*. Influenza viruses resistant to the antiviral drug oseltamivir: transmission studies in ferrets. *J Infect Dis* **2004**; 190: 1627–30.

6. Yen HL, Ilyushina NA, Salomon R, Hoffmann E, Webster RG, Govorkova EA. Neuraminidase inhibitor-resistant recombinant A/Vietnam/1203/04 (H5N1) influenza viruses retain their replication efficiency and pathogenicity in vitro and in vivo. *J Virol* **2007**; 81: 12418–12426.

7. Nicoll A, Ciancio B, Kramarz P; Influenza Project Team. Observed oseltamivir resistance in seasonal influenza viruses in Europe interpretation and potential implications. *Euro Surveill* **2008**; 13: pii8025.

8. van der Vries E, van den Berg B, Schutten M. Fatal oseltamivir-resistant influenza virus infection. *N Engl J Med* **2008**; 359: 1074–1076.

9. Templeton KE, Scheltinga SA, Beersma MF, Kroes AC, Claas EC. Rapid and sensitive method using multiplex real-time PCR for diagnosis of infections by influenza A and influenza B viruses, respiratory syncytial virus, and parainfluenza viruses 1, 2, 3, and 4. *J Clin Microbiol* **2004**; 42: 1564–1569.

10. Gooskens J, Kuiken T, Claas EC, *et al*. Severe influenza resembling hemorrhagic shock and encephalopathy syndrome. *J Clin Virol* **2007**; 39: 136–40.

11. Potier M, Mameli L, Belisle M, Dallaire L, Melancon SB. Fluorometric assay of neuraminidase with a sodium (4-methylumbelliferyl-alpha-D-N acetylneuraminate) substrate. *Anal Biochem* **1979**; 94: 287–296.

12. Hayden F. Developing new antiviral agents for influenza treatment: what does the future hold? *Clin Infect Dis* **2009**; 48: S3–S13.

Chapter 7

Mass spectrometry-based comparative sequence analysis for the genetic monitoring of influenza A(H1N1)pdm09 virus

Gooskens J [1], Zevenhoven-Dobbe JC[1], Claas ECJ[1],
Kroes ACM[1], Posthuma CC[1]

PLoS One **2014**; *9: e92970*

1 Department of Medical Microbiology
Leiden University Medical Center, Leiden, the Netherlands

Abstract

The pandemic influenza A (H1N1) 2009 virus (pH1N1) contains novel gene segments of zoonotic origin that lack virulence and antiviral resistance markers. We aimed to evaluate the applicability and accuracy of mass spectrometry-based comparative sequence analysis (MSCSA) to detect genetic mutations associated with increased virulence or antiviral resistance in pH1N1. During the 2009 H1N1 pandemic, routine surveillance specimens and clinical antiviral resistance monitoring specimens were analyzed. Routine surveillance specimens obtained from 70 patients with pH1N1 infection were evaluated for mutations associated with increased virulence (PB1-F2, PB2 and NS1 genes) or antiviral resistance (neuraminidase gene, NA) using MSCSA and Sanger sequencing. MSCSA and Sanger sequencing results revealed a high concordance (nucleotides >99%, SNPs ~94%). Virulence or resistance markers were not detected in routine surveillance specimens: all identified SNPs encoded for silent mutations or non-relevant amino acid substitutions. In a second study population, the presence of H275Y oseltamivir-resistant virus was identified by real-time PCR in 19 of 35 clinical antiviral resistance monitoring specimens obtained from 4 immunocompromised patients with ≥14 days prolonged pH1N1 excretion. MSCSA detected H275Y in 24% (4/19) of positive specimens and Sanger sequencing in 89% (17/19). MSCSA only detected H275Y when the mutation was dominant in the analyzed specimens. In conclusion, MSCSA may be used as a rapid screening tool during molecular surveillance of pH1N1. The low sensitivity for the detection of H275Y mutation in mixed viral populations suggests that MSCSA is not suitable for antiviral resistance monitoring in the clinical setting.

Introduction

Influenza A and B viruses cause seasonal epidemics of febrile respiratory illness in the human population. The negative-sense RNA genome of influenza A viruses consists of eight segments which encode at least 13 proteins following transcription and translation [1-3]. Seasonal epidemics arise by antigenic drift through frequent mutations in hemagglutinin (HA) and neuraminidase (NA) surface glycoprotein genes enabling seasonal host immune evasion by the virus. In rare occasions, human influenza A virus gene segments re-assort with other influenza A viruses from avian, human, and swine origins which can result in new antigenic subtypes that may cause severe human influenza pandemics [4].

In 2009, the influenza A (H1N1)pdm09 virus (pH1N1) with a relatively new antigenic subtype of zoonotic-origin emerged in Mexico and caused an influenza pandemic followed by rapid global spread in the human population [5-7]. The virus was likely generated through multiple reassortment events in pigs, and contained gene segments from North American "classical" swine influenza virus A/H1N1 (HA, NP, NS), North American avian influenza virus A/H1N1 (PB2, PA), human seasonal influenza virus A/H3N2 (PB1) and Eurasian swine influenza virus A/H1N1 (M, NA) [8]. The overall clinical presentation in humans appeared unexpectedly mild which can not be explained by residual host immunity in the human population. A minority of children and adults had preexisting cross-protective antibody titers against pH1N1 [9]. Only ~18% of influenza A CD8+ cytotoxic T lymphocyte epitopes derived from previous seasonal influenza A viruses are conserved in pH1N1 [10]. The novel pH1N1 virus contained gene segments of zoonotic origin that lack virulence and antiviral resistance markers due to favorable genetic polymorphisms or truncations in PB2, PB1-F2, NS1 and NA genes [11-14]. Novel adaptive mutations may emerge in PB2, PB1-F2 and NS1 genes with the potential to increase viral replication and host pathogenicity of pH1N1 [14-16]. In addition, oseltamivir-resistant virus may emerge during selective pressure [17, 18].

Wide-scale molecular surveillance is warranted but conventional sequencing methods are laborious and inefficient. Mass spectrometry-based comparative sequence analysis (MSCSA) enables rapid multi-genomic sequence analysis with automated data analysis and could improve the availability of relevant pH1N1 genomic sequences [19, 20]. Rapid identification of relevant pH1N1 mutations in the community or in clinical settings may guide early prevention and intervention strategies. MSCSA was previously successfully

used for the analysis of human cytomegalovirus sequence polymorphisms in UL97 gene and for detection of genetic mutations associated with antiviral resistance in clinical specimens [19]. In this study, we evaluated the applicability and accuracy of MSCSA to detect pH1N1 genetic mutations associated with increased virulence (PB1-F2, PB2 and NS1 genes) or antiviral resistance (NA gene).

Methods

Active Case Finding and Ethics Statement. During the 2009 H1N1 pandemic, human pH1N1 infection cases became obligatory notifiable by law in the Netherlands on 29th April 2009 (RIVM website. Available: www.rivm.nl/bibliotheek/rapporten/215011006.pdf; access verified March 3rd, 2013). A nationwide case definition was determined based on the European Union case definition [21] and contained clinical and epidemiological criteria upon which routine diagnostic testing was indicated and subsequent control measures could be applied. Diagnostic specimens were obtained from suspect cases by municipal health services, general practitioners or hospitals following influenza pandemic procedures from the National Institute of Public Health and the Environment (RIVM) responsible for the control of infectious diseases. In the region of Leiden, the first pandemic wave (weeks 27 through 35) inferred a normal (Gaussian) distribution of which the peak occurred in week 31 when 72% of positive patients were observed **(Figure 1)**. All specimens included in this study were collected in the region of Leiden for routine influenza diagnostic testing following national pandemic procedures. The specimens were sent directly to the regional reference outbreak assistance laboratory accompanied by a written diagnostic request from the responsible phycisians and results were reported back to the medical facility who requested them. After performing routine influenza diagnostic testing, the reference outbreak assistance laboratory evaluated the additional presence of virulence and resistance markers for technical and epidemiological reasons. Additional influenza molecular characterization was not considered to be fully part of the initial diagnostic request, therefore all specimens were anonymized for precautionary reasons after receiving them from the requesting medical facilities and phycisians. There was no access to patient identifying information or clinical data during additional molecular characterization following ethical principles expressed in the Declaration of Helsinki. Approval from the institutional review board and the use of informed consents were not necessary for influenza diagnostic testing on specimens sent to the laboratory for this specific purpose.

Study Populations. Two study populations with pH1N1 infection are under evaluation in this study. Routine surveillance specimens were obtained from 344 suspect cases in the region of Leiden during the early phase of the 2009 H1N1 pandemic (June 29th through August 30th, 2009) and submitted to the regional reference outbreak assistance laboratory at Leiden University Medical Center. Influenza A virus was detected in 75 specimens (70 pH1N1, 4 seasonal H1N1, 1 seasonal H3N2). A total of 70 specimens from 70 patients with pH1N1 infection (43 male; median age 21 years, range 1 to 63 years) were included in this study. A second collection of 35 clinical antiviral resistance monitoring specimens was obtained from hospitalized immunocompromised patients with ≥14 days prolonged pH1N1 excretion (panH1 Ct values ≤37.0) during widespread pH1N1 circulation (November 3rd 2009 through January 13th, 2010). Real-time PCR confirmed H275Y mutated virus in 19 of 35 specimens obtained from 3 of 4 immunocompromised patients and these results were compared to MSCSA and Sanger sequencing findings of the NA gene.

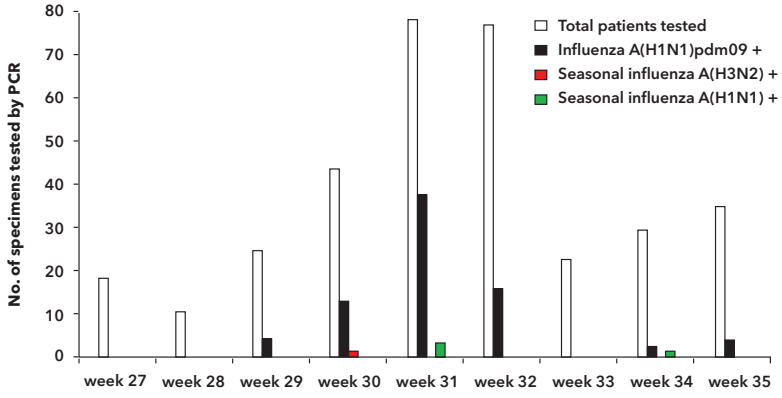


Figure 1. A graphic representation of the first wave of the 2009 H1N1 pandemic in the region of Leiden (The Netherlands).

Influenza A Virus Detection and Molecular Typing. RNA was extracted from clinical specimens using the automated MagNA Pure nucleic acid isolation system (Roche Diagnostics, Almere, The Netherlands). Influenza A virus screening was performed by real-time PCR using InfA primers and a probe targeting the matrix gene and following procedures validated by the World Health Organization (WHO) and Centers for Disease Control and Prevention (CDC) as described previously (WHO

website: http://www.who.int/csr/resources/publications/swineflu/CDCrealtimeRTPCR protocol_20090428.pdf; access verified March 3rd, 2013). The presence of pH1N1 was confirmed by PCR using panH1 and MexFluN1 protocols targeting the HA and NA genes [22, 23]. Influenza A virus specimens that were negative for pH1N1 were evaluated for seasonal influenza A (H1N1) and A (H3N2) viruses using H1-RF and H3-RF protocols targeting the HA gene [24] (http://www.who.int/csr/resources/publications/swineflu/WHO_Diagnostic_RecommendationsH1N1_20090521.pdf; access verified March 3rd, 2013).

MSCSA, Sanger Sequencing and H275Y Real-time PCR. MSCSA and Sanger sequencing were performed on all specimen collections containing influenza A virus, whereas H275Y real-time PCR was only carried out on the clinical antiviral resistance monitoring specimens after which H275Y positive findings were correlated to MSCSA and Sanger sequencing results. H275Y real-time PCR was performed as described previously [22]. MSCSA using the MassARRAY/iSEQ™ – Comparative Sequence Analysis technique (Sequenom, San Diego, USA) is based on the PCR amplification of a genomic target and *in vitro* transcription to produce RNA strands. These transcripts are cleaved to produce a sequence-specific set of fragments for analysis by mass spectrometry [20]. As the MSCSA protocol starts from DNA, a reverse transcription reaction using SuperScriptII (Invitrogen) with random primers (Promega; 1 mg final concentration; incubations at 25 ˚C for 10 min, at 40 ˚C for 90 min and 70 ˚C for 15 min) was implemented prior to PCR amplification. We designed T7 promoter-tagged (CAGTAATACGACTCACTATAGGGAGAAGGCT) forward primers and SP6-tagged (CGATTTAGGTGACACTATAGAAGAGAGGCT) reverse primers for the specific amplification of NA, PB1-F2, PB2 and NS1 targets **(Table 1)**. The pH1N1 primers were designed to cover potential markers of virulence (PB2 gene positions 271, 590, 591, 627, 701; PB1-F2 gene position 66; NS1 gene positions 227–230), protein segment truncation (PB1-F2 gene positions 12, 58, 88; NS1 gene position 220) and antiviral resistance (NA gene positions 116, 117, 119, 136, 150, 151, 199, 223, 275 and 295) in the viral genome **(Table 2)** [11-14, 25, 26]. PCR amplification of the targets was performed using 2 μl of RT product, with the tagged primers in 10 μl volume in 384 well plates as previously described [19]. The sample was processed by shrimp alkaline phosphatase treatment, *in vitro* transcription, and C- or U-specific RNaseA cleavage, according to the manufacturer's instructions and using a MassARRAY Liquid Handler (Matrix+Fusio™ Chip Module, Sequenom). The fragments resulting from RNA cleavage were diluted in double-distilled water and desalted with Clean Resin (Sequenom), transferred to a SpectroCHIP array (Sequenom), and analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry

(MassARRAY Compact Analyzer, Sequenom). Sanger sequencing was done on 2 μl of the PCR amplified products that were produced during the MSCSA procedure. Sanger sequencing reactions were performed in a volume of 10 μl using Big Dye terminator v 1.1 with sequencing buffer (Applied Biosystems) and 1 mM primer. The primers for Sanger sequencing were identical to the primers used for MSCSA **(Table 1)**, but without the T7- and SP6-containing tails. Nucleotide sequence analysis was performed on an ABI Prism 3100 Genetic Analyser (Applied Biosystems).

Table 1. Overview of pH1N1 Sanger sequencing and MSCSA primers and amplicon sizes.

Gene	Forward primer	Oligonucleotide sequence	Reverse primer	Oligonucleotide sequence	Amplicon size
NA	Flu-NA_F (274) $	tgccctgttagtggatggc	Flu-NA_SP6R (988) #	cattagggcgtggattgtctc	715 Bp
PB1-F2	Flu-PB1-F2_F (1) $	atggatgtcaatccgactctac	Flu-PB1-F2_SP6R (476) #	tcattagctgttaggccattcg	476 Bp
PB2	Flu-PB2_F (1764) $	cagaagccggtacagtggattc	Flu-PB2_SP6R (2201) #	accaacactacgtccccttgc	438 Bp
NS1	Flu-NS1_F (211) $	cttgaaagaggaatccagcgag	Flu-NS1_SP6R (740) #	caatctgtgccgcatttcttc	530 Bp

Bp, base pairs.
$ 5′ position of the first nucleotide of the forward primer in the corresponding gene.
# 5′ position of the last nucleotide of the reverse primer in the corresponding gene.

Table 2. Surveillance of pH1N1 genetic markers associated with virulence or antiviral resistance.

Gene	Antiviral resistance	Increased virulence	Protein truncation
NA	V116, I117, E119, Q136, K150, D151, D199, I223, H275, N295	none	none
PB1-F2	none	N66	Stop12, Stop58, Stop88
PB2	none	A271, S590, R591, E627, D701	none
NS1	none	G227, T228, E229, I230	Stop220

Relevant amino acid genetic positions are depicted for each corresponding gene.

Data Analysis. For MSCSA, the mass spectra of four amplicon transcript cleavage products per sample were matched against cleavage patterns calculated from an imported set of reference sequences [20]. The reference database was created by the authors and included a set of 193 NA gene, 67 PB2 gene, 210 NS1 gene and 53 PB1-F2 gene sequences imported from a resource database in September 2009 (PubMed website. Available: http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html; access verified September 3rd, 2009). Data processing was performed using iSEQ Software Version 1.0.0.2 to assemble amplicon

sequences. MSCSA and Sanger sequences were aligned to influenza A/California/04/2009 (H1N1) and single nucleotide polymorphisms (SNPs) were defined as nucleotide variations to this reference sequence.

Results

MSCSA Primers and Amplicons. Primers utilized for MSCSA were specific for pH1N1 as they did not amplify seasonal influenza A(H1N1 or H3N2). Among 70 routine surveillance specimens, the primers amplified 265 of in total 280 amplicons (95%) including NA (63/70), PB1-F2 (65/70), PB2 (68/70) and NS1 genes (69/70). PCR amplification of PB1-F2, PB2 and NS1 genes only failed in specimens with low viral levels (mean InfA PCR Ct 36.2), whereas amplification was observed in specimens containing high viral levels (mean InfA PCR Ct 26.5). The amplification of the NA gene failed in 4 specimens with high viral levels (range Ct 22.3 to 28.1), suggesting that occasionally the primer sequence did not correspond to the actual viral sequence.

MSCSA and Sanger Sequencing of Routine Surveillance Specimens. Using the resource database in September 2009 as reference database for MSCSA, 99.9% of the total number of nucleotides (n= 130204) matched between both methods. A total of 93.6% (456/487) of all SNPs were identified by both methods **(Table 3)**. More accurate results were obtained for the NA, PB2 and NS1 amplicons (93.2%, 98.7% and 90.1%, respectively) compared to the PB1-F2 amplicon (82.9%). In a small number of cases, a SNP detected by both methods could not be pinpointed to an exact position within an eleven nucleotide region by MSCSA (17/487, 3.5%). These inconclusive sequence data resulted in <0.1% uncertain nucleotide positions and were not considered as mismatches. A high degree of concordance was observed for the detection of SNPs via MSCSA and Sanger sequencing. The majority of SNPs (246/456) resulted in silent mutations and few SNPs (210/456) encoded for non-relevant amino acid substitutions in NA gene (V106I (n= 61), V203M (n = 1), N248D (n= 62), S286G (n =1)), PB1-F2 gene (T34A (n= 1), V113A(n = 1)), PB2 gene (K660R (n =2)) and NS1 gene segments (I123V (n= 61), N133D (n= 16), S135N (n= 1), G154R (n= 1), V194I (n= 1), D207N (n = 1)). The degree of discordance was limited (31/487 SNPs) and only 8 amino acid substitutions differed among the results generated by MSCSA and Sanger sequencing. We detected no mutations associated with increased virulence or with reversion of protein segment truncation by either method. All surveillance specimens had wild-type sequences in virulence markers (PB2 gene A271, S590, R591,

E627, D701; PB1-F2 gene N66; NS1 gene G227, T228, E229 and I230) and stop codons in protein truncation markers (PB1-F2 stop12, stop58, stop88; NS1 gene stop220). MSCSA and Sanger sequencing detected no mutations associated with antiviral resistance (NA gene V116, I117, E119, Q136, K150, D151, D199, I223, H275 and N295).

Table 3. MSCSA and Sanger sequencing of 70 pH1N1 virus specimens.

Target	Evaluation	Total	NA gene	PB1-F2 gene	PB2 gene	NS1 gene
Nucleotides	Genetic sequence	130204	42525	27216	26860	33603
	Match (%) #	130173 (99,98%)	42512 (99,97%)	27210 (99,98%)	26858 (99,99%)	33593 (99,97%)
Amplicons	Genetic sequence	263	63	63 $	68	69
	Match (%) #	239 (90,9%)	54 (85,7%)	58 (92,1%)	66 (97,1%)	61 (88,4%)
SNPs	Genetic sequence	487	192	35	154	106
	Match (%) #	456 (93,6%)	179 (93,2%)	29 (82,9%)	152 (98,7%)	96 (90,1%)
	SNPs in MSCSA 1	13	8	0	0	5
	SNPs in Sanger 2	18	5	6	2	5

$ Sanger sequencing was succesful in 63 of 65 specimens determined by MSCSA.
# Sequence match using MSCSA and Sanger sequencing.
1 SNPs in MSCSA and not in Sanger sequencing;
2 SNPs in Sanger and not MSCSA.

Clinical Antiviral Resistance Monitoring Specimens. Oseltamivir-resistant H275Y mutated virus was confirmed in 19 of 35 specimens clinical antiviral resistance monitoring specimens by H275Y real-time PCR and these findings were compared to MSCSA and Sanger sequencing results. MSCSA detected NA gene H275Y mutation in 4 of 19 samples (24%) while the mutation was observed in 17/19 samples by Sanger sequencing (89%). MSCSA detected NA gene H275Y mutation in specimens with fully mutant virus populations (4/4) but not in specimens with mixed wild-type and mutant populations (0/15). By visual evaluation of NA gene mass spectrometry spectra, the presence of peaks derived from the wild type sequence as well as from the H275Y-associated mutation were observed in many samples. In this way, we were able to infer the presence of H275Y minor populations in 14 pH1N1 samples, which led to a 95% correspondence between MSCSA and real-time PCR (18 of 19 samples).

Discussion

Molecular surveillance of human influenza viruses is important to monitor viral evolution. Genetic mutations occur frequently due to the lack of proofreading by the influenza virus RNA polymerase and may emerge during recurrent interspecies transmission or wide-scale antiviral selective pressure [1, 7, 14-17]. Rapid identification of relevant mutations associated with increased virulence or antiviral resistance during active surveillance of pH1N1 may guide early prevention and treatment strategies. MSCSA enables medium- to high-throughput genetic sequence analysis of amplified targets of genomic DNA or RNA with automated data analysis [20]. During the 2009 H1N1 pandemic, we evaluated influenza A-positive routine surveillance specimens for virulence or resistance markers using MSCSA and Sanger sequencing and included H275Y real-time PCR on clinical antiviral resistance monitoring specimens. MSCSA did not cover the complete pH1N1 genome since we chose to focus on gene segments **(Table 2)** that contain relevant virulence or resistance markers (with the exception of the HA gene) [11-14, 25, 26].

We observed a high concordance between MSCSA and Sanger sequencing (nucleotides >99%, SNPs 93.6%) in 70 routine surveillance specimens and detected 487 SNPs. Most SNPs encoded for silent mutations and few encoded for non-relevant amino acid changes compared to reference influenza A/California/04/2009 (H1N1) virus. The most frequently detected non-relevant amino acid changes in the NA gene (V106I (n = 61), N248D (n = 62)) and NS1 gene segments (I123V (n = 61), N133D (n = 16)) are well described by others [27]. Genetic mutations associated with increased virulence (PB2 gene positions 271, 590, 591, 627, 701; PB1-F2 gene position 66; NS1 gene positions 227–230), reversion of protein segment truncation (PB1-F2 gene positions 12, 58, 88; NS1 gene position 220) or antiviral resistance (NA gene positions V116, I117, E119, Q136, K150, D151, D199, I223, H275 and N295) were not detected by either method in routine surveillance specimens [11-14, 25, 26]. Amino acid replacement at residue S334 associated with increased oseltamivir resistance was not monitored in the NA amplicon. This is not of great concern because mutations at amino acid S334 can only increase oseltamivir resistance in the presence of H275Y mutation but does not lead to phenotypic antiviral resistance by itself [26]. Genetic sequences of pH1N1 circulating in the region of Leiden appeared genetically stable, similar to pH1N1 during the early phase of the 2009 H1N1 pandemic in Mexico and the United States [7]. MSCSA could not accurately pinpoint the exact position of a small number of SNPs in spite of the reference database. Relevant

SNPs detected by MSCSA always need to be confirmed by Sanger sequencing, pyrosequencing or real-time PCR to limit potential misinterpretations during molecular surveillance. We emphasize that the reference database must be continuously updated with recent virus sequences for MSCSA to remain accurate. The MSCSA reference database has not been updated since the 2009 H1N1 pandemic, therefore newer versions of the reference database were not available to re-analyze the data.

Influenza reverse transcription-polymerase chain reaction/electro-spray ionization (RT-PCR/ESI-MS) assay is a similar mass spectrometry-based automated method which is capable of measuring amplicon-derived masses. The benefit of MSCSA over RT-PCR/ESI-MS is the possibility to analyze larger amplicon sizes (500–800 versus 150 nucleotides) and its capability to detect all nucleotide variations within the analyzed target region rather than providing genomic signatures [28, 29]. Some methods for antiviral resistance detection, like real-time PCR, are fast, but only allow for the analysis of fixed genome positions known to be involved in antiviral drug resistance.

In this study, a second collection of clinical antiviral resistance monitoring specimens was included and real-time PCR detected oseltamivir-resistant H275Y mutated virus in 19 of 35 specimens obtained from 3 of 4 immunocompromised patients. We detected no other mutations that are associated with antiviral resistance including NA gene positions V116, I117, E119, Q136, K150, D151, D199, I223 and N295 [25,26]. Previous influenza studies have shown that immunocompromised patients with prolonged viral excretion are at increased risk for developing neuraminidase inhibitor-resistant virus during continued oseltamivir treatment [30, 31]. Frequent development of antiviral-resistant viruses among 3 of 4 (75%) immunocompromised patients is in agreement with previous studies [32-34]. MSCSA detected H275Y in 24% (4/19) of positive specimens and Sanger sequencing in 89% (17/19). MSCSA only detected H275Y when the mutation was dominant in the analyzed specimens. The ability to infer the presence of H275Y (18 of 19 samples) by visual evaluation of the mass spectrometry spectra indicated that iSEQ software improvement may result in improved detection of minor variants in mixed populations. However, pyrosequencing and real-time PCR currently remain the designated methods for continued antiviral resistance monitoring in clinical settings [22, 25].

In conclusion, MSCSA may be used as a rapid screening tool to monitor fixed nucleotide changes and potential virulence markers in the pH1N1 genetic background. MSCSA does

not seem applicable for continued antiviral resistance monitoring in the clinical setting since detection of the H275Y mutation was limited by a very low sensitivity in the presence of minor variants and mixed genotypes.

Conflict of interest

There was no financial support for the study. None of the authors have associations that might pose a conflict of interest.

Acknowledgements

We thank Eric Snijder (Molecular Virology Laboratory) for valuable assistance and advice. Dennis Kremer, Bas Heijmans, and Eline Slagboom (Department of Molecular Epidemiology) provided valuable MS-based sequencing support.

References

1. Ghedin E, Sengamalay NA, Shumway M, *et al.* Large-scale sequencing of human influenza reveals the dynamic nature of viral genome evolution. *Nature* **2005**; 437: 1162–6.

2. Wise HM, Foeglein A, Sun J, *et al.* A complicated message: Identification of a novel PB1-related protein translated from influenza A virus segment 2 mRNA. *J Virol* **2009**; 83: 8021–31.

3. Jagger BW, Wise HM, Kash JC, *et al.* An overlapping protein-coding region in influenza A virus segment 3 modulates the host response. *Science* **2012**; 337: 199–204.

4. Kilbourne ED. Influenza pandemics of the 20th century. *Emerg Infect Dis* **2006**; 12: 9–14.

5. Centers for Disease Control and Prevention (CDC). Outbreak of swine-origin influenza A (H1N1) virus infection - Mexico, March-April 2009. *MMWR Morb Mortal Wkly Rep* **2009**; 58: 467–70.

6. Writing Committee of the WHO Consultation on Clinical Aspects of Pandemic (H1N1) 2009 Influenza, Bautista E, Chotpitayasunondh T, Gao Z, *et al.* Clinical aspects of pandemic 2009 influenza A (H1N1) virus infection. *N Engl J Med* **2010**; 362: 1708–19.

7. Garten RJ, Davis CT, Russell CA, *et al.* Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. *Science* **2009**; 325: 197–201.

8. World Health Organization website. Pandemic (H1N1) 2009-update 112 (2010) Available: http://www.who.int/entity/csr/don/2010_08_06/en/index.html; access verified on Mar 3rd, **2013**.

9. Hancock K, Veguilla V, Lu X, *et al.* Cross-reactive antibody responses to the 2009 pandemic H1N1 influenza virus. *N Engl J Med* **2009**; 361: 1945–52.

10. Tu W, Mao H, Zheng J, *et al.* Cytotoxic T lymphocytes established by seasonal human influenza cross-react against 2009 pandemic H1N1 influenza virus. *J Virol* **2010**; 84: 6527–35.

11. Neumann G, Noda T, Kawaoka Y. Emergence and pandemic potential of swine-origin H1N1 influenza virus. *Nature* **2009**; 459: 931–9.

12. McAuley JL, Chipuk JE, Boyd KL, Van De Velde N, Green DR, McCullers JA. PB1-F2 proteins from H5N1 and 20 century pandemic influenza viruses cause immunopathology. *PLoS Pathog* **2010**; 6: e1001014.

13. Liu Q, Qiao C, Marjuki H, *et al.* Combination of PB2 271A and SR polymorphism at positions 590/591 is critical for viral replication and virulence of swine influenza virus in cultured cells and in vivo. *J Virol* **2012**; 86: 1233–7.

14. Ozawa M, Basnet S, Burley LM, Neumann G, Hatta M, Kawaoka Y. Impact of amino acid mutations in PB2, PB1-F2, and NS1 on the replication and pathogenicity of pandemic (H1N1) 2009 influenza viruses. *J Virol* **2011**; 85: 4596–601.

15. Yamada S, Hatta M, Staker BL, *et al.* Biological and structural characterization of a host-adapting amino acid in influenza virus. *PLoS Pathog* **2010**; 6: e1001034.

16. Octaviani CP, Li C, Noda T, Kawaoka Y. Reassortment between seasonal and swine-origin H1N1 influenza viruses generates viruses with enhanced growth capability in cell culture. *Virus Res* **2011**; 156: 147–50.

17. Moore C, Galiano M, Lackenby A, *et al*. Evidence of person-to-person transmission of oseltamivir-resistant pandemic influenza A(H1N1) 2009 virus in a hematology unit. *J Infect Dis* **2011**; 203: 18–24.

18. Seibert CW, Kaminski M, Philipp J, *et al*. Oseltamivir-resistant variants of the 2009 pandemic H1N1 influenza A virus are not attenuated in the guinea pig and ferret transmission models. *J Virol* **2010**; 84: 11219–26.

19. Posthuma CC, van der Beek MT, van der Blij-de Brouwer CS, *et al*. Mass spectrometry-based comparative sequencing to detect ganciclovir resistance in the UL97 gene of human cytomegalovirus. *J Clin Virol* **2011**; 51: 25–30.

20. Honisch C, Chen Y, Mortimer C, *et al*. Automated comparative sequence analysis by base-specific cleavage and mass spectrometry for nucleic acid-based microbial typing. *Proc Natl Acad Sci U S A* **2007**; 104: 10649–54.

21. European Commission. Commission Decision of 30 April 2009 amending Decision 2002/253/EC laying down case definitions for reporting communicable diseases to the Community network under Decision No 2119/98/EC of the European Parliament and of the Council. L 110/58. 1.5.2009. *Official Journal of the European Union*. **2009**. Available: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:110:0058:0059:EN:PDF; access verified on March 3rd, 2013.

22. van der Vries E, Jonges M, Herfst S, *et al*. Evaluation of a rapid molecular algorithm for detection of pandemic influenza A (H1N1) 2009 virus and screening for a key oseltamivir resistance (H275Y) substitution in neuraminidase. *J Clin Virol* **2010**; 47: 34–7.

23. Meijer A, Beerens A, Claas E, *et al*. Preparing the outbreak assistance laboratory network in the Netherlands for the detection of the influenza virus A(H1N1) variant. *J Clin Virol* **2009**; 45: 179–84.

24. Rahamat-Langendoen JC, Tutuhatunewa ED, Schölvinck EH, *et al*. Influenza in the immediate post-pandemic era: a comparison with seasonal and pandemic influenza in hospitalized patients. *J Clin Virol* **2012**; 54: 135–40.

25. Deyde VM, Sheu TG, Trujillo AA, *et al*. Detection of molecular markers of drug resistance in 2009 pandemic influenza A (H1N1) viruses by pyrosequencing. *Antimicrob Agents Chemother* **2010**; 54: 1102–10.

26. Pizzorno A, Bouhy X, Abed Y, Boivin G. Generation and characterization of recombinant pandemic influenza A(H1N1) viruses resistant to neuraminidase inhibitors. *J Infect Dis* **2011**; 203: 25–31.

27. Pascalis H, Temmam S, Wilkinson DA, *et al*. Molecular evolutionary analysis of pH1N1 2009 influenza virus in Reunion Island, South West Indian Ocean region: a cohort study. *PLoS One* **2012**; 7: e43742.

28. Deyde VM, Sampath R, Garten RJ, *et al*. Genomic signature-based identification of influenza A viruses using RT-PCR/electro-spray ionization mass spectrometry (ESI-MS) technology. *PLoS One* **2010**; 5: e13293.

29. Jeng K, Massire C, Zembower TR, *et al*. Monitoring seasonal influenza A evolution: rapid 2009 pandemic H1N1 surveillance with an reverse transcription-polymerase chain reaction/electro-spray ionization mass spectrometry assay. *J Clin Virol* **2012**; 54: 332–6.

30. Gooskens J, Jonges M, Claas EC, Meijer A, Kroes AC. Prolonged influenza virus infection during lymphocytopenia and frequent detection of drug-resistant viruses. *J Infect Dis* **2009**; 199: 1435–41.

31. Carr S, Ilyushina NA, Franks J, *et al.* Oseltamivir-resistant influenza A and B viruses pre- and post-antiviral therapy in children and young adults with cancer. *Pediatr Infect Dis* J **2011**; 30: 284–8.

32. Harvala H, Gunson R, Simmonds P, *et al.* The emergence of oseltamivir-resistant pandemic influenza A (H1N1) 2009 virus amongst hospitalised immunocompromised patients in Scotland, November-December, 2009. *Euro Surveill* **2010**; 15. pii: 19536.

33. Tramontana AR, George B, Hurt AC, *et al.* Oseltamivir resistance in adult oncology and hematology patients infected with pandemic (H1N1) 2009 virus, Australia. *Emerg Infect Dis* **2010**; 16: 1068–75.

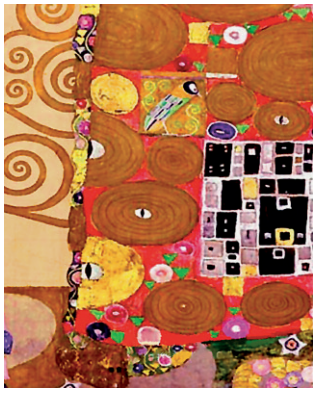
34. Renaud C, Boudreault AA, Kuypers J, *et al.* H275Y mutant pandemic (H1N1) 2009 virus in immunocompromised patients. *Emerg Infect Dis* **2011**; 17: 653–60

Chapter 8

Host immune responses dictate influenza A(H1N1)pdm09 infection outcome in hematology-oncology patients

Gooskens J [1], Marijt WAF [2], van Essen EHR [3],
Rimmelzwaan GF [4], Kroes ACM [1]

Bone Marrow Transplantation **2015**; Advance online publication.



1 Department of Medical Microbiology
Leiden University Medical Center, Leiden, the Netherlands
2 Department of Hematology
Leiden University Medical Center, Leiden, the Netherlands
3 Department of Intensive Care
Leiden University Medical Center, Leiden, the Netherlands
4 Department of Viroscience
Erasmus University Medical Center, Rotterdam, the Netherlands

Introduction

Hematology-oncology patients can develop remarkably prolonged influenza virus excretion with an enigmatic wide clinical spectrum [1-6]. These patients often develop mild virus-associated symptoms and occasional bacterial or fungal pneumonic coinfections, but significant numbers of cases develop severe influenza virus-associated lower respiratory tract infection (LRTI) and acute respiratory distress syndrome (ARDS). Risk factors for influenza LRTI include profound lymphopenia, lack of early antiviral treatment and old age [5, 6]. Pathogenesis of severe viral LRTI and ARDS is unclear and may include virus-induced pathology or excessive immunopathology [7]. Impaired influenza-specific host immune responses are well-established in hematology-oncology patients but surprisingly little is known about the interactions with virus-associated clinical manifestations and outcomes. The recent introduction of influenza A (H1N1)pdm09 virus in the human population with limited preexistent immunity provided the opportunity to evaluate the role of innate and adaptive host immune findings in determining virus-specific symptoms and viral clearance among hematology-oncology patients with prolonged viral excretion.

Methods

Patients. In this observational study, adult hematology-oncology patients hospitalized with ≥14 days prolonged A(H1N1)pdm09 virus excretion between November 2009 and April 2013 were eligible for inclusion. The institutional review board approved the pre-established study protocol and informed consent forms that were obtained from all subjects. Patients or legal representatives signed informed consent for voluntary study participation and confidentiality. Permission was granted for clinical data collection, blood draw of a research specimen and immunologic studies. Exclusion criteria were patients aged < 18 years, patients deemed unfit by the treating physician (for example, owing to severe underlying bleeding disorders, religious background including Jehova's witnesses, altered mental or emotional status) and patients or legal representatives not wishing to enter the study.

Clinical data and virus diagnostics. Medical records were reviewed for relevant clinical findings, virus-associated symptoms and outcomes. Respiratory specimens were assessed for A(H1N1)pdm09, neuraminidase gene H275Y mutation encoding oseltamivir

resistance and other respiratory viruses using real-time PCR and viral culture [8]. Pulmonary imaging (chest radiography or computed tomography), bronchoalvolar lavage microbiology results and broad-spectrum antimicrobial treatment regimens were evaluated to confirm severe A(H1N1)pdm09- associated LRTI and ARDS requiring invasive mechanical ventilation and to exclude concomitant infections (viral, bacterial or fungal) and non-infectious cardiopulmonary causes (lung embolism, pneumothorax or congestive cardiac failure). Influenza A (H1N1)pdm09 virus isolates were routinely cultured in established lines of monkey kidney cells (LLCMK2) and Madin-Darby canine kidney cells and further characterized using duplicate hemagglutinin inhibition (HI) tests to confirm antigenic similarity with the corresponding vaccine strain.

Humoral and cell-mediated immune responses. Humoral responses were determined against homologous virus and reference A/California/007/09 vaccine strain using serum duplicate HI tests, turkey erythrocytes and four hemagglutinin units of virus. Seroconversion was defined as a fourfold rise of HI titers and HI titers ≥80 were considered seroprotective. White blood cell differential counts were evaluated. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation and lymphocyte subsets were quantified by flow cytometry using CD3, CD4, CD8, CD16, CD19 and CD56 fluorochrome-labeled antibodies [9]. Minimum absolute cell count references were defined for granulocytes (500/mm^3), monocytes (100/mm^3), lymphocytes (1000/mm^3), $CD3^+CD4+$ (560/mm^3) T cells, $CD3^+CD8^+$ (260/mm^3) T cells, $CD3^-CD16^+$ and/or $CD56^+$ nature killer (NK) cells (40/mm^3) and $CD19^+$ B cells (60/mm^3). T-cell counts ≤20% of minimum reference were defined as profound T-cell lymphopenia [10]. Defined surrogate antibody-dependent cell-mediated cytotoxicity (ADCC) markers included the combined presence of seroprotective HI titers with $CD16^+$ FcIgG receptor-bearing cytotoxic NK cells and monocytes in blood. The presence of virus-specific T cells was evaluated by flow cytometry. In brief, thawed PBMCs were stimulated with conserved peptides derived from the nucleoprotein and M1 protein, live influenza A/Netherlands/602/09 (H1N1)pdm09 virus or reference strain Resvir-9 (H3N2), 1 µg/ml Staphylococcus enterotoxin B (SEB; Sigma-Aldrich, Zwijndrecht, the Netherlands), or left untreated. The cells were permeabilized and incubated with antibodies directed against differentiation (CD3, αCD3 PerCP BD Biosciences (Breda, The Netherlands) # 345766; CD4, αCD4 Pacific blue BD Biosciences # 558116; CD8, αCD8 PECy7 eBioscience # 25-0088-42), activation (CD69, αCD69 APC BD Biosciences # 340560) and intracellular cytokine expression (IFNγ, αIFN-γ FITC eBioscience (Vienna, Austria) # 11-7319-82) markers and analyzed by flow cytometry (~ 1 ×10^6 cells per sample). Dead cell

staining excluded cells with non-specific results. SEB was used as a positive control and to monitor functional integrity of T cells. Functional virus-specific T cells were confirmed by duplicate detection of $CD3^+CD4^+CD69^+IFN\gamma^+$ or $CD3^+CD8^+CD69^+IFN\gamma^+$ cells [9, 11], excluding profound T-cell lymphopenia. Virus- and SEB-specific T cells were calculated by subtracting $CD4^+IFN\gamma^+$ and $CD8^+IFN\gamma^+$ cell percentages observed after incubation with medium only.

Results

Enrolled patients. Six adult hematology-oncology patients (age, range 39–67 years) hospitalized with prolonged A(H1N1)pdm09 excretion (duration, 29 to >90 days) were enrolled (Figure 1a). Patient 1 (cutaneous T-cell lymphoma) and patient 2 (acute undifferentiated leukemia) received immunosuppressive agents for the prophylaxis or treatment of GvHD following allogeneic hematopoietic stem cell transplantation (allo-HSCT) 7 and 22 months earlier. Patient 3 (chronic lymphocytic leukemia) received high-dose steroids for leukemic hyperleukocytosis. Pre-allo-HSCT conditioning regimens were provided to patient 4 (progressive multiple myeloma), patient 5 (refractory T-cell non-Hodgkin lymphoma) and patient 6 (refractory B-cell non-Hodgkin lymphoma). Never was the decision taken to proceed with allo-HSCT during any knowledge of active A (H1N1)pdm09 infection. Four patients received a well-matched influenza vaccine (patients 1, 3, 4 and 5) during the corresponding season but had no seroprotective HI Ab titers during onset (Figure 1b). Oseltamivir or zanamivir antiviral treatment was provided to all patients (n=2, ≤48 h; n=4, >48 h). Four patients developed H275Y resistant virus (Figure 1a) and variable clinical outcomes (2 mild, 2 severe).

Prolonged A(H1N1)pdm09 virus excretion. All six patients displayed prolonged viral excretion during $CD4^+$ and $CD8^+$ T-cell lymphopenia (Figure 2a). Virus excretion duration was not influenced by antiviral treatment, seroprotective HI titers (Figures 1a, 1b) or ADCC markers (data not shown).

Virus-associated symptoms. The six patients developed a wide spectrum of virus-associated symptoms ranging from mild URTI to severe LRTI and ARDS (Figure 1a). Intercurrent pulmonary co-infections and non-infectious cardiopulmonary diagnoses (congestive cardiac failure, pneumothorax) were excluded or effectively treated and did not seem to confound virus-associated measures. Two cases (patients 1 and 2) displayed

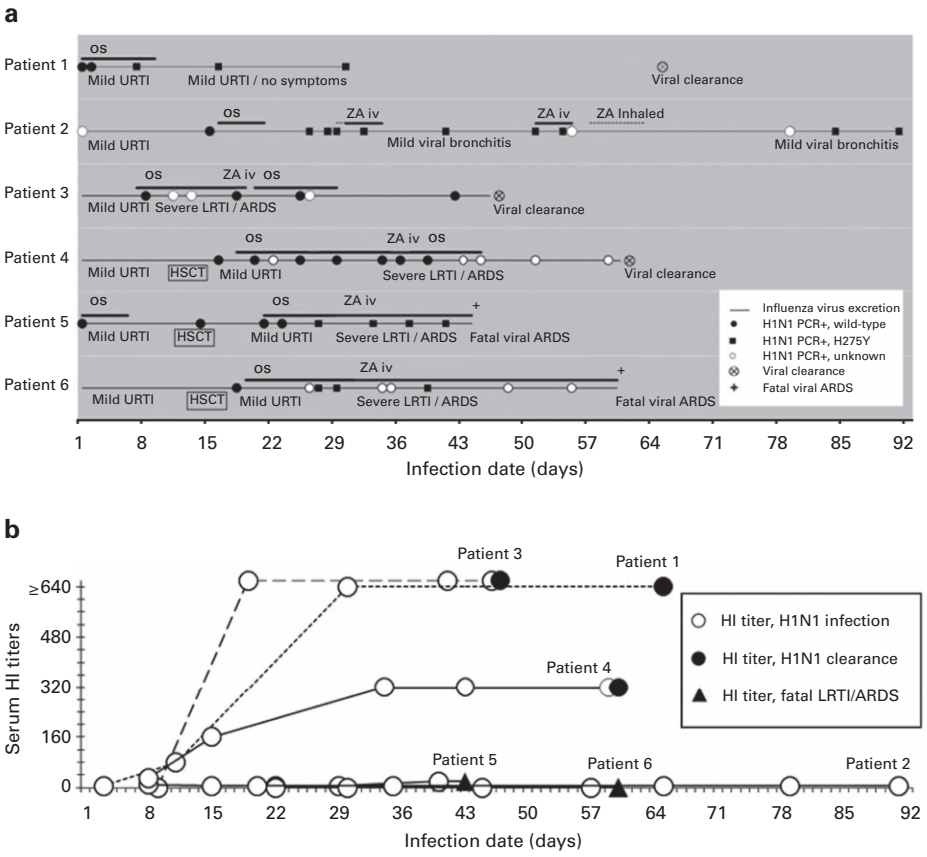


Figure 1. (a) Timeline of prolonged A(H1N1)pdm09 virus infection and antiviral treatment. (b) Humoral responses during A(H1N1)pdm09 virus infection.

HI titers are expressed as the reciprocals of the highest serum dilution that inhibited homologous virus hemagglutination.

mild symptoms during the presence of CD8+ T cells (Figure 2a). Mild symptoms of patient 1 completely alleviated in the presence of ADCC markers whereas patient 2 manifested sustained mild bronchitis and recurrent Haemophilus influenzae broncho-pneumonia during absence of seroprotective antibodies. Four cases (patients 3, 4, 5 and 6) developed severe viral LRTI during profound CD4+ and CD8+ T-cell lymphopenia (Figures 1a, 2a) and intercurrent combined absence of ADCC markers. The onset of severe viral LRTI and ARDS during profound CD4+ and CD8+ T-cell lymphopenia coincided with innate cell-mediated immune reconstitution in all four patients

(Figure 2b). Patients 4, 5 and 6 manifested granulocyte, monocyte and NK cell reconstitution after recent allo-HSCT and patient 3 displayed granulocyte reconstitution during leukemic hyperleukocytosis.

Viral clearance. Complete viral clearance occurred strictly during $CD4^+$ and $CD8^+$ T-cell reconstitution and functional virus-specific T-cell responses (Figure 2a) in patient 1 ($CD3^+CD4^+CD69^+IFN\gamma^+$ 0.02%; $CD3^+CD8^+CD69^+IFN\gamma^+$ 0.12%), patient 3 ($CD4^+CD69^+IFN\gamma^+$ 0.47%; $CD3^+CD8^+CD69^+IFN\gamma^+$ 0.20%) and patient 4 ($CD4^+$ T-cell reconstitution with homologous virus seroconversion and emergence of $CD8^+$ T cells).

Discussion

The findings from this study confirm that influenza virus-infected hematology-oncology patients develop a wide clinical spectrum ranging from mild [1] to severe respiratory symptoms [2-4] during prolonged viral excretion. Innate and adaptive host immune responses appear to be major determinants of virus-associated outcome and viral clearance [3, 6, 7]. Earlier studies report that undefined lymphopenia increases the risk for prolonged influenza virus excretion [5, 6] and that lymphocyte reconstitution is associated with viral clearance [3]. Our findings show that prolonged viral excretion more specifically correlated with T-cell lymphopenia. It is generally assumed that T cells induce viral clearance [7], but this has not clearly been demonstrated in human influenza cases. The role of $CD8^+$ T cells has frequently been characterized in influenza animal models but the role of $CD4^+$ T cells remains unclear [12]. We confirm that viral clearance occurs during $CD4^+$ and $CD8^+$ T-cell reconstitution in the presence of functional virus-specific T cells (Figure 2a). Viral clearance did not occur during profound $CD4^+$ T-cell and $CD8^+$ T-cell lymphopenia (patients 5 and 6) or during profound $CD4^+$ T-cell lymphopenia with a low-level $CD8^+$ T-cell count (patient 2). This observation supports the assumption that both $CD4^+$ and $CD8^+$ T-cell responses determine complete viral clearance [12].

The wide clinical spectrum among six hematology-oncology patients with prolonged influenza virus excretion prompted an investigation into the protective role of host immune responses and pathogenesis. Mild symptoms (patients 1 and 2) correlated with a low-level presence of $CD8^+$ T cells even during absence of seroprotective HI titers (patient 2). In contrast, severe viral LRTI (patients 3, 4, 5 and 6) manifested during profound $CD4^+$ and $CD8^+$ T-cell lymphopenia and intercurrent absence of ADCC markers

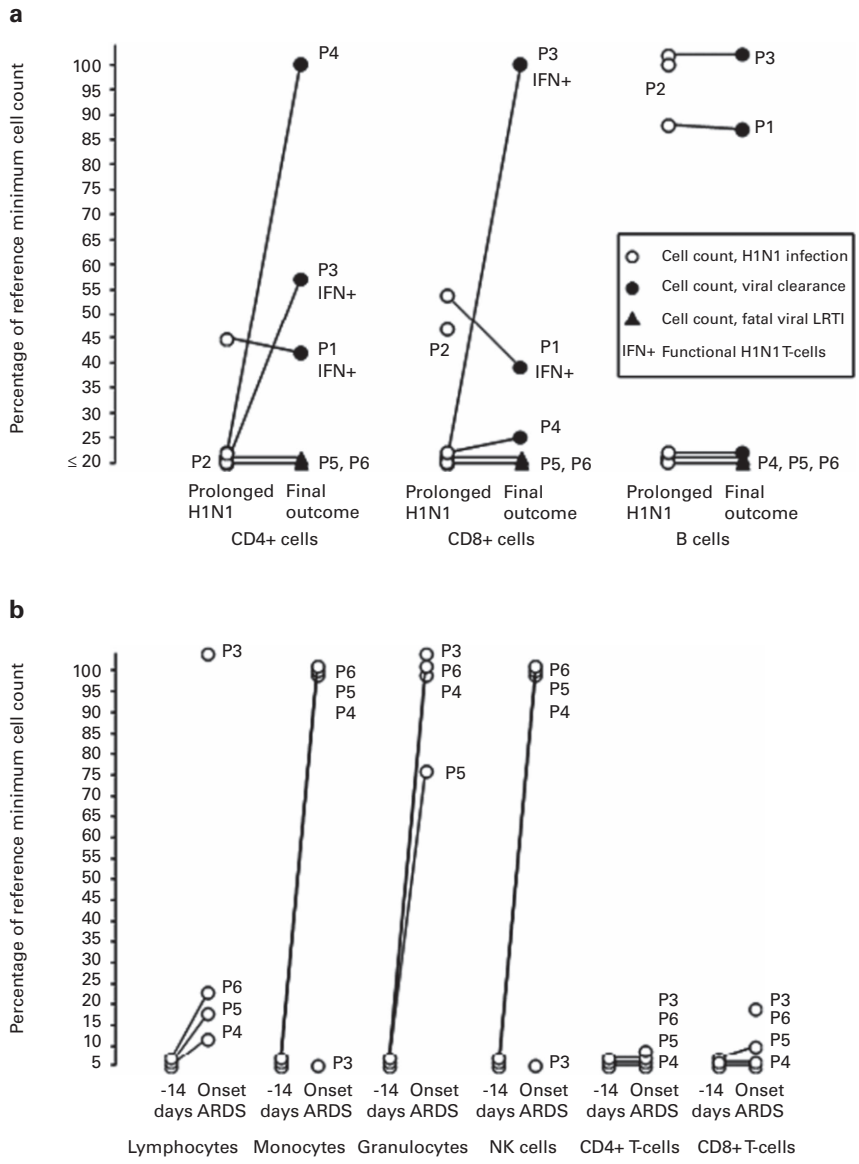


Figure 2. (a) T- and B-cell counts during prolonged A(H1N1)pdm09 virus infection and final outcome. (b) Cell-mediated responses during a 2-week development period of ARDS.

even during the presence of seroprotective HI titers (patients 3 and 4). Patients 3 and 4 developed seroprotective HI titers despite transient profound $CD4^+$ T-cell lymphopenia. High virus-specific Ab titers did not prevent the development of severe viral LRTI and ARDS probably due to intercurrent absence of ADCC effector cells. Altogether, these results support the hypothesis that $CD8^+$ T cells independently mediate clinical protection [7, 11] and that ADCC provides additional clinical protection [13]. The pathogenesis of severe influenza LRTI and ARDS remains unclear and is likely multifactorial. Four cases (patients 3, 4, 5 and 6) with (transient) profound T-cell lymphopenia and absence of ADCC markers developed severe virus-associated LRTI and ARDS during innate cell reconstitution. Our findings suggest that profound $CD4^+$ and $CD8^+$ T-cell lymphopenia and (transient) absence of ADCC markers may have provided a window of opportunity for the virus to reach lower alveolar compartments and trigger severe immunopathology by the excessive influx of neutrophils and macrophages [14]. Timely antiviral treatment is therefore important when protective immune responses are still lacking and early IV zanamivir therapy may benefit patients who are most at risk [15]. Study limitations include a small sample size due to the rare occurrence of prolonged influenza virus excretion, which does not permit statistical analysis. Additional limitations include technical difficulties of measuring T-cell and ADCC responses, unblinded clinical and outcome assessments by the clinical investigator and incomplete detection of existing antiviral resistance mutations. Despite these limitations, the study provides new insights into the role of host immune responses in determining influenza infection outcomes. Our findings underline the importance of influenza prevention strategies in hematology-oncology patients and show that vaccine improvements are needed to raise immunogenicity in this vulnerable patient group.

In conclusion, prolonged influenza virus excretion is associated with T-cell lymphopenia in hematology-oncology patients. $CD8^+$ T cells and ADCC markers afford clinical protection and combined $CD4^+$ and $CD8^+$ T-cell responses mediate viral clearance. Pathogenesis of severe viral LRTI and ARDS is likely the result of virus reaching lower compartments of the lung during a lack of combined T-cell- and ADCC-mediated immunological protection followed by excessive immunopathology triggered by innate cell-mediated responses. More insight into the role of influenza host immune responses can improve the clinical management of infected hematology-oncology patients and may limit the emergence of antiviral-resistant viruses.

Conflict of interest

There was no financial support for the study. None of the authors have associations that might pose a conflict of interest.

Acknowledgements

We thank Eric Claas (Department of Medical Microbiology, Leiden) and Geeske Brouwer-Mandema (Department of Hematology, Leiden) for valuable assistance and advice. We are indebted to Martina Geelhoed-Mieras, Carel van Baalen and Tamara Roelofse (Department of Virology, Rotterdam) for excellent technical support. None of these persons were financially compensated for providing assistance and technical support.

References

1. Redelman-Sidi G, Sepkowitz KA, Huang CK, *et al*. 2009 H1N1 influenza infection in cancer patients and hematopoietic stem cell transplant recipients. *J Infect* **2010**; 60: 257–63.

2. Nichols WG, Guthrie KA, Corey L, Boeckh M. Influenza infections after hematopoietic stem cell transplantation: risk factors, mortality, and the effect of antiviral therapy. *Clin Infect Dis* **2004**; 39: 1300–6.

3. Gooskens J, Jonges M, Claas EC, Meijer A, Kroes AC. Prolonged influenza virus infection during lymphocytopenia and frequent detection of drug-resistant viruses. *J Infect Dis* **2009**; 199: 1435–41.

4. Tramontana AR, George B, Hurt AC, *et al*. Oseltamivir resistance in adult oncology and hematology patients infected with pandemic (H1N1) 2009 virus, Australia. *Emerg Infect Dis* **2010**; 16: 1068–75.

5. Ljungman P, de la Camara R, Perez-Bercoff L, *et al*; Infectious Diseases Working Party, European Group for Blood and Marrow Transplantation; Infectious Complications Subcommittee, Spanish Group of Haematopoietic Stem-cell Transplantation. Outcome of pandemic H1N1 infections in hematopoietic stem cell transplant recipients. *Haematologica* **2011**; 96: 1231–5.

6. Choi SM, Boudreault AA, Xie H, Englund JA, Corey L, Boeckh M. Differences in clinical outcomes after 2009 influenza A/H1N1 and seasonal influenza among hematopoietic cell transplant recipients. *Blood* **2011**; 117: 5050–6.

7. Hillaire M, Rimmelzwaan G, Kreijtz J. Clearance of influenza virus infections by T cells: risk of collateral damage? *Curr Opin Virol* **2013**; 3: 430–7.

8. van der Vries E, Jonges M, Herfst S, *et al*. Evaluation of a rapid molecular algorithm for detection of pandemic influenza A (H1N1) 2009 virus and screening for a key oseltamivir resistance (H275Y) substitution in neuraminidase. *J Clin Virol* **2010**; 47: 34–7.

9. Bodewes R, Fraaij PL, Geelhoed-Mieras MM, *et al*. Annual vaccination against influenza virus hampers development of virus-specific CD8 T cell immunity in children. *J Virol* **2011**; 85: 11995–2000.

10. Hakki M, Riddell SR, Storek J, *et al*. Immune reconstitution to cytomegalovirus after allogeneic hematopoietic stem cell transplantation: impact of host factors, drug therapy, and subclinical reactivation. *Blood* **2003**; 102: 3060–7.

11. Hillaire ML, van Trierum SE, Bodewes R, *et al*. Characterization of the human CD8 T cell response following infection with 2009 pandemic influenza H1N1 virus. *J Virol* **2011**; 85: 12057–61.

12. Thomas PG, Keating R, Hulse-Post DJ, Doherty PC. Cell-mediated protection in influenza infection. *Emerg Infect Dis* **2006**; 12: 48–54.

13. Jegaskanda S, Weinfurter JT, Friedrich TC, Kent SJ. Antibody-Dependent Cellular Cytotoxicity Is Associated with Control of Pandemic H1N1 Influenza Virus Infection of Macaques. *J Virol* **2013**; 87: 5512–22.

14. Narasaraju T, Yang E, Samy RP, *et al*. Excessive neutrophils and neutrophil extracellular traps contribute to acute lung injury of influenza pneumonitis. *Am J Pathol* **2011**; 179: 199–210.

15. Fraaij PL, van der Vries E, Beersma MF, *et al*. Evaluation of the antiviral response to zanamivir administered intravenously for treatment of critically ill patients with pandemic influenza A (H1N1) infection. *J Infect Dis* **2011**; 204: 777–82.

Chapter 9

General discussion

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 [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 [13, 14]. The two children in this study had clinical manifestations that were similar to severe IAE cases in Japan [15]. Previous studies report occasional detection of influenza RNA in cerebrospinal fluids but neuro-invasion remains controversial [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 [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 [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 [18]. Recent studies confirm that influenza RNA can be detected in the blood of patients with severe illness [22, 23]. The lack of virus replication in the brain implicates pro-inflammatory cytokines in the pathogenesis [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 [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 [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 [70-73]. Previous assumptions that NAI-resistance invariably compromised virus fitness were no longer valid [74]. 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 [75-77]. 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 [41, 59-61]. 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 [78, 79]. We underscore that antiviral susceptibility monitoring is important to guide influenza treatment and control [46, 70]. Unfortunately, oseltamivir treatment is hampered by drug-resistant viruses due to treatment failures [53, 63, 64]. Inhaled zanamivir is effective for mild [63] but not for severe influenza LRTI due to the risks of bronchospasms and clogging of ventilator tubes [80-82]. 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 **(Figure 1)** [83, 84]. Future antiviral resistance surveillance studies should monitor permissive and antiviral resistance mutations in the clinical and community setting.

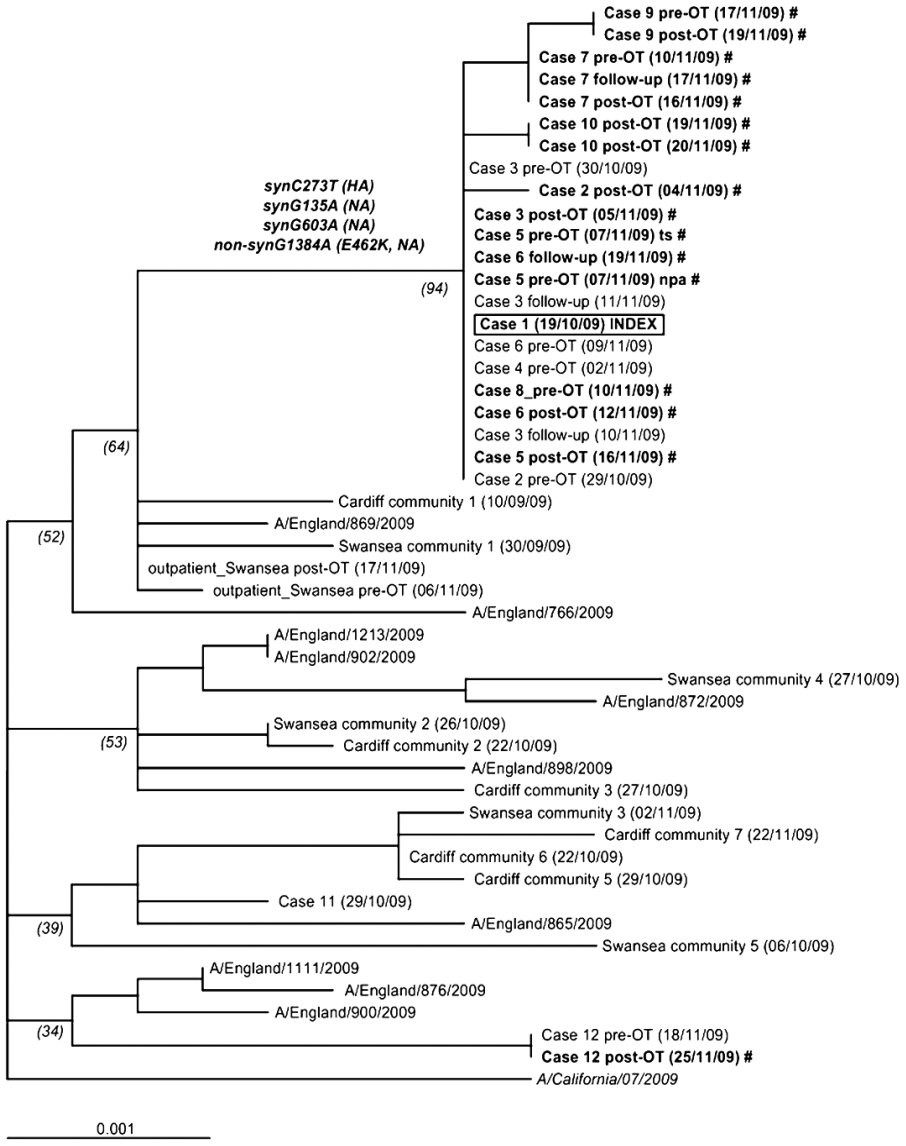


Figure 1. Maximum-likelihood tree of concatenated HA and NA genes of A(H1N1)pdm09 hospital outbreak strains, unlinked clinical strains and community surveillance strains in Wales and the United Kingdom.

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

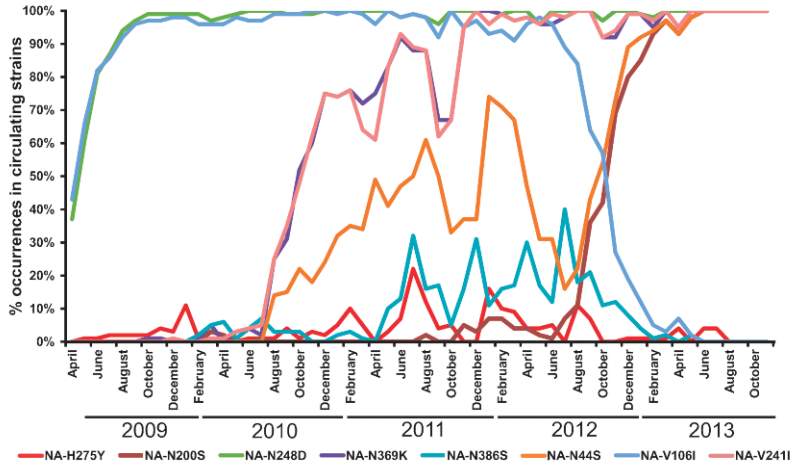


Figure 2. Emergence of V241I and N369K permissive mutations in circulating A(H1N1)pdm09 viruses.
Source: adapted from [108].

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].

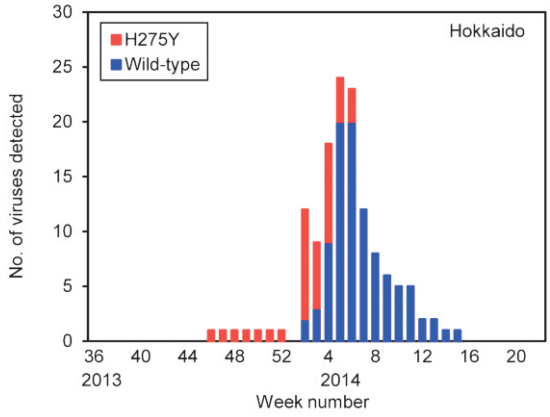


Figure 3. Circulating H275Y virus was replaced by wild-type A(H1N1)pdm09 virus in Hokkaido, Japan.
Source: adapted from [107].

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 [115, 116]. Our findings corroborate with virus challenge studies in healthy volunteers that correlated virus-specific T-cells with influenza host immunity [117] and with human CMV studies that correlated virus-specific T-cells with viral clearance [118]. $CD4^+$ and $CD8^+$ influenza virus-specific T-cell responses circulate for only ~30 days after infection and are associated with recent active infection **(Figure 4)** [119]. 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 [120, 121]. 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 [122]. 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 [123, 124]. Recent studies show that severe human influenza infections manifest hypercytokinemia [24-26, 125, 126]. New treatments using sphingosine analogs effectively temper immune pathology in animal models and may be promising in humans [127, 128]. 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.

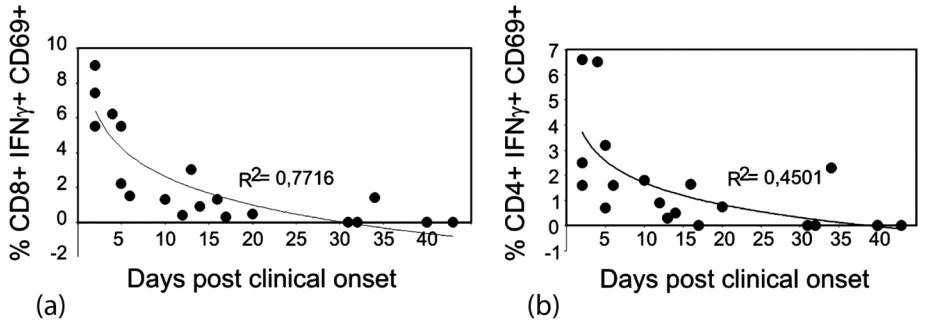


Figure 4. The percentages of virus-specific CD8⁺ (a) and CD4⁺ (b) influenza (H1N1)pdm09 virus-specific T-cells in individual patients at different time points after infection onset.
Source: adapted from [119].

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 hematology-oncology 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 hemagglutin 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

1. Black RE, Cousens S, Johnson HL, *et al*; Child Health Epidemiology Reference Group of WHO and UNICEF. Global, regional, and national causes of child mortality in 2008: a systematic analysis. *Lancet* **2010**; 375: 1969–87.

2. Nair H, Brooks WA, Katz M, *et al*. Global burden of respiratory infections due to seasonal influenza in young children: a systematic review and meta-analysis. *Lancet* **2011**; 378: 1917–30.

3. Gill PJ, Ashdown HF, Wang K, *et al*. Identification of children at risk of influenza-related complications in primary and ambulatory care: a systematic review and meta-analysis. *Lancet Respir Med* **2015**; 3: 139–49.

4. Garcia-Garcia ML, Calvo C, Pozo F, Villadangos PA, Prez-Brea P, Casas I. Spectrum of respiratory viruses in children with community-acquired pneumonia. *Pediatr Infect Dis J* **2012**, 31: 808–13.

5. Laurent C, Dugu AE, Brouard J, *et al*. Viral epidemiology and severity of respiratory infections in infants in 2009: a prospective study. *Pediatr Infect Dis J* **2012**, 31: 827–31.

6. Conway NT, Wake ZV, Richmond PC, *et al*, on Behalf of the Western Australia Influenza Vaccine Effectiveness (WAIVE) Team. Clinical Predictors of Influenza in Young Children: The Limitations of "Influenza-Like Illness". *J Ped Infect Dis* **2013**; 2: 21–9.

7. Kasper MR, Wierzba TF, Sovann L, Blair PJ, Putnam SD. Evaluation of an influenza-like illness case definition in the diagnosis of influenza among patients with acute febrile illness in Cambodia. *BMC Infect Dis* **2010**; 10: 320.

8. Aziz M, Vasoo S, Aziz Z, Patel S, Eltoukhy N, Singh K. Oseltamivir overuse at a Chicago hospital during the 2009 influenza pandemic and the poor predictive value of influenza-like illness criteria. *Scand J Infect Dis* **2012**; 44: 306–11.

9. Rhedin S, Hamrin J, Naucler P, *et al*. Respiratory viruses in hospitalized children with influenza-like illness during the h1n1 2009 pandemic in Sweden [corrected]. *PLoS One* **2012**; 7: e51491.

10. Messacar K, Schreiner TL, Maloney JA, *et al*. A cluster of acute flaccid paralysis and cranial nerve dysfunction temporally associated with an outbreak of enterovirus D68 in children in Colorado, USA. Lancet. **2015**; 385: 1662–71.

11. Pierangeli A, Scagnolari C, Selvaggi C, *et al*. Virological and clinical characterization of respiratory infections in children attending an emergency department during the first autumn-winter circulation of pandemic A (H1N1) 2009 influenza virus. *Clin Microbiol Infect* **2012**, 18: 366–73.

12. Ruf BR, Knuf M. The burden of seasonal and pandemic influenza in infants and children. *Eur J Pediatr* **2014**; 173: 265–76.

13. Centers for Disease Control and Prevention (CDC). Update: Influenza activity—United States and worldwide, 2006-07 season, and composition of the 2007-08 influenza vaccine. *MMWR Morb Mortal Wkly Rep* **2007**; 56: 789–94.

14. Louie JK, Schechter R, Honarmand S, *et al*. Severe pediatric influenza in California, 2003–2005: implications for immunization recommendations. *Pediatrics* **2006**; 117: e610–8.

15. Morishima T, Togashi T, Yokota S, *et al*. Encephalitis and encephalopathy associated with an influenza epidemic in Japan. *Clin Infect Dis* **2002**; 35: 512–7.

16. Fujimoto S, Kobayashi M, Uemura O, *et al*. PCR on cerebrospinal fluid to show influenza-associated acute encephalopathy or encephalitis. *Lancet* **1998**; 352: 873–5.

17. Ito Y, Ichiyama T, Kimura H, *et al*. Detection of influenza virus RNA by reverse transcription-PCR and proinflammatory cytokines in influenza-virus-associated encephalopathy. *J Med Virol* **1999**; 58: 420–5.

18. Steininger C, Popow-Kraupp T, Laferl H, *et al*. Acute encephalopathy associated with influenza A virus infection. *Clin Infect Dis* **2003**; 36: 567–74.

19. Louria DB, Blumenfeld HL, Ellis JT, Kilbourne ED, Rogers DE. Studies on influenza in the pandemic of 1957-1958. II. Pulmonary complications of influenza. *J Clin Invest* **1959**; 38: 213–65.

20. Nakajima N, Sato Y, Katano H, *et al*. Histopathological and immunohistochemical findings of 20 autopsy cases with 2009 H1N1 virus infection. *Mod Pathol* **2012**; 25: 1–13.

21. Nara A, Nagai H, Yamaguchi R, Yoshida K, Iwase H, Mizuguchi M. An unusual autopsy case of cytokine storm-derived influenza-associated encephalopathy without typical histopathological findings: autopsy case report. *Am J Forensic Med Pathol* **2015**; 36: 3–5.

22. Tse H, To KK, Wen X, *et al*. Clinical and virological factors associated with viremia in pandemic influenza A/H1N1/2009 virus infection. *PLoS One* **2011**; 6: e22534.

23. Choi SM, Xie H, Campbell AP, *et al*. Influenza viral RNA detection in blood as a marker to predict disease severity in hematopoietic cell transplant recipients. *J Infect Dis* **2012**; 206: 1872–7.

24. Kawada J, Kimura H, Ito Y, *et al*. Systemic cytokine responses in patients with influenza-associated encephalopathy. *J Infect Dis* **2003**; 188: 690–8.

25. Mizuguchi M, Yamanouchi H, Ichiyama T, Shiomi M. Acute encephalopathy associated with influenza and other viral infections. *Acta Neurol Scand* **2007**; 115: 45–56.

26. Hasegawa S, Matsushige T, Inoue H, Shirabe K, Fukano R, Ichiyama T. Serum and cerebrospinal fluid cytokine profile of patients with 2009 pandemic H1N1 influenza virus-associated encephalopathy. *Cytokine* **2011**; 54: 167–72.

27. Kawashima H, Togashi T, Yamanaka G, *et al*. Efficacy of plasma exchange and methylprednisolone pulse therapy on influenza-associated encephalopathy. *J Infect* **2005**; 51: E53–6.

28. Monto AS, Hornbuckle K, Ohmit SE. Influenza vaccine effectiveness among elderly nursing home residents: a cohort study. *Am J Epidemiol* **2001**; 154: 155–60.

29. Hayward AC, Harling R, Wetten S, *et al*. Effectiveness of an influenza vaccine programme for care home staff to prevent death, morbidity, and health service use among residents: cluster randomised controlled trial. *BMJ* **2006**; 333: 1241.

30. Bridges CB, Harper S. The full-court press for influenza prevention in elderly persons. *Clin Infect Dis* **2004**; 39: 465–7

31. Chartrand C, Leeflang MM, Minion J, Brewer T, Pai M. Accuracy of rapid influenza diagnostic tests: a meta-analysis. *Ann Intern Med* **2012**; 156: 500–11.

32. Hernes SS, Quarsten H, Hagen E, *et al*. Swabbing for respiratory viral infections in older patients: a comparison of rayon and nylon flocked swabs. *Eur J Clin Microbiol Infect Dis* **2011**; 30: 159–65.

33. van der Sande MA, Ruijs WL, Meijer A, Cools HJ, van der Plas SM. Use of oseltamivir in Dutch nursing homes during the 2004–2005 influenza season. *Vaccine* **2006**; 24: 6664–9.

34. Coles FB, Balzano GJ, Morse DL. An outbreak of influenza A (H3N2) in a well immunized nursing home population. *J Am Geriatr Soc* **1992**; 40: 589–92.

35. Hill T, Platzer A, Reyes C. Influenza deaths in spite of immunization and prophylaxis. *Clin Infect Dis* **2005**; 40: 492–3.

36. Gorišek Miksić N, Uršič T, Simonović Z, *et al*. Oseltamivir prophylaxis in controlling influenza outbreak in nursing homes: a comparison between three different approaches. *Infection* **2015**; 43: 73–81.

37. Cools HJ, Gussekloo J, Remmerswaal JE, Remarque EJ, Kroes AC. Benefits of increasing the dose of influenza vaccine in residents of long-term care facilities: a randomized placebo-controlled trial. *J Med Virol* **2009**; 81: 908–14.

38. DiazGranados CA, Dunning AJ, Kimmel M, *et al*. Efficacy of high-dose versus standard-dose influenza vaccine in older adults. *N Engl J Med* **2014**; 371: 635–45.

39. Ekiert DC, Wilson IA. Broadly neutralizing antibodies against influenza virus and prospects for universal therapies. *Curr Opin Virol* **2012**; 2: 134–41.

40. Friesen RH, Lee PS, Stoop EJ, *et al*. A common solution to group 2 influenza virus neutralization. *Proc Natl Acad Sci U S A* **2014**; 111: 445–50.

41. Nichols WG, Guthrie KA, Corey L, Boeckh M. Influenza infections after hematopoietic stem cell transplantation: risk factors, mortality, and the effect of antiviral therapy. *Clin Infect Dis* **2004**; 39: 1300–6.

42. McGeer A, Green KA, Plevneshi A, *et al*; Toronto Invasive Bacterial Diseases Network. Antiviral therapy and outcomes of influenza requiring hospitalization in Ontario, Canada. *Clin Infect Dis* **2007**; 45: 1568–75.

43. Ives JA, Carr JA, Mendel DB, *et al*. The H274Y mutation in the influenza A/H1N1 neuraminidase active site following oseltamivir phosphate treatment leave virus severely compromised both in vitro and in vivo. *Antiviral Res* **2002**; 55: 307–17.

44. Herlocher ML, Truscon R, Elias S, *et al*. Influenza viruses resistant to the antiviral drug oseltamivir: transmission studies in ferrets. *J Infect Dis* **2004**; 190: 1627–30.

45. Yen HL, Ilyushina NA, Salomon R, Hoffmann E, Webster RG, Govorkova EA. Neuraminidase inhibitor-resistant recombinant A/Vietnam/1203/04 (H5N1) influenza viruses retain their replication efficiency and pathogenicity in vitro and in vivo. *J Virol* **2007**; 81: 12418–26.

46. Bright RA, Shay DK, Shu B, Cox NJ, Klimov AI. Adamantane resistance among influenza A viruses isolated early during the 2005-2006 influenza season in the United States. *JAMA* **2006**; 295: 891–4.

47. Hauge SH, Dudman S, Borgen K, Lackenby A, Hungnes O. Oseltamivir-resistant influenza viruses A (H1N1), Norway, 2007-08. *Emerg Infect Dis* **2009**; 15: 155–62.

48. Jonges M, van der Lubben IM, Dijkstra F, Verhoef L, Koopmans M, Meijer A. Dynamics of antiviral-resistant influenza viruses in the Netherlands, 2005–2008. *Antiviral Res* **2009**; 83: 290–7.

49. Englund JA, Champlin RE, Wyde PR, *et al*. Common emergence of amantadine- and rimantadine-resistant influenza A viruses in symptomatic immunocompromised adults. *Clin Infect Dis* **1998**; 26: 1418–24.

50. Weinstock DM, Gubareva LV, Zuccotti G. Prolonged shedding of multidrug-resistant influenza A virus in an immunocompromised patient. *N Engl J Med* **2003**; 348: 867–8.

51. Ison MG, Gubareva LV, Atmar RL, Treanor J, Hayden FG. Recovery of drug-resistant influenza virus from immunocompromised patients: a case series. *J Infect Dis* **2006**; 193: 760–4.

52. Baz M, Abed Y, McDonald J, Boivin G. Characterization of multidrug-resistant influenza A/H3N2 viruses shed during 1 year by an immunocompromised child. *Clin Infect Dis* 2006; 43: 1555–61.

53. van der Vries E, Stittelaar KJ, van Amerongen G, *et al*. Prolonged influenza virus shedding and emergence of antiviral resistance in immunocompromised patients and ferrets. *PLoS Pathog* **2013**; 9: e1003343.

54. Harvala H, Gunson R, Simmonds P, *et al*. The emergence of oseltamivir-resistant pandemic influenza A (H1N1) 2009 virus amongst hospitalised immunocompromised patients in Scotland, November-December, 2009. *Euro Surveill* **2010**; 15. pii: 19536.

55. Bautista E, Chotpitayasunondh T, Gao Z, *et al*. Clinical aspects of pandemic 2009 influenza A (H1N1) virus infection. *N Engl J Med* **2010**; 362: 1708–19.

56. Khanna N, Steffen I, Studt JD, *et al*. Outcome of influenza infections in outpatients after allogeneic hematopoietic stem cell transplantation. *Transpl Infect Dis* **2009**; 11: 100–5.

57. Renaud C, Boudreault AA, Kuypers J, *et al*. H275Y mutant pandemic (H1N1) 2009 virus in immunocompromised patients. *Emerg Infect Dis* **2011**; 17: 653–60.

58. Schnell D, Mayaux J, de Bazelaire C, *et al*. Risk factors for pneumonia in immunocompromised patients with influenza. *Respir Med* **2010**; 104: 1050–6.

59. Chemaly RF, Ghosh S, Bodey GP, *et al*. Respiratory viral infections in adults with hematologic malignancies and human stem cell transplantation recipients: a retrospective study at a major cancer center. *Medicine (Baltimore)* **2006**; 85: 278–87.

60. Ljungman P, de la Camara R, Perez-Bercoff L, *et al*. Outcome of pandemic H1N1 infections in hematopoietic stem cell transplant recipients. *Haematologica* **2011**; 96: 1231–5.

61. Choi SM, Boudreault AA, Xie H, Englund JA, Corey L, Boeckh M. Differences in clinical outcomes after 2009 influenza A/H1N1 and seasonal influenza among hematopoietic cell transplant recipients. *Blood* **2011**; 117: 5050–6.

62. van der Vries E, Anber J, van der Linden A, *et al*. Molecular assays for quantitative and qualitative detection of influenza virus and oseltamivir resistance mutations. *J Mol Diagn* **2013**; 15: 347–54.

63. Kawai N, Ikematsu H, Hirotsu N, *et al*. Clinical effectiveness of oseltamivir and zanamivir for treatment of influenza A virus subtype H1N1 with the H274Y mutation: a Japanese, multicenter study of the 2007-2008 and 2008-2009 influenza seasons. *Clin Infect Dis* **2009**; 49: 1828–35.

64. Saito R, Sato I, Suzuki Y, *et al*. Reduced effectiveness of oseltamivir in children infected with oseltamivir-resistant influenza A (H1N1) viruses with His275Tyr mutation. *Pediatr Infect Dis J* **2010**; 29: 898–904.

65. Petersen E, Keld DB, Ellermann-Eriksen S, *et al*. Failure of combination oral oseltamivir and inhaled zanamivir antiviral treatment in ventilator- and ECMO-treated critically ill patients with pandemic influenza A (H1N1)v. *Scand J Infect Dis* **2011**; 43: 495–503.

66. Escuret V, Cornu C, Boutitie F, *et al*. Oseltamivir-zanamivir bitherapy compared to oseltamivir monotherapy in the treatment of pandemic 2009 influenza A(H1N1) virus infections. *Antiviral Res* **2012**; 96: 130–7.

67. South East Asia Infectious Disease Clinical Research Network. Effect of double dose oseltamivir on clinical and virological outcomes in children and adults admitted to hospital with severe influenza: double blind randomised controlled trial. *BMJ* **2013**; 346: f3039.

68. Dixit R, Khandaker G, Hay P, *et al*. A randomized study of standard versus double dose oseltamivir for treating influenza in the community. *Antivir Ther* **2014**.

69. Kim WY, Young Suh G, Huh JW, *et al*; Korean Society of Critical Care Medicine H1N1 Collaborative. Triple-combination antiviral drug for pandemic H1N1 influenza virus infection in critically ill patients on mechanical ventilation. *Antimicrob Agents Chemother* **2011**; 55: 5703–9.

70. Meijer A, Lackenby A, Hungnes O, *et al*; European Influenza Surveillance Scheme. Oseltamivir-resistant influenza virus A (H1N1), Europe, 2007-08 season. *Emerg Infect Dis* **2009**; 15: 552–60.

71. Hurt AC, Ernest J, Deng Y, *et al*. Emergence and spread of oseltamivir-resistant A(H1N1) influenza viruses in Oceania, South East Asia and South Africa. *Antiviral Res* **2009**; 83: 90–3.

72. Working Group for Influenza Virus Surveillance in Japan. Oseltamivir-resistant influenza viruses A (H1N1) during 2007–2009 influenza seasons, Japan. *Emerg Infect Dis* **2010**; 16: 926–35.

73. Dharan NJ, Gubareva LV, Meyer JJ, *et al*; Oseltamivir-Resistance Working Group. Infections with oseltamivir-resistant influenza A(H1N1) virus in the United States. *JAMA* **2009**; 301: 1034–41.

74. Weinstock DM, Zuccotti G. The evolution of influenza resistance and treatment. *JAMA* **2009**; 301: 1066–9.

75. Bloom JD, Gong LI, Baltimore D. Permissive secondary mutations enable the evolution of influenza oseltamivir resistance. *Science* **2010**; 328: 1272–5.

76. Rameix-Welti MA, Munier S, Le Gal S, *et al*. Neuraminidase of 2007-2008 influenza A(H1N1) viruses shows increased affinity for sialic acids due to the D344N substitution. *Antivir Ther* **2011**; 16: 597–603.

77. Bouvier NM, Rahmat S, Pica N. Enhanced mammalian transmissibility of seasonal influenza A/H1N1 viruses encoding an oseltamivir-resistant neuraminidase. *J Virol* **2012**; 86: 7268–79.

78. Mohty B, Thomas Y, Vukicevic M, *et al*. Clinical features and outcome of 2009 influenza A (H1N1) after allogeneic hematopoietic SCT. *Bone Marrow Transplant* **2012**; 47: 236–42.

79. Tramontana AR, George B, Hurt AC, *et al*. Oseltamivir resistance in adult oncology and hematology patients infected with pandemic (H1N1) 2009 virus, Australia. *Emerg Infect Dis* **2010**; 16: 1068–75.

80. Williamson JC, Pegram PS. Respiratory distress associated with zanamivir. *N Engl J Med* **2000**; 342: 661–2.

81. Glaxo Wellcome Inc. *Relenza (zanimivir for inhalation)* [Package insert]. Research Triangle Park, NC: Glaxo Wellcome, Inc.; **2009**.

82. Kiatboonsri S, Kiatboonsri C, Theerawit P. Fatal respiratory events caused by zanamivir nebulization. *Clin Infect Dis* **2009**; 50: 620.

83. Chen LF, Dailey NJ, Rao AK, *et al*. Cluster of oseltamivir-resistant 2009 pandemic influenza A (H1N1) virus infections on a hospital ward among immunocompromised patients—North Carolina, 2009. *J Infect Dis* **2011**; 203: 838–46.

84. Moore C, Galiano M, Lackenby A, *et al*. Evidence of person-to-person transmission of oseltamivir-resistant pandemic influenza A(H1N1) 2009 virus in a hematology unit. *J Infect Dis* **2011**; 203: 18–24.

85. Subbarao EK, London W, Murphy BR. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. *J Virol* **1993**; 67: 1761–4.

86. Steel J, Lowen AC, Mubareka S, Palese P. Transmission of influenza virus in a mammalian host is increased by PB2 amino acids 627K or 627E/701N. *PLoS Pathog* **2009**; 5: e1000252.

87. Mehle A, Doudna JA. Adaptive strategies of the influenza virus polymerase for replication in humans. *Proc Natl Acad Sci U S A* **2009**; 106: 21312–6.

88. Herfst S, Chutinimitkul S, Ye J, *et al*. Introduction of virulence markers in PB2 of pandemic swine-origin influenza virus does not result in enhanced virulence or transmission. *J Virol* **2010**; 84: 3752–8.

89. Jagger BW, Memoli MJ, Sheng ZM, *et al*. The PB2-E627K mutation attenuates viruses containing the 2009 H1N1 influenza pandemic polymerase. *MBio* **2010**; 1. pii: e00067–10.

90. Hai R, Schmolke M, Varga ZT, *et al*. PB1-F2 expression by the 2009 pandemic H1N1 influenza virus has minimal impact on virulence in animal models. *J Virol* **2010**; 84: 4442–50.

91. Pena L, Vincent AL, Loving CL, *et al*. Restored PB1-F2 in the 2009 pandemic H1N1 influenza virus has minimal effects in swine. *J Virol* **2012**; 86: 5523–32.

92. Bussey KA, Bousse TL, Desmet EA, Kim B, Takimoto T. PB2 residue 271 plays a key role in enhanced polymerase activity of influenza A viruses in mammalian host cells. *J Virol* **2010**; 84: 4395–406.

93. Zhu W, Zhu Y, Qin K, *et al*. Mutations in polymerase genes enhanced the virulence of 2009 pandemic H1N1 influenza virus in mice. *PLoS One* **2012**; 7: e33383.

94. Zhou B, Li Y, Halpin R, Hine E, Spiro DJ, Wentworth DE. PB2 residue 158 is a pathogenic determinant of pandemic H1N1 and H5 influenza a viruses in mice. *J Virol* **2011**; 85: 357–65.

95. Zhao Z, Yi C, Zhao L, *et al*. PB2-588I enhances 2009 H1N1 pandemic influenza virus virulence by increasing viral replication and exacerbating PB2 inhibition of beta interferon expression. *J Virol* **2014**; 88: 2260–7.

96. Ramirez-Gonzalez JE, Gonzalez-Duran E, Alcantara-Perez P, *et al*. Oseltamivir-resistant pandemic (H1N1) 2009 virus, Mexico. *Emerg Infect Dis* **2011**; 17: 283–6.

97. Shetty AK, Ross GA, Pranikoff T, *et al*. Oseltamivir-resistant 2009 H1N1 influenza pneumonia during therapy in a renal transplant recipient. *Pediatr Transplant* **2012**; 16: E153–7.

98. Centers for Disease Control and Prevention (CDC). Oseltamivir-resistant 2009 pandemic influenza A (H1N1) virus infection in two summer campers receiving prophylaxis—North Carolina, 2009. *MMWR Morb Mortal Wkly Rep* **2009**; 58: 969–72.

99. Le QM, Wertheim HF, Tran ND, van Doorn HR, Nguyen TH, Horby P; Vietnam H1N1 Investigation Team. A community cluster of oseltamivir-resistant cases of 2009 H1N1 influenza. *N Engl J Med* **2010**; 362: 86–7.

100. Hurt AC, Deng YM, Ernest J, *et al*. Oseltamivir-resistant influenza viruses circulating during the first year of the influenza A(H1N1) 2009 pandemic in the Asia-Pacific region, March 2009 to March 2010. *Euro Surveill* **2011**; 16. pii: 19770.

101. Longtin J, Patel S, Eshaghi A, *et al*. Neuraminidase-inhibitor resistance testing for pandemic influenza A (H1N1) 2009 in Ontario, Canada. *J Clin Virol* **2011**; 50: 257–61.

102. Ujike M, Ejima M, Anraku A, *et al*; Influenza Virus Surveillance Group of Japan. Monitoring and characterization of oseltamivir- resistant pandemic (H1N1) 2009 virus, Japan, 2009-2010. *Emerg Infect Dis* **2011**; 17: 470–9.

103. Calatayud L, Lackenby A, Reynolds A, *et al*. Oseltamivir-resistant pandemic (H1N1) 2009 virus infection in England and Scotland, 2009-2010. *Emerg Infect Dis* **2011**; 17: 1807–15.

104. Lackenby A, Moran Gilad J, Pebody R, *et al*. Continued emergence and changing epidemiology of oseltamivir-resistant influenza A(H1N1)2009 virus, United Kingdom, winter 2010/11. *Euro Surveill* **2011**; 16. pii: 19784.

105. Storms AD, Gubareva LV, Su S, *et al*; US Antiviral Resistance Surveillance Working Group. Oseltamivir-resistant pandemic (H1N1) 2009 virus infections, United States, 2010-11. *Emerg Infect Dis* **2012**; 18: 308–11.

106. Hurt AC, Hardie K, Wilson NJ, *et al*. Community transmission of oseltamivir-resistant A(H1N1)pdm09 influenza. *N Engl J Med* **2011**; 365: 2541–2.

107. Takashita E, Kiso M, Fujisaki S, *et al*; The Influenza Virus Surveillance Group of Japan. Characterization of a large cluster of influenza A(H1N1)pdm09 virus cross-resistant to oseltamivir and peramivir during the 2013/2014 influenza season in Japan. *Antimicrob Agents Chemother* **2015**. pii: AAC.04836–14.

108. Butler J, Hooper KA, Petrie S, *et al*. Estimating the fitness advantage conferred by permissive neuraminidase mutations in recent oseltamivir-resistant A(H1N1)pdm09 influenza viruses. *PLoS Pathog* **2014**; 10: e1004065.

109. Abed Y, Pizzorno A, Bouhy X, Rhéaume C, Boivin G. Impact of potential permissive neuraminidase mutations on viral fitness of the H275Y oseltamivir-resistant influenza A(H1N1)pdm09 virus in vitro, in mice and in ferrets. *J Virol* **2014**; 88: 1652–8.

110. Takashita E, Meijer A, Lackenby A, *et al*. Global update on the susceptibility of human influenza viruses to neuraminidase inhibitors, 2013-2014. *Antiviral Res* **2015**; 117: 27–38.

111. Fordyce SL, Bragstad K, Pedersen SS, *et al*. Genetic diversity among pandemic 2009 influenza viruses isolated from a transmission chain. *Virol J* **2013**; 10: 116.

112. de la Rosa-Zamboni D, Vázquez-Pérez JA, Avila-Ríos S, *et al*. Molecular characterization of the predominant influenza A(H1N1)pdm09 virus in Mexico, December 2011–February 2012. *PLoS One* **2012**; 7: e50116.

113. Van den Hoecke S, Verhelst J, Vuylsteke M, Saelens X. Analysis of the genetic diversity of influenza A viruses using next-generation DNA sequencing. *BMC Genomics* **2015**; 16: 79.

114. Sleeman K, Guo Z, Barnes J, Shaw M, Stevens J, Gubareva LV. R292K substitution and drug susceptibility of influenza A(H7N9) viruses. *Emerg Infect Dis* **2013**; 19: 1521–4.

115. Hobson D, Curry RL, Beare AS, Ward-Gardner A. The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses. *J Hyg (Lond)* **1972**; 70: 767–77.

116. Potter CW, Oxford JS. Determinants of immunity to influenza infection in man. *Br Med Bull* **1979**; 35: 69–75.

117. McMichael AJ, Gotch FM, Noble GR, Beare PA. Cytotoxic T-cell immunity to influenza. *N Engl J Med* **1983**; 309: 13–7.

118. Gratama JW, Boeckh M, Nakamura R, *et al*. Immune monitoring with iTAg MHC Tetramers for prediction of recurrent or persistent cytomegalovirus infection or disease in allogeneic hematopoietic stem cell transplant recipients: a prospective multicenter study. *Blood* **2010**; 116: 1655–62.

119. Hillaire ML, van Trierum SE, Bodewes R, *et al*. Characterization of the human $CD8^+$ T cell response following infection with 2009 pandemic influenza H1N1 virus. *J Virol* **2011**; 85: 12057–61.

120. Moscona A. Global transmission of oseltamivir-resistant influenza. *N Engl J Med* **2009**; 360: 953–6.

121. Smee DF, Tarbet EB, Furuta Y, Morrey JD, Barnard DL. Synergistic combinations of favipiravir and oseltamivir against wild-type pandemic and oseltamivir-resistant influenza A virus infections in mice. *Future Virol* **2013**; 8: 1085–1094.

122. Hillaire ML, Rimmelzwaan GF, Kreijtz JH. Clearance of influenza virus infections by T cells: risk of collateral damage? *Curr Opin Virol* **2013**; 3: 430–7.

123. Teijaro JR, Walsh KB, Rice S, Rosen H, Oldstone MB. Mapping the innate signaling cascade essential for cytokine storm during influenza virus infection. *Proc Natl Acad Sci U S A* **2014**; 111: 3799–804.

124. Brandes M, Klauschen F, Kuchen S, Germain RN. A systems analysis identifies a feedforward inflammatory circuit leading to lethal influenza infection. *Cell* **2013**; 154: 197–212.

125. de Jong MD, Simmons CP, Thanh TT, *et al*. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. *Nat Med* **2006**; 12: 1203–7.

126. Bermejo-Martin JF, Ortiz de Lejarazu R, Pumarola T, *et al*. Th1 and Th17 hypercytokinemia as early host response signature in severe pandemic influenza. *Crit Care* **2009**; 13: R201.

127. Walsh KB, Teijaro JR, Wilker PR, *et al*. Suppression of cytokine storm with a sphingosine analog provides protection against pathogenic influenza virus. *Proc Natl Acad Sci U S A* **2011**; 108: 12018–23.

128. Teijaro JR, Walsh KB, Long JP, *et al*. Protection of ferrets from pulmonary injury due to H1N1 2009 influenza virus infection: immunopathology tractable by sphingosine-1-phosphate 1 receptor agonist therapy. *Virology* **2014**; 452-453: 152–7.

129. Yassine HM, Boyington JC, McTamney PM, *et al*. Hemagglutinin-stem nanoparticles generate heterosubtypic influenza protection. *Nat Med* **2015** Sep; 21: 1065–70.

130. Impagliazzo A, Milder F, Kuipers H, *et al*. A stable trimeric influenza hemagglutinin stem as a broadly protective immunogen. *Science* **2015**; 349: 1301–6.

131. DiLillo DJ, Tan GS, Palese P, Ravetch JV. Broadly neutralizing hemagglutinin stalk-specific antibodies require Fc R interactions for protection against influenza virus in vivo. *Nat Med* **2014**; 20: 143–51.

Samenvatting (Summary in Dutch)

Curriculum Vitae

List of Publications

Acknowledgements

Samenvatting (Summary in Dutch)

Influenza of griep is bij de mens een veelvoorkomende infectieziekte van de luchtwegen die meestal wordt veroorzaakt door influenzavirussen type A en B. Humane influenza A virussen worden onderverdeeld in subtypen op basis van de oppervlakte-eiwitten hemagglutine (H1, H2, H3) en neuraminidase (N1, N2). Daarnaast circuleren twee influenza B virus genetische lijnen. Influenza A(H1N1), A(H3N2), B(Yamagata) en B(Victoria) virussen veroorzaken jaarlijks een seizoensgebonden epidemie in de herfst- en winterperioden en ontwijken de immuniteit van de mens door mutaties en veranderingen in de oppervlakte-eiwitten, ook *antigene drift* genoemd. Een pandemie ontstaat wanneer een dierlijk influenzavirus overspringt naar de mens (meestal vanuit varkens) en zich efficiënt verspreidt in een bevolking zonder of met een beperkte immuniteit, zoals bij de pandemieën van 1918 A(H1N1), 1957 A(H2N2), 1968 A(H3N2) en 2009 A(H1N1). In 2009 ontstond een relatief milde pandemie die werd veroorzaakt door een nieuw influenza A(H1N1)pdm09 virus dat genetisch bestond uit elementen afkomstig uit Noord-Amerikaanse varkens influenza, Euro-Aziatische varkens influenza, Noord-Amerikaanse aviaire influenza en een menselijk griepvirus. Dergelijke grote veranderingen van influenza A virus worden aangeduid als *antigene shift*. Het influenza A(H1N1)pdm09 virus bevatte genetische elementen waarin bekende virulentie en antivirale resistentie kenmerken echter ontbraken.

Seizoensgebonden influenza verloopt meestal ongecompliceerd. Acute symptomen beginnen met koorts en keelpijn, gevolgd door spierpijn, hoofdpijn en andere luchtwegklachten. De patiënt voelt zich beroerd maar de klachten gaan bijna altijd binnen 7 dagen vanzelf over. Ouderen (leeftijd ≥65 jaar) en mensen met een chronische ziekte (hart- en vaatziekten, longaandoeningen, ernstige nierinsufficiëntie, verminderde weerstand en diabetes mellitus) behoren tot risicogroepen met een verhoogd risico op pneumonie complicaties, ziekenhuisopnames en sterfte door de gevolgen van griep. Risicogroepen krijgen daarom jaarlijks een gratis griepvaccinatie aangeboden in het kader van het Nationaal Programma Griepppreventie. Influenzainfecties verlopen bij tevoren gezonde kinderen en jongvolwassen mensen meestal ongecompliceerd en zelflimiterend, waardoor vaccinatie en antivirale behandeling bij deze groep niet noodzakelijk is. Antivirale neuraminidaseremmers kunnen de ziekteduur en complicaties verminderen indien de behandeling binnen 48 uur na aanvang van de klachten is gestart. In het geval van ernstige influenzainfectie bij patienten in het ziekenhuis verlaagt het middel het risico op mortaliteit indien binnen 96 uur is gestart. Het stellen van de diagnose op basis

van symptomen blijkt weinig betrouwbaar en de conventionele diagnostiekmethoden als serologie en viruskweek zijn traag en weinig gevoelig. Hierdoor is er een toenemende behoefte aan de ontwikkeling en het gebruik van moleculaire diagnostiek in de klinische praktijk. Dit proefschrift beschrijft een breed scala aan onderwerpen die gerelateerd zijn aan de toepassing van influenza moleculaire diagnostiek en de interpretatie van de gevolgen van seizoensgebonden en pandemische influenza, bij patiënten met een verschillende klinische achtergrond.

De achtergrond van influenzavirussen en aanleiding tot dit proefschrift

In **hoofdstuk 1** wordt een introductie gegeven van de geschiedenis en achtergrond humane van influenzavirussen, de epidemiologie van seizoensgebonden en pandemische influenza en het klinisch spectrum bij gezonde mensen en risicogroepen. De ontwikkelingen op het gebied van influenzavirusdiagnostiek, antivirale behandeling en vaccinatie worden beschreven evenals de beperkte kennis over antivirale resistentievorming en virale pathogenese bij immuungecompromiteerde patiënten. Tenslotte staan de doelstellingen en indeling van dit proefschrift beschreven. Deze doelstellingen zijn: (1) het evalueren van real-time PCR diagnostiek methoden en beschrijven van klinische aspecten van influenza, (2) het onderzoeken van de precisie en toepasbaarheid van massaspectrometrie en moleculaire technieken voor de detectie van resistentie en virulentie mutaties en (3) het correleren van het gastheerimmuunsysteem met viruseliminatie, symptomen, en antivirale resistentievorming.

Moleculaire detectie van acute respiratoire virusinfecties bij kinderen

Het klinische spectrum van influenza en andere respiratoire virussen is wetenschappelijk onderbelicht bij jonge kinderen met acute luchtweg klachten in het ziekenhuis. **Hoofdstuk 2** beschrijft een studie waarbij 274 kinderen met acute luchtwegklachten in het ziekenhuis gedurende twee winterseizoenen in 2006 en 2007 op respiratoire virussen werden onderzocht met het gebruik van multiplex real-time PCR. De meeste kinderen (n=236) waren jonger dan 3 jaar en een virusinfectie kon bij 82% van alle kinderen worden bewezen. Individuele virusinfecties (59%) werden vaker aangetoond dan gemengde virusinfecties (23%). Het respiratoir syncytieel virus en humaan rhinovirus werden nadrukkelijk het meest aangetoond en waren significant vaker aantoonbaar bij jonge kinderen dan influenza ($p<0.0001$). In een subanalyse werden virus-geassocieerde symptomen en klinische uitkomsten onderling vergeleken bij kinderen jonger dan 3 jaar. Individuele

virusinfecties konden niet gekoppeld worden met specifieke symptomen omdat verschillende virussen dezelfde symptomen deelden en gemengde virusinfecties frequent voorkwamen. Virus-geassocieerde klinische uitkomsten toonden dat het respiratoir syncytieel virus en humaan rhinovirus respectievelijk vaker in verband konden worden gebracht met zuurstoftoediening en ernstige complicaties. De conclusies waren dat respiratoire virussen frequent worden gevonden bij kinderen met acute luchtwegklachten in het ziekenhuis. PCR diagnostiek is nodig voor het stellen van de diagnose omdat klinische symptomen niet onderscheidend zijn. Respiratoir syncytieel virus en humaan rhinovirusinfecties geven de hoogste ziektelast bij jonge kinderen. De resultaten suggereren dat influenzavirusinfecties bij kinderen in het ziekenhuis relatief mild verlopen, maar algemene conclusies kunnen niet geformuleerd worden door de lage aantallen. Het is bekend dat levensbedreigende influenzavirusinfecties kunnen voorkomen bij kinderen.

Kinderen met ernstige influenzaviruspneumonie

De pathogenese van ernstige influenzavirusinfecties bij kinderen is grotendeels onbekend. In **hoofdstuk 3** worden de klinische beelden en pathologische bevindingen beschreven van twee kinderen die zijn overleden aan een ziektebeeld met influenzaviruspneumonie, diffuse intravasale stolling, encephalopathie en multi-orgaanfalen. Na specifieke toestemming van ouders zijn histopathologische en moleculaire studies uitgevoerd om de aanwezigheid van influenzavirus in longen en andere organen en gerelateerde weefselschade te bestuderen. Immuunhistochemische kleuringen en innovatieve moleculaire bepalingen zijn uitgevoerd om de aanwezigheid van replicerend virus te onderzoeken in de luchtwegen en andere orgaan weefsels. Pathologie resultaten toonden een bronchitis en vroege diffuse alveolaire schade in de longen met histopathologisch en moleculair bewijs voor virus replicatie in de bronchiën en enkele alveolaire cellen. Influenzavirus RNA werd sporadisch aangetoond in organen buiten de longen zonder histopathologische of moleculaire aanwijzingen voor virusreplicatie. Wij concludeerden dat ernstige influenza bij kinderen zich kan manifesteren met diffuse intravasale stolling, encephalopathie en multi-orgaanfalen en dat bij soortgelijke klinische presentaties influenza dient te worden uitgesloten. De afwezigheid van virus replicatie buiten de longen onderbouwt de hypothese dat fulminante symptomen worden veroorzaakt door een systemische afgifte van proinflammatoire cytokinen. Het aanbieden van influenzavaccins aan kinderen moet worden overwogen tijdens seizoenen, wanneer zeldzame virulente influenza A drift varianten ontstaan door intra-subtypische genetische reassortering, zoals waargenomen in 2003 en 2004 met influenza A/H3N2/Fujian/411/02 virus.

Moleculaire detectie van influenzavirusinfecties in verpleegtehuizen

De studie in **hoofdstuk 4** vergelijkt de gevoeligheid van influenzavirus real-time PCR, virus kweek en antigeen sneltesten op diverse respiratoire materialen verkregen uit 6 verpleegtehuizen met influenza verspreiding in 2005 en 2006. De meerwaarde van logistieke ondersteuning door Gemeentelijke gezondheidsdienst (GGD) 'outbreak teams' werd tevens geëvalueerd. Met behulp van PCR werd influenzavirus in 80% van de 85 verkregen materialen en bij 81% van de 47 onderzochte bewoners aangetoond. De gevoeligheid van viruskweek (54%) en antigeen sneltest (22%) waren laag vergeleken met PCR en hiermee werd influenza verspreiding in enkele verpleegtehuizen gemist. De semi-quantitatieve PCR gevoeligheid van nasopharynx spoelsels en nasopharynx uitstrijken waren vergelijkbaar maar wel 10–100 keer gevoeliger dan bij keel uitstrijken. Influenza-geïnfecteerde ouderen waren meestal gevaccineerd (82%) en gekweekte virussen kwamen overeen met het vaccin. Nasopharynx spoelsels waren niet goed uitvoerbaar bij ouderen. Met GGD logistieke ondersteuning werd een uitslag eerder gegenereerd (gemiddeld na 28.2 uur) dan zonder ondersteuning (gemiddeld na 84 uur) (P = 0.05). Wij concludeerden dat PCR diagnostiek uitgevoerd op nasopharynx uitstrijken met GGD logistieke ondersteuning het meest praktisch, gevoelig en snel is. De studie resultaten dragen bij aan een structurele verbetering van influenzadiagnostiek in verpleeghuizen. De resultaten tonen aan dat conventionele influenzavaccins een lage bescherming bieden aan ouderen en dat hogere dosis influenzavaccins dienen te worden overwogen.

Langdurige influenzavirusinfecties bij immuungecompromiteerden

Hoofdstuk 5 beschrijft een studie waarin ziekenhuis patiënten met ≥14 dagen langdurige influenzavirusinfectie klinisch en virologisch werden geëvalueerd tussen 2005 en 2008. Er werden 8 immuungecompromiteerde patiënten geïdentificeerd met een hematologisch (n=1) of hemato-oncologisch (n=7) ziektebeeld. Langdurig virus excretie kon worden geassocieerd met lymfocytopenie (8/8 patiënten) en niet met granulocytopenie (2/8 patiënten) of monocytopenie (2/8 patiënten). Zes patiënten ontwikkelden een zuurstof-afhankelijke influenza-geassocieerde lage luchtweginfectie en antivirale therapie met neuraminidase-remmers (meestal oseltamivir) was gecorreleerd met klinische verbetering (P = .02). Virus-eliminatie was geassocieerd met lymfocyten reconstitutie (P = .04) maar niet met oseltamivir behandeling. Twee van de 3 patiënten die aanvullend onderzocht werden ontwikkelden een resistent virus tijdens antivirale therapie. Wij concludeerden dat lymfocytopenie geassocieerd is met een langer bestaande

influenzavirusinfectie, het ontwikkelen van influenza-geassocieerde lage luchtweginfecties en met het ontstaan van antivirale resistente virussen. Antivirale behandeling is geassocieerd met klinische verbetering maar niet met virus-eliminatie. Virus-eliminatie lijkt afhankelijk van de gastheerimmuniteit waarin lymfocyten een sleutelrol spelen. Deze nieuwe klinische inzichten zijn belangrijk voor het herkennen van risicofactoren voor het ontwikkelen van respiratoire complicaties en antivirale resistentie.

Oseltamivir-resistent influenzavirus in het ziekenhuis

In **hoofdstuk 6** wordt de verspreiding van een oseltamivir-resistent seizoensgebonden influenza A(H1N1) virus beschreven. In februari 2008 werd een epidemiologisch cluster van patiënten met ernstige influenzavirus symptomen waargenomen binnen het ziekenhuis. Sequentieanalyse van de hemagglutinine en neuraminidase genen van influenza A(H1N1) virussen van de 4 patiënten werden vergeleken met de sequentiegegevens van ongerelateerde virussen binnen het ziekenhuis, de Leidse regio en in de Nederlandse samenleving. De moleculaire gegevens toonden een fylogenetisch cluster van oseltamivir-resistente virussen met een H274Y mutatie (volgens N2 nummering) in het neuraminidase gen wat bewijzend was voor de verspreiding van het virus binnen het ziekenhuis. Drie patiënten met lymfocytopenie ontwikkelden tijdens infectie een influenzapneumonie, waaraan 2 overleden. Histopathologische, moleculaire en microbiologische bevindingen waren consistent met een influenzaviruspneumonie bij een van de patiënten. Wij concludeerden dat seizoensgebonden oseltamivir-resistent influenza A(H1N1) virus met een H274Y neuraminidasegenmutatie evident overdraagbaar is en ernstig ziekmakend kan zijn bij hoog-risico patiënten. De nieuwe klinische inzichten van deze studie zijn dat unieke influenzavirus clusters moleculair kunnen worden aangetoond in het ziekenhuis en dat oseltamivir-resistente influenzavirussen niet altijd intrinsiek verzwakt en niet-overdraagbaar zijn, zoals eerder werd aangenomen.

Moleculaire surveillance van influenza A(H1N1)pdm09 virus

In **hoofdstuk 7** onderzochten wij de toepasbaarheid en precisie van 'massaspectrometrie gebaseerde vergelijkende sequentie analyse' (mass spectrometry-based comparative sequence analysis, MSCSA) tijdens de influenzapandemie van 2009. De genetische virulentie en resistentie kenmerken van het influenza A(H1N1)pdm09 virus werden met MSCSA geëvalueerd bij patiënten die in een vroeg stadium van de influenzapandemie besmet waren. Bij MSCSA, wordt het virus RNA na enzymatische amplificatie omgezet

naar DNA, waarna het DNA met restrictie-enzymen wordt geknipt. Met behulp van een massaspectrometrie worden de lading en massa van de kleine DNA stukjes semiautomatisch afgelezen. Softwarematig worden lading en massa omgerekend en vergeleken met virus database sequenties om mutaties op te sporen. Bij dit onderzoek werden surveillancematerialen van 70 patiënten verkregen tijdens de eerste pandemische golf uit Leiden en omgeving in de zomer van 2009. De materialen werden onderzocht op bekende virulentiemutaties in PB1-F2, PB2 en NS1 genen en resistentie mutaties in het neuraminidase (NA) gen. Sanger sequencing en MSCSA resultaten werden met elkaar vergeleken en waren vergelijkbaar. Er werden geen nieuwe virulentie of resistentie kenmerken gevonden. Aanvullend werden 35 materialen verkregen van 4 immuungecompromiteerde patiënten met ≥14 dagen langdurige virus excretie onderzocht op NA gen H275Y oseltamivir resistentie mutaties (volgens N1 nummering) met behulp van H275Y real-time PCR, Sanger sequencing en MSCSA. H275Y mutatie werd met behulp van real-time PCR gedetecteerd in 19 van de 35 materialen, terwijl de gevoeligheid van Sanger sequencing 89% (17/19) en MSCSA 24% (4/19) beduidend lager was. MSCSA software kon de aanwezigheid van H275Y mutaties niet goed interpreteren in patiëntmaterialen met gemengde viruspopulaties. Wij concludeerden dat MSCSA gebruikt kan worden voor de moleculaire surveillance van influenza A(H1N1)pdm09 virus maar dat de gevoeligheid te laag is voor het detecteren van H275Y mutaties in patiëntenmateriaal.

Gastheerimmuniteit bij langdurige influenzavirusinfectie

In **hoofdstuk 8** onderzochten wij de klinische betekenis van humorale en cellulaire gastheerimmuniteit bij het ontwikkelen van virus-geassocieerde symptomen en viruseliminatie bij hematologie-oncologie patiënten met langdurige influenzavirusinfectie. In totaal werden 6 volwassen hematologie-oncologie patiënten met ≥14 dagen influenza A(H1N1)pdm09 virusinfectie prospectief geïncludeerd tussen november 2009 en april 2013. Realtime-PCR diagnostiek werd verricht naar influenza A(H1N1)pdm09 virus en neuraminidase gen H275Y oseltamivir resistentie mutatie. Leukocyten differentiatie, lymfocyten subsets, virus-specifieke hemagglutine-inhibitie (HI) antistof titers en virusspecifieke T-cellen werden vergeleken met virus-geassocieerde symptomen en viruseliminatie. Beschermende HI titers (HI ≥80) tegen homoloog virus werden samen met aanwezige $CD16^+$ effector cellen gecombineerd beschouwd als een surrogaatbepaling voor de aanwezigheid van antistof-afhankelijke cellulaire cytotoxiciteit (antibody-dependent cell-mediated cytotoxicity, ADCC). Wij concludeerden dat langdurige influenzavirusinfectie specifiek geassocieerd was met T-cel lymfocytopenie. $CD8^+$ T-cel en

ADCC activiteit bieden klinische bescherming tijdens langdurige virale excretie. Definitieve virus-eliminatie kwam tot stand door gecombineerde virus-specifieke $CD4^+$ en $CD8^+$ T-cel activiteit en kon niet worden geassocieerd met antivirale therapie, HI seroconversie of ADCC activiteit. Longinsufficiëntie door 'acute respiratory distress syndrome' werd klinisch waarschijnlijk veroorzaakt door excessieve inflammatoire reacties aangezet door de aangeboren niet-specifieke cellulaire immuniteit, nadat het virus in de afwezigheid van T-cel- en ADCC-gemedieerde bescherming de lagere compartimenten van de luchtwegen heeft bereikt.

Conclusie

De studies in dit proefschrift tonen aan dat moleculaire diagnostiek van grote waarde is voor het aantonen van influenzavirusinfecties en antivirale resistentie in zorginstellingen. Enkele nieuwe inzichten betreffende influenzavirusdiagnostiek en klinische kenmerken beschreven in dit proefschrift zijn: (1) moleculaire diagnostiek van influenzavirus is superieur aan conventionele diagnostiek, (2) virus replicatie kan moleculair in weefsels worden aangetoond, (3) virus verspreiding kan moleculair worden bewezen, (4) oseltamivir-resistente virussen kunnen overdraagbaar en ziekmakend zijn, (5) $CD4^+$ en $CD8^+$ T-cel lymfocyten zijn bepalend voor virus-eliminatie, (6) $CD8^+$ T-cel en ADCC activiteit bieden klinische bescherming tijdens langdurige virale infectie en (7) virusgeassocieerde longinsufficiëntie wordt waarschijnlijk voor een belangrijk deel aangezet door de aangeboren niet-specifieke cellulaire immuniteit van de gastheer. Moleculaire technieken zijn nu onmisbaar voor het snel en accuraat detecteren en karakteriseren van influenzavirusinfecties in de klinische praktijk. Het is aanbevolen dat moleculaire technieken in de toekomst toenemend gebruikt worden om de klinisch relevante kenmerken van dit veranderlijk virus goed te kunnen vaststellen.

List of publications

1. **Gooskens J**, Marijt WA, van Essen EH, Rimmelzwaan GF, Kroes AC. Host immunity dictates influenza A(H1N1)pdm09 infection outcome in hematology-oncology patients. *Bone Marrow Transplant* **2015**. Advance online publication.

2. **Gooskens J**, van der Ploeg V, Sukhai RN, Vossen AC, Claas EC, Kroes AC. Clinical evaluation of viral acute respiratory tract infections in children presenting to the emergency department of a tertiary referral hospital in the Netherlands. *BMC Pediatr* **2014**; 14: 297.

3. **Gooskens J**, Zevenhoven-Dobbe JC, Claas EC, Kroes AC, Posthuma CC. Mass spectrometry-based comparative sequence analysis for the genetic monitoring of influenza A(H1N1)pdm09 virus. *PLoS One* **2014**; 9: e92970.

4. **Gooskens J**, Jonges M, Claas EC, Meijer A, van den Broek PJ, Kroes AC. Morbidity and mortality associated with nosocomial transmission of oseltamivir-resistant influenza A(H1N1) virus. *JAMA* **2009**; 301: 1042–6.

5. **Gooskens J**, Jonges M, Claas EC, Meijer A, Kroes AC. Prolonged influenza virus infection during lymphocytopenia and frequent detection of drug-resistant viruses. *J Infect Dis* **2009**; 199: 1435–41.

6. **Gooskens J**, Swaan CM, Claas EC, Kroes AC. Rapid molecular detection of influenza outbreaks in nursing homes. *J Clin Virol* **2008**; 41: 7–12.

7. **Gooskens J**, Coenraad MJ, Groeneveld JH, Prins FA, Smit VT. Clinical challenges and images in GI. Syphilitic sigmoiditis complicated by membranous nephropathy. *Gastroenterology* **2008**; 135: 363, 716.

8. **Gooskens J**, Kuiken T, Claas EC, Harinck HI, Thijssen JC, Baelde HJ, Kroes AC. Severe influenza resembling hemorrhagic shock and encephalopathy syndrome. *J Clin Virol* **2007**; 39: 136–40.

9. **Gooskens J**, Templeton KE, Claas EC, van Bussel MJ, Smit VT, Kroes AC. Quantitative detection of herpes simplex virus DNA in the lower respiratory tract. *J Med Virol* **2007**; 79: 597–604.

10. van Nieuwkoop C, **Gooskens J**, Smit VT, Claas EC, van Hogezand RA, Kroes AC, Kroon FP. Lymphogranuloma venereum proctocolitis: mucosal T cell immunity of the rectum associated with chlamydial clearance and clinical recovery. *Gut* **2007**; 56: 1476–7.

11. **Gooskens J**, Templeton KE, Claas EC, van Dam AP. Evaluation of an internally controlled real-time PCR targeting the ospA gene for detection of Borrelia burgdorferi sensu lato DNA in cerebrospinal fluid. *Clin Microbiol Infect* **2006**; 12: 894–900.

12. **Gooskens J**, Neeling AJ, Willems RJ, Wout JW, Kuijper EJ. Streptococcal toxic shock syndrome by an iMLS resistant M type 77 Streptococcus pyogenes in the Netherlands. *Scand J Infect Dis* **2005**; 37: 85–9.

13. Hatta M, Goris MG, Heerkens E, **Gooskens J**, Smits HL. Simple dipstick assay for the detection of Salmonella typhi-specific IgM antibodies and the evolution of the immune response in patients with typhoid fever. *Am J Trop Med Hyg* **2002**; 66: 416–21.

14. Hatta M, Smits HL, Gussenhoven GC, **Gooskens J**. Introduction of a rapid dipstick assay for the detection of Leptospira-specific immunoglobulin m antibodies in the laboratory diagnosis of leptospirosis in a hospital in Makassar, Indonesia. *Southeast Asian J Trop Med Public Health* **2000**; 31: 515–20.

Curriculum Vitae

Jairo Gooskens was born on October 7th, 1974 in Oranjestad, Aruba. After having graduated at Colegio Arubano (VWO) in Aruba, he attended Santa Clara University (Santa Clara, California, USA) in 1992 as a pre-medical undergraduate student with a Biology major. In 2001, he received his medical degree from the Faculty of Medicine of the University of Amsterdam based at the Academic Medical Center in Amsterdam, the Netherlands. During his medical studies, Jairo performed his undergraduate clinical and scientific internships at Instituto de Gastroenterología Boliviano – Japonés (Cochabamba, Bolivia), the department of Medical Microbiology of Hasanuddin University (Makassar, Indonesia) and the Royal Tropical Institute (Amsterdam, the Netherlands). He completed his residency at the department of Medical Microbiology of Leiden University Medical Center under supervision of Prof. Dr. Louis Kroes. In 2006, Jairo continued working as a registered medical specialist and research fellow on the molecular detection of respiratory virus infections. In April 2009, his PhD work focused on influenza research and he worked as an investigator for clinical research studies involving the molecular diagnosis and antiviral treatment of influenza virus infections in high-risk patients. Research collaborations were established with the department of Virology and the National Influenza Center at Erasmus Medical Center in Rotterdam, with the Public Health Services GGD Hollands Midden in Leiden and with the Section of Virology at the RIVM National Institute for Public Health and the Environment in Bilthoven.

Acknowledgements

This thesis would not have been possible without the support of many people who were involved in this research project over the past years.

I would first like to express my gratitude to my promotor Louis Kroes for giving me this opportunity to extend my academic development and for maintaining confidence in my work. Your patience and guidance allowed for this dissertation to become a reality. I am grateful to Eric Claas for sharing his enthusiasm and knowledge on influenza. You remind me that humor, knowledge and creative thinking are crucially important in science. I am thankful to Robbert Webster for his influenza master class. I have been privileged to work with leading influenza virus researchers Thijs Kuiken, Adam Meijer and Guus Rimmelzwaan. Thank you very much for your intellectual support and willingness to collaborate. I thank Peterhans van den Broek for sharing his knowledge on hospital infection prevention and control issues. A special thanks goes to Marcel Jonges with whom I shared a genuine interest for the emergence and transmission of antiviral resistant influenza viruses. Your dedication is admirable and I hope we can extend our scientific alliance. I express my appreciation to my co-authors for their commitment and to all colleagues mentioned in the acknowledgements of each chapter for their generous assistance. I thank Pulak Goswami for sharing his eloquence. I admire the dedication of the staff and nurses of the participating clinical departments, nursing homes and Public Health Services. I express my appreciation to Osvaldo Anaya, Mochammad Hatta and Henk Smits for their formative medical and scientific guidance.

I am grateful to my (staff) colleagues Ed, Sandra, Karin-Ellen, Martha, Ann, Mariet, Jutte, Herman, Anneloes, Els, Eric S., Clara, Hans, Thijs, Kate, Alje and Willy for their encouragement and willingness to support my scientific work. I thank my colleagues from the departments of Infectious Diseases, Pediatrics, Pathology, Intensive Care and Hematology for an ever-inspiring working atmosphere. I thank all colleagues and technicians from the clinical and research laboratories for their interest and assistance. My office mates Herman and Sandra are thanked for the pleasant conversations and moments of silence during writing. My previous office mates Ingrid, Janke, Jayant, Norbert, Suzanne, Nathalie, Martha, Sunita and Pulak are thanked for sharing learning experiences. I am now privileged to work with residents who inspire me to be a dedicated reflective clinical teacher with a concise and comprehensive approach.

On a personal note, I am grateful for the support from my family, in-laws and friends. Menno, Ervin, Kirk, Otmar and Dan, thank you for your encouragement and loyal friendship. I am grateful to Esther and Marc for their kind interest and to Ellen for her welcoming heart. I express a deep appreciation to my parents Hein and Beatriz for their confidence and devotion. My warmest affection and gratitude go to Diana and my children. Diana, thank you for your unconditional love and magnificent support during the past years. Sophia, daughter and sister of our dreams, we love you! Lucas, Simon and Maurits, you are the tropical sunshines in my life and you fill my heart with limitless joy and happiness!