INFLUENZA RESISTANCE TO ANTIVIRAL DRUGS

VIRUS CHARACTERIZATION, MECHANISM AND CLINICAL IMPACT

Erhard van der Vries

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Erhard van der Vries / 2014

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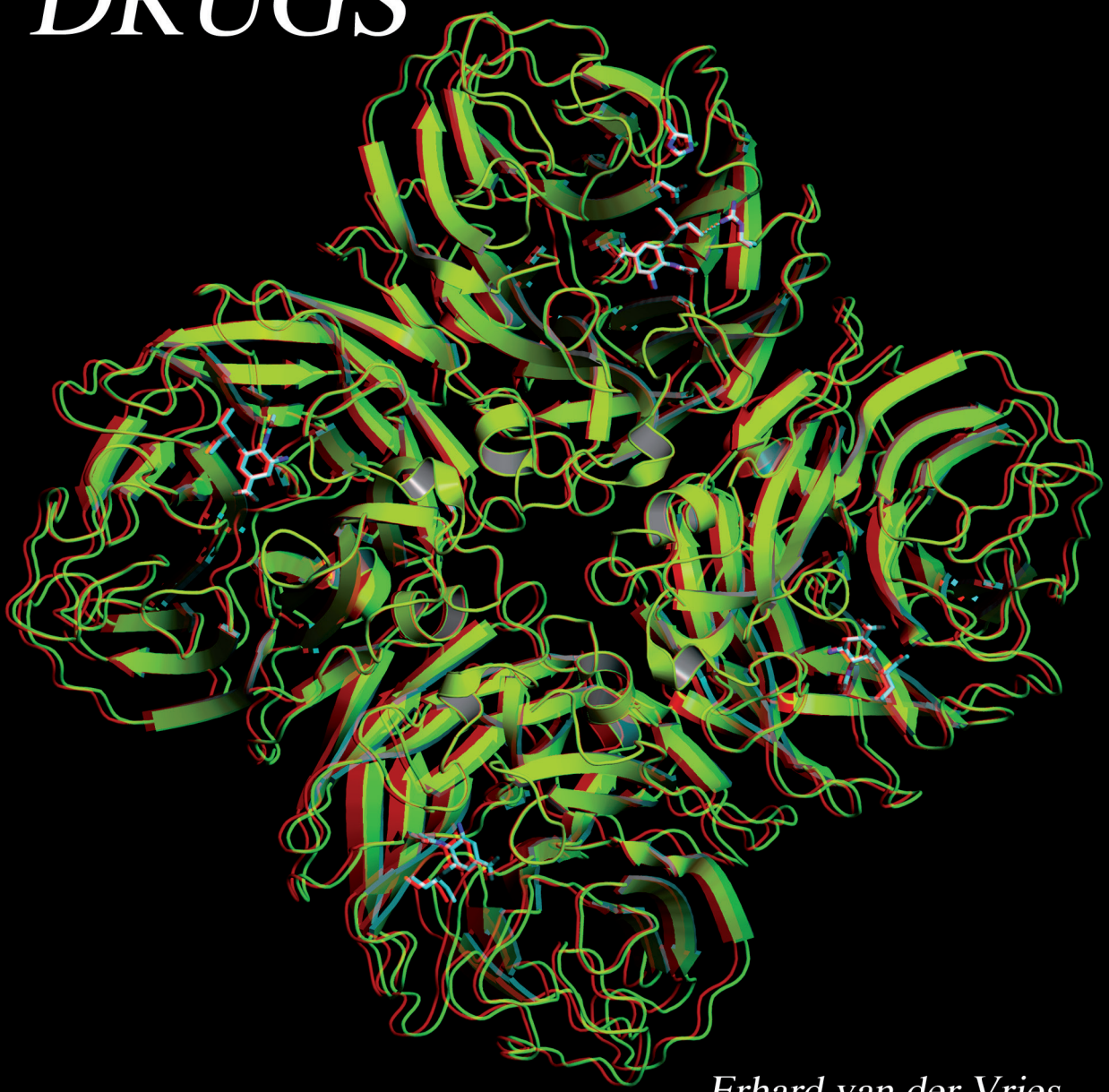
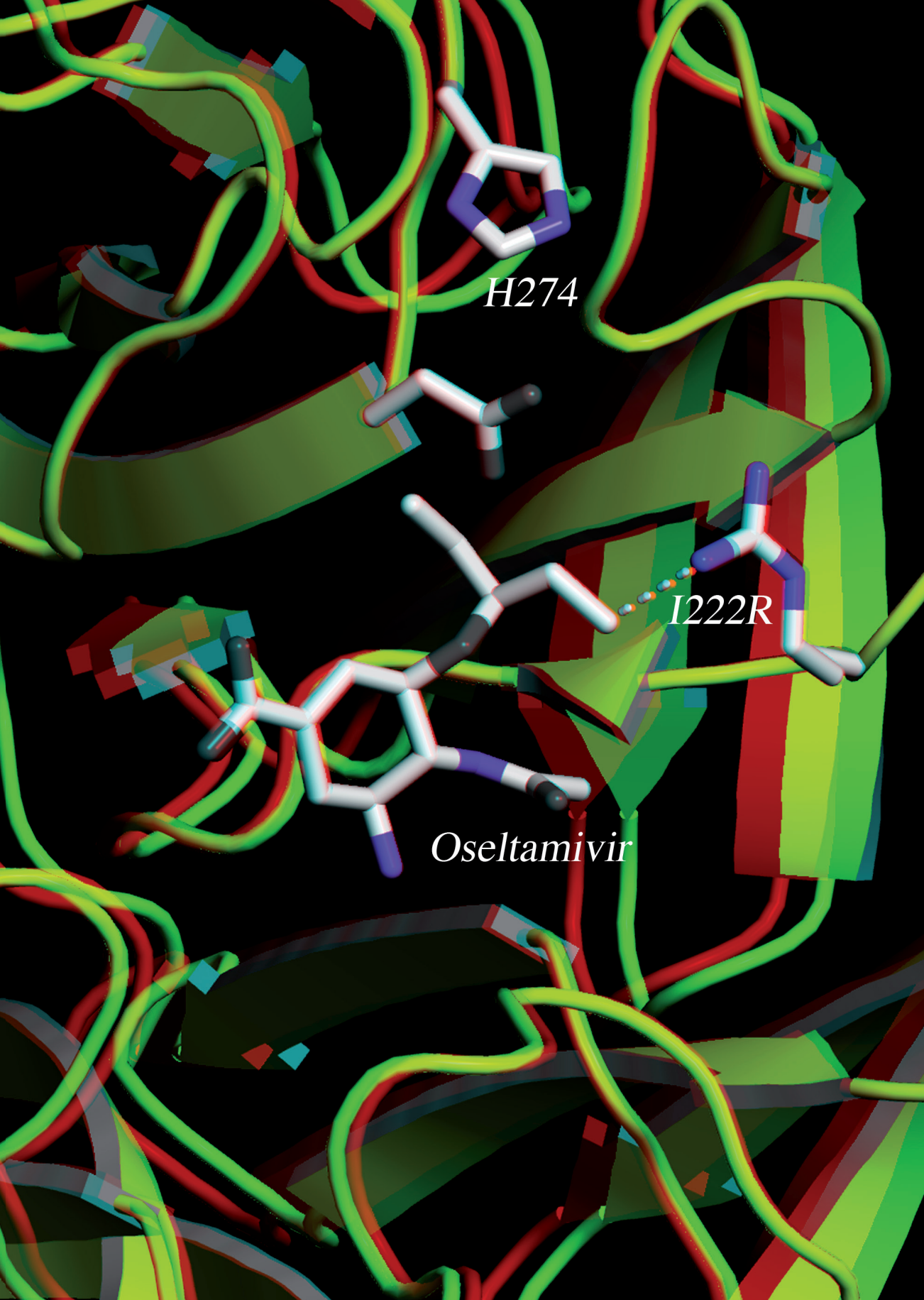


Erhard van der Vries

Active site of I222R neuraminidase mutant with oseltamivir


H274
I222R
Oseltamivir

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Cover
Electron photograph of purified egg-grown influenza viruses A/WSN/33 (Courtesy of Lesley J. Calder, NIMR).

Pages one to three
Anaglyph cartoon presentations of a H5N1 neuraminidase tetramer in complex with oseltamivir (Tamiflu). In the upper monomer the I222R neuraminidase inhibitor resistance change and its interaction with oseltamivir is presented. Image was created using PYMOL and protein databank files 2HU0 and 4B7J.

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Cartoon drawn in 1933 by P.F. Poy (1874-1948). Published with permission from J.J. Skehel, MRC-National Institute of Medical Research, Mill Hill, UK.

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Chapter 1

GENERAL INTRODUCTION

Partially based on:

Influenza virus resistance to antiviral therapy
E. van der Vries, M. Schutten, P.L. Fraaij, C.A.B. Boucher and A.D.M.E. Osterhaus
Advances in Pharmacology (2013); 67:217-46 (Review)

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Current Opinion Infectious Diseases (2011); 24: 599-604 (Review)

Satisfying the need for rapid diagnosis of new variant influenza A/H1N1
E. van der Vries and M. Schutten
Expert Reviews on Molecular Diagnostics (2010): 251-253 (Review)

1 Introduction

Before the discovery in 1933 of a viral agent causing '*the flu*' it was assumed a bacterial disease. However, when virologists Wilson Smith and colleagues had collected throat-washings from patients with influenza, and tested these samples on ferrets, the animals became ill exhibiting influenza like symptoms [1] (Figure 1). Since these respiratory samples had been passed through a filter, impermeable for bacteria, influenza had to be caused by a virus. From one of these ferrets, infected with a sample from Dr. W. Smith himself [2], an influenza virus was isolated. This virus strain was named W.S. and still is one of the most utilized laboratory strains around the world (A/WSN/33).

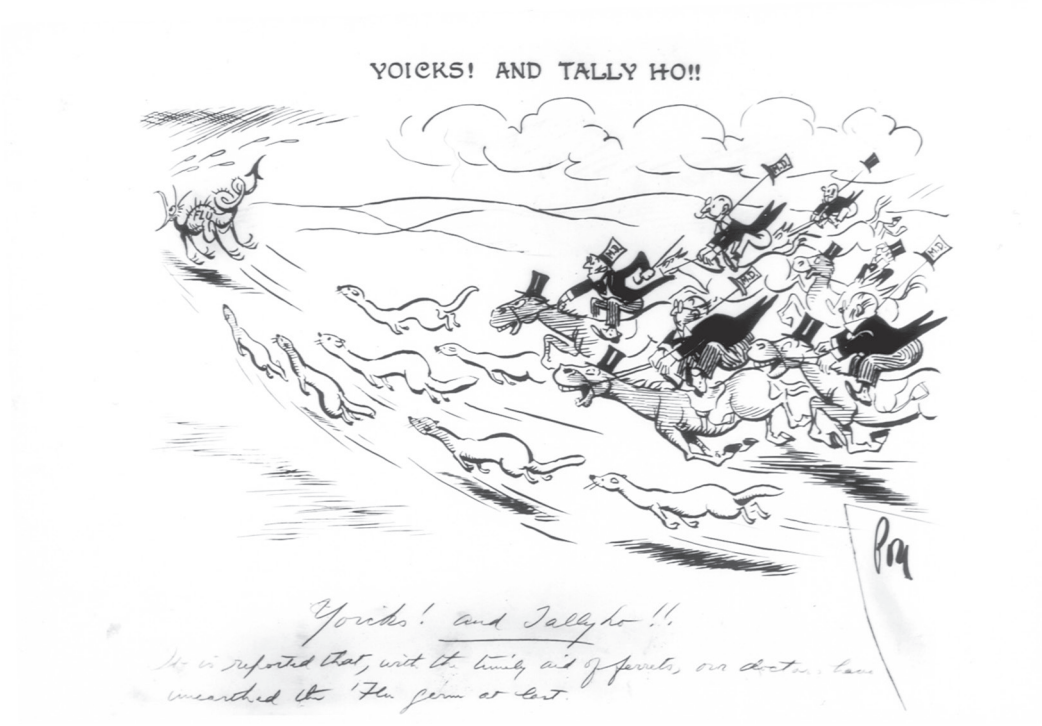

Figure 1 Yoicks and Tally Ho!!
Cartoon publiished in 1933 in the Evening News (London) after as it was published in 1933 in the Evening News (London) after the discovery of the influenza virus. Legend: "Yoicks! And tally ho!! It is reported that, with the timely aid of ferrets, our doctors have unearthed the 'Flu germ at last."

1.1 Virus types and subtypes

Influenza virus types A, B and C are enveloped viruses with a segmented negative-sense RNA genome [3]. They belong to the family of *Orthomyxoviridae* together with Thogoto virus and Isavirus. They are about 80 to 120nm in size. An electron microscopic image of influenza A/WSN/33 virus is presented on the cover of this thesis. Influenza A viruses are subtyped by their hemagglutinin (HA) and neuraminidase (see also Figure 2) (NA) [3]. These are two integral membrane glycoproteins and are the spike-like structures on the virus surface [4]. So far, 17 HAs (H1-H17) and 9

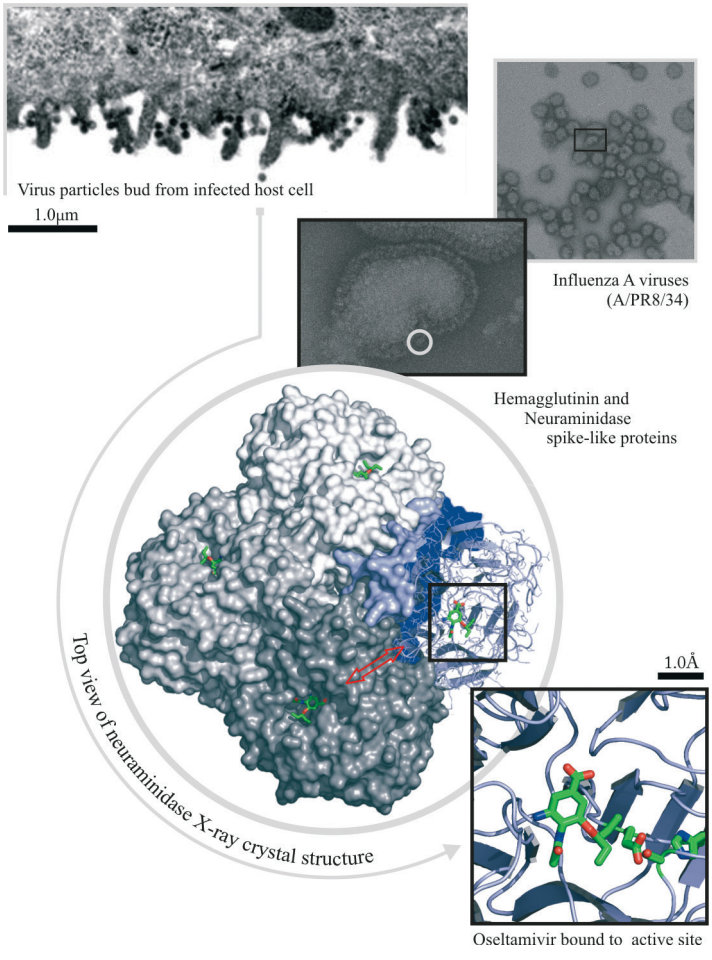

Figure 2 Influenza virus structure by electron microscopy and X-ray crystallography
Influenza viruses can be seen by electron microscopy with high magnification. Beyond the technical limits of the microscope detailed data of protein structure can by obtained by X-ray protein crystallography. (Electron microscopic images were kindly provided by Lesley J. Calder).

NAs (N1-N10) have been identified. Recently, H17 was identified on a virus isolated from a bat [5]. It also contained a novel neuraminidase-like protein "N10" which did not appear to have sialidase activity however [6-8]. HA subtypes 1 to 16 and NA1 to 9 have been isolated from a wide variety of other animals, including horses, seals, pigs and birds [9]. The latter are considered the natural host of these viruses. When transmitted to humans a new influenza A virus may spread and potentially cause an influenza pandemic [10].

1.2 Replication cycle

The influenza virus replication cycle starts when an influenza virus particle binds to receptors on the epithelial cell wall along the respiratory tract. These are sialic acid terminated sugars found abundantly on glycosylated membrane proteins [11]. Binding to sialic acids is established at the receptor binding site (RBS) of HA [12]. After binding the virus becomes internalized by endocytosis. A critical step of the replication cycle is at the stage when the virion-trapped endosome acidifies [3]. Acidification induces an influx of protons into the virion across the M_2-channel. This triggers uncoating of the viral RNA (vRNA) [13,14]. Furthermore, the decreased pH in the endosome induces HA to undergo a conformational change, which forces fusion of the virus and endosome membranes. The uncoated vRNA is then released into the cytoplasm and transported into the nucleus [14]. In the nucleus vRNA is transcribed into copy RNA (cRNA) and messenger RNA (mRNA). Messenger RNA is used for the translation of viral proteins and cRNA serves as a template for viral RNA synthesis. At the final stage, just before newly formed virus particles bud off from the infected cell, removal of sialic acids from both the virus and cellular glycoproteins prevents premature HA binding and aggregation of progeny viruses. At this step sialidase activity of NA is required [15].

1.3 Influenza: the disease

Each year, approximately 5 to 10% of the world population is infected with the influenza virus. In the United States (US) influenza virus infections result in an estimated 200,000 hospitalizations each year and 3,000 to 49,000 influenza related deaths [16]. The highest fatality rates are found among adults over 65 years [17]. These rates have slowly increased over the past decade, probably a consequence of an ageing population [16]. Therefore, the World Health Organization (WHO) recommends vaccination of people over 65 and those at risk of developing severe influenza [18,19]. In addition to vaccination, antiviral drugs are available for both treatment and prophylaxis.

Of the three virus types only influenza virus A and B are considered of major clinical importance, although type C virus infections have been associated with severe infections in children [20,21]. In healthy individuals influenza virus infections usually cause a self-limiting infection of the upper respiratory tract, which is controlled by innate and adaptive immune responses. Influenza like symptoms typically include headache, sore throat, muscle ache, fatigue and fever. Although the total course of illness usually does not take more than a week complete recovery to normal daily activities may take much longer. However, influenza virus infections are well known to cause complications, ranging from bronchitis and otitis media to death [22,23]. Those complications usually start when replication of the virus expands into the lower respiratory tract and more or less pneumonia develops [24]. This may eventually lead to severe damage of the lungs, hypoxemia, shock, renal failure and acute respiratory disease syndrome (ARDS) [25,26]. Individuals at increased risk of severe complications include children below 2yrs of age, pregnant women, elderly and patients of any age with a concomitant morbidity [27].

1.4 Seasonal and pandemic influenza

Influenza A viruses currently circulating in the human population are of the A/H3N2 and A/H1N1 subtypes [21,28]. These have been responsible for the last two influenza pandemics of 1968 (H3N2) and 2009 (H1N1). The 2009 influenza pandemic was the first pandemic of the 21st century and replaced another A/H1N1 subtype, which had caused the pandemic of 1918 that re-emerged in 1977 [29]. The other pandemic of the last century took place in 1957 (H2N2). Pandemic waves are usually associated with a relatively high case fatality rate. Subsequent waves then become milder with increasing population (herd) immunity against this virus and form the seasonal epidemics.

In addition to influenza A, two antigenically distinct influenza B virus lineages are currently circulating in humans. These are represented by the virus strains B/Victoria/2/87 and B/Yamagata/16/88 [30,31]. Influenza B viruses are more restricted to the human host, although an influenza B virus has also been isolated from other mammalian species [32].

1.5 Human cases of highly pathogenic avian influenza virus infections

Until the 2009 pandemic all eyes had been on the Far and Middle East in anticipation of a new influenza pandemic. Since 1997, the number of cases reported to the World Health Organisation (WHO) of fatal human infections with a highly pathogenic

avian influenza A/H5N1 virus had been accumulating [33]. Case fatality rates have been suggested to be as high as 60%, although this percentage may be somewhat overestimated [34]. Apart from the threat of a H5N1 pandemic more than 40 fatal cases have been reported in China in 2013 due to another avian influenza A/H7N9 virus [35,36]. The threat posed by these introductions with pandemic potential has prompted the WHO to recommend stockpiling of antiviral drugs as part of national programs of pandemic preparedness plans.

1.6 Molecular influenza virus diagnostics

In the last decade, the traditional diagnostic armamentarium for influenza (antigen-based rapid tests, virus culture and antibody tests) has shifted more towards polymerase chain reaction (PCR) based assays. Several real-time reverse-transcriptase (RT) PCR assays are available, which allow processing of large numbers of samples on a daily basis. With the addition of internal and external assay controls, RT-PCR based assays are capable of generating quantitative and reproducible results. Since PCR machines have become standard equipment in most laboratories around the world, and consumables are relatively cheap, RT-PCR assays can easily be implemented all around the world. In addition, the technique can also be used for the detection of influenza antiviral resistance markers.

1.7 Current antiviral drugs

Currently two classes of antiviral drugs are available for therapy. These are the adamantane and the neuraminidase inhibitor classes of antiviral drugs.

Adamantanes

The adamantanes, amantadine and rimantadine, have been around for many years [37]. However, these drugs are hardly prescribed nowadays, because of their limited effectiveness against influenza B virus, their substantial side effects and the naturally occurring adamantane resistance of virtually all circulating influenza A viruses [19,38,39]. Both drugs act similarly by binding to the M_2-channel and blocking proton transport across the membrane [40,41] (Figure 3). The major amino acid changes causing adamantane cross-resistance are at positions 26, 27, 30, 31 and 34 of the M_2-channel. These destabilize M_2-channel assembly and lower adamantane binding affinity or they restore membrane proton transport at the acidification step during virus replication [41], but they do not affect virus spread and pathogenicity. Several high resolution structures of M_2 protein channels have been published recently

[40-43]. These structures may drive the development of new effective M_2-proton channel inhibitors.

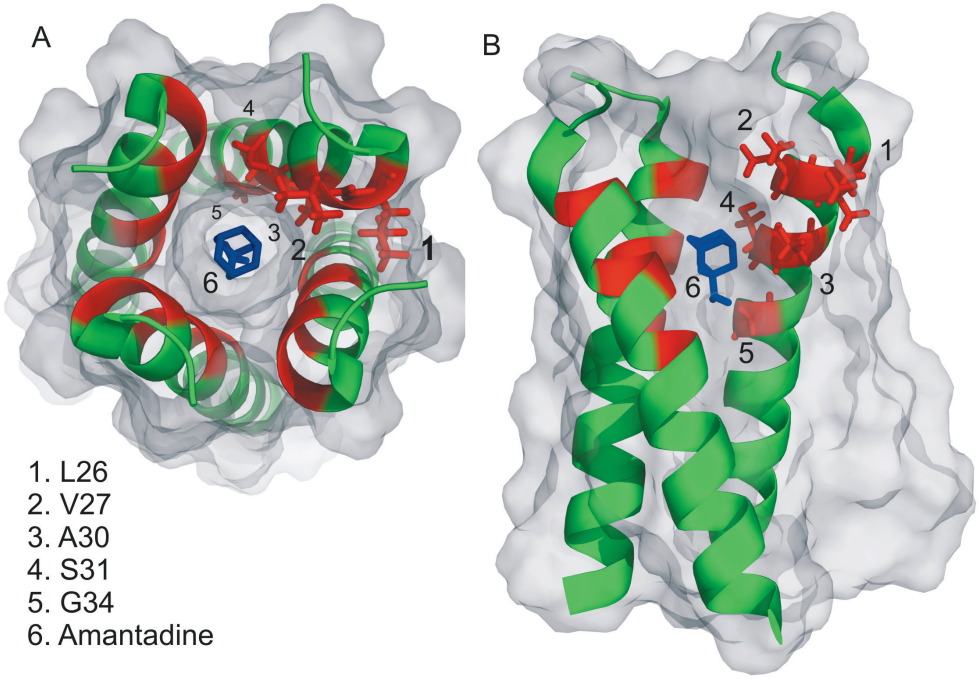

Figure 3 Amino acid residues associated with adamantane resistance
Top view (A) and side view (B) of the (homo) tetrameric M_2-proton channel membrane domain (PDB code 2KQT). Both protein surface (grey) and helices are displayed (green). The positions of amino acid residues 26, 27, 30, 31 and 34, (numbered 1-5) which cause adamantane resistance, are displayed in red and side chains are displayed as sticks on a single helix only. Amantadine (number 6) (blue) located at the proposed binding site [40]. One membrane domain was left out from (B). Image was generated using PYMOL.

Neuraminidase inhibitors

Removal of sialic acids from the infected cell surface by NA is an essential and final step in the influenza virus replication cycle. If sialic acid removal is inhibited viruses tend to aggregate and remain attached to the cell surfaces [44]. The development of the neuraminidase inhibitors (NAIs) was accelerated by resolving of the first NA crystal structure in 1983 [15]. They were designed on the basis of an early transition state analogue of sialic acid, 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (DANA) (Figure 4). This compound was recognized as a weak neuraminidase inhibitor in the

1970s [45-47]. Subsequently, structure based design of these initial weak binding compounds lead to the synthesis of zanamivir (ZA) and OSC phosphate (OSP) [48,49]. Since 1999, these two drugs have been approved by the US Food and Drug Administration (FDA) and later by other regulatory bodies for the treatment and prophylaxis of influenza A and B virus infections. Guidelines for their prescription are found elsewhere [19].

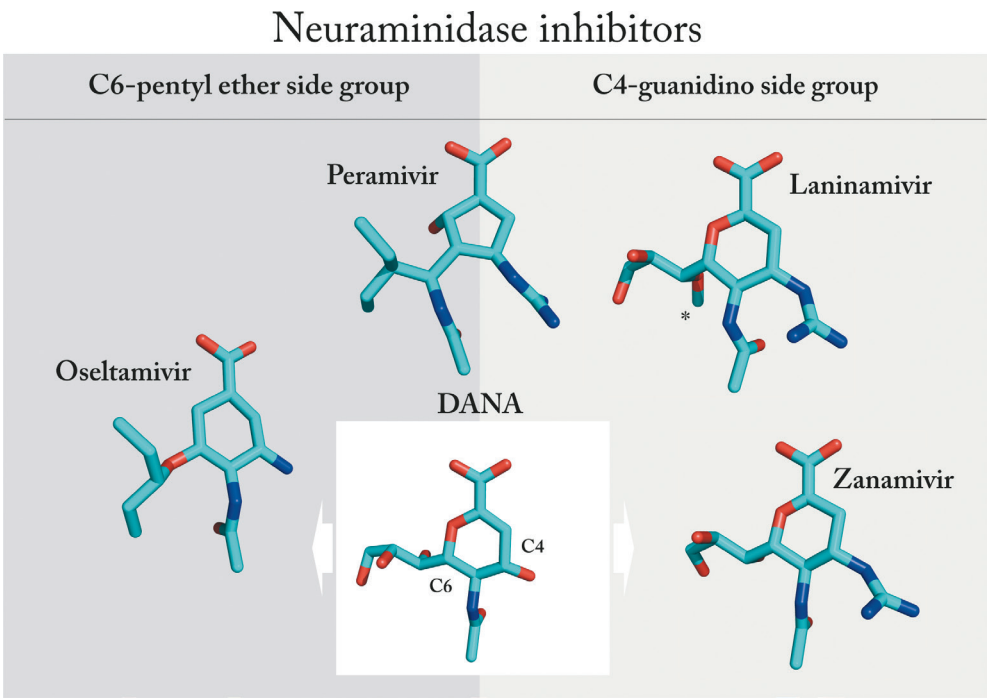

Figure 4 Chemical structure of DANA with neuraminidase inhibitors
As compared to a transition state analogue of sialic acid, DANA (2,3-dehydro-2-deoxy-N-acetylneuraminic acid), OSC has a hydrophobic pentyl ether side group the C6-carbon of the cyclohexane ring where DANA has a polar glycerol. At the C4-carbon, the hydroxyl of DANA is substituted for a guanidino group in both ZA and laninamivir. Peramivir has a cylopentane ring with both a hydrophobic as well as a guanidino side group. As compared to ZA, laninamivir has an additional methoxyl group at the C7-carbon (marked with an asterisk). Adapted from [50,51]. Image was generated using PYMOL.

As compared to the initial sialic acid transition state analogue DANA, ZA has a 4-guanidino group [52]. In the structure of OSC carboxylate (OSC), the active form of OSP, this 4-guanidino group is substituted by a 4-amino group and the 6-glycerol

by a hydrophobic pentyl ether side chain. These adjustments resulted in enhanced binding to the NA active site by approximately 10.000-fold [51]. OSP shows better bioavailability compared to ZA, is administered orally and converted in the liver to the active form OSC [53]. Oral bioavailability of ZA is poor and therefore it is administered by inhalation [54]. However, for those patients who are critically ill, in particular patients on mechanical ventilation, this administration route may be challenging [55,56]. Therefore, alternative intravenous ZA and OSP formulations are in phase III clinical trials [57-60]. Some of these formulations were available on emergency compassionate use during the 2009 influenza pandemic.

More recently, peramivir (PER) and laninamivir octanoate (LANO) were added to the NAI class of drugs. So far, PER has only been approved in Japan and South Korea [60,61]. It has the 4-guanidino group of ZA and the hydrophobic pentoxyl group of OSC and is administered intravenously [60]. LANO has been developed as a long-lasting prodrug with the 4-guanidino and polar glycerol groups of ZA. This prodrug is administered by a single inhalation and is converted directly to the active form LAN in the respiratory tract [62]. LANO has been approved in Japan only, but is entering phase II studies in the US [63,64].

1.8 Resistance to the neuraminidase inhibitors

In the first few years after the approval of the NAIs it was generally believed that selection of antiviral resistance would go hand in hand with loss of virus pathogenicity and transmission [65,66]. Therefore, antiviral resistance was thought not to be of major clinical significance. Indeed, viruses that were isolated early after the introduction of the NAIs were indeed attenuated [65,66]. Unexpectedly, however, a dramatic increase of OS-resistant viruses was seen during the influenza season of 2007/2008 [67]. This virus did not seem to be attenuated in its ability to spread [68], in fact it had completely replaced the OS-sensitive variant by the end of 2008 [69,70]. In the discussion of this thesis a hypothesis is proposed, which may explain its emergence and spread.

1.9 Mechanisms of resistance to neuraminidase inhibitors

The active site of the NA is highly conserved among different influenza viruses [71]. However, the combination of small differences in the active sites of N1, N2 and B NAs and those between the NAIs (Figure 4), lead to different options for development of resistance. Most amino acid changes causing NAI resistance are located in or are in

close proximity to the active site [72]. All amino acids described in the introduction are numbered according to those in N2 NA.

N1 neuraminidase

In type 1 NA, the major amino acid change causing antiviral resistance is a histidine to tyrosine change at position 274 (H274Y) (Table 1). This change causes an increase of the 50% inhibitor constant (IC_{50}) of about 150-fold for OSC and 80-fold for PER [73], but no increase in IC_{50} for ZA. This mutation is the major OS-resistance change in influenza A/H1N1 and A/H5N1 viruses [74-77].

Table 1 N1 neuraminidase mutations causing reduced sensitivity to NAIs

Influenza (sub)type	NA mutation [a]	Virus source/ NAI used for selection	Phenotype in NA inhibition assays [b]			References
			OSC	ZA	PER	
A(H1N1)	Q136K	In vitro (clinic?)/none	S	HRI	ND	[78,79]
	H274Y	Clinic/OSC	HRI	S	HRI	[80]
2009 A(H1N1)	E119V	Reverse genetics	RI	HRI	RI	[81]
	E119G	Reverse genetics	S	HRI	RI	[81]
	I222R	Clinic/OSC	RI	RI	ND	[82]
	I222R/H274Y	Clinic/OSC	HRI	RI	HRI	[83]
	I222V/H274Y	Clinic/OSC	HRI	S	ND	[84]
	S246N/H274Y	Clinic/OSC	HRI	S	HRI	[85]
	H274Y	Clinic/OSC	HRI	S	HRI	[86-88]
	N294S	Reverse genetics	HRI	S	RI	[81]
A(H5N1)	E119G	In vitro/ZA	S	HRI	RI/HRI	[89]
	D198G	In vitro/ZA	RI	RI	S	[89]
	H274Y	Clinic/OSC	HRI	S	HRI	[90]
	N294S	Clinic/OSC	RI	S	S	[90]

[a]Numbers indicate the position of the substituted residue in the NA amino acid sequence (N2 numbering).
[b]S:<10-fold increase in IC_{50} over wild type); RI: 10–100-fold increase in IC_{50} over wild type); HRI: >100-fold increase in IC_{50} over wild type. ND: Not determined. A(H1N1): seasonal H1N1 viruses, A(H1N1) pdm09: Swine origin H1N1 viruses responsible for the 2009 pandemic. Table adapted from [73].

For binding of the pentoxyl group of OSC into the hydrophobic pocket reorientation is required of a glutamic acid residue at position 276 (E276) towards H274 (Figure 5). In case of the H274Y change this reorientation is blocked by the larger tyrosine residue [50,91]. Since the C6-group of ZA is different it does not involve reorientation

of E276 upon binding [50,71,91]. The I222R and S246N changes alter the hydrophobic pocket also and make binding of OSC to a H274Y mutant even more difficult [50,91]. A glutamine to lysine change at position 136 (Q136K) was previously shown to cause ZA (300-fold) and peramivir (70-fold) resistance in H1N1 viruses [79]. More recently it was shown that the presence of the Q136K mutation seemed to be an artefact of virus propagation in Madin Darby Canine Kidney (MDCK) cell cultures [78].

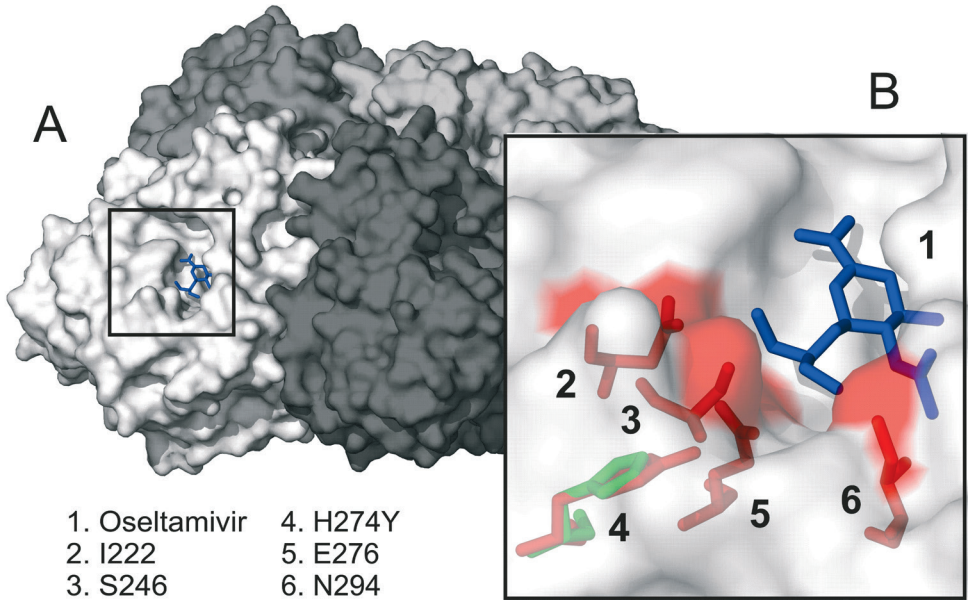

Figure 5 Amino acid residues in N1 neuraminidase associated with NAI resistance
Surface presentation of (homo) tetrameric NA (grey) in complex with OSC (blue) (A) (PDB ID code 2HU0). In an enlarged image of the active site (B, inset), OSC and amino acids associated with NAI resistance are presented as red sticks (1-6). These are located close to the active site of NA to which OSC is bound. At position 274 a histidine (H, green) to tyrosine (Y, red) change, prevents reorientation of glutamic acid (E) side chain at position 276 upon OSC binding. At position 294 (5), an asparagine (N, green) to serine (S, red) substitution weakens OSC binding affinity. Surface was made transparent to visualize otherwise hidden residues and colored red if a residue is a surface occupant. Image was generated using PYMOL.

N2 Neuraminidase

The major neuraminidase inhibitor resistance changes are the glutamic acid to valine change at position 119 (E119V) and the arginine to lysine change at position 292 (R292K) (Table 2). These mutations cause resistance to OSC only and not to

ZA. Compared to the H274Y change in N1 neuraminidase these mutations are less frequently detected in virus isolates [92]. Similar to the resistance mechanism of H274Y, the R292K change also prevents rotation of glutamic acid at position 276 [93]. For the E119V OSC resistant mutant no X-ray crystal structure is available to determine its exact resistance mechanism. However, another neuraminidase structure of a glutamic acid to glycine mutant (E119G) reveals that this change involves interaction with the C4 groups of ZA (C4-guanidino) and OSC (C4-amino) [93].

Table 2 N2 neuraminidase mutations causing reduced sensitivity to NAIs

Influenza Subtype	NA mutation (a)	Virus source/ NAI used for selection	Phenotype in NA inhibition assays (b)			References
			OSC	ZA	PER	
A(H3N2)	E119V	Clinic/OSC	HRI	S	S	[94]
	E119V/I222V	Clinic/OSC	HRI	S	S	[95]
	Q136K	Clinic/none	S	RI	ND	[96]
	D151A/D	Clinic?/none	S	HRI	ND	[97]
	R224K	Reverse genetics	HRI	HRI	ND	[98]
	Del. 245-248	Clinic/OSC	HRI	S	S	[99]
	R292K	Clinic/OSC	HRI	ND	ND	[65,97]
		Reverse genetics	HRI	RI	ND	[98]
	N294S	Clinic/OSC	HRI	S	ND	[100]
	R371K	Reverse genetics	RI	RI	ND	[98]

[a]Numbers indicate the position of the substituted residue in the NA amino acid sequence (N2 numbering).

[b]S:<10-fold increase in IC_{50} over wild type; RI: 10–100-fold increase in IC_{50} over wild type; HRI: >100-fold increase in IC_{50} over wild type. ND: Not determined. Table adapted from [73].

B neuraminidase

Several OSC-resistant B viruses have been isolated from patients [101-103] (Table 3). OSC resistance may be caused by amino acid changes, D198N, R371K [97,104] and by an isoleucine change at position 222 (I221V/T, B numbering) [103,105]. These mutations cause only a two to three fold increase in IC_{50} to the NAIs. Recently, an influenza B H274Y mutant virus was isolated from an untreated patient causing OSC and PER resistance [106]. An arginine to lysine change at position 152 (R152K) was identified in an influenza B virus infected patient on ZA [107].

Table 3 B neuraminidase mutations causing reduced sensitivity to NAIs

Influenza Type	NA mutation (a)	Virus source/ NAI used for selection	Phenotype in NA inhibition assays (b)			References
			OSC	ZA	PER	
B	R371K	Clinic/none	HRI	RI	ND	[97]
	N294S	Clinic/none	HRI	ND	ND	[108]
	D198N	Clinic/OSC	HRI	HRI	S	[94]
	R152K	Clinic/zanamivir	HRI	RI	HRI	[94]
		Reverse genetics	HRI	RI	HRI	[103]
	E119A	Reverse genetics	HRI	HRI	HRI	[109]
	E119D	Reverse genetics	HRI	HRI	HRI	[109]
	E119G	Reverse genetics	HRI	RI	HRI	[109]
	E119V	Reverse genetics	HRI	S	HRI	[109]
	R292K	Reverse genetics	RI	RI	HRI	[109]
	E105K	Clinical/none	RI	S	HRI	[110]
	H274Y	Clinical/?	RI	S	RI	[110]
	I222T	Clinical/none	RI	S	ND	[111]

[a]Numbers indicate the position of the substituted residue in the NA amino acid sequence (N2 numbering).
[b]S:<10-fold increase in IC_{50} over wild type; RI: 10–100-fold increase in IC_{50} over wild type; HRI: >100-fold increase in IC_{50} over wild type. ND: Not determined. Table adapted from [73].

1.10 Effectiveness of the neuraminidase inhibitors

There has been a lively discussion in the scientific community regarding the clinical effectiveness of the neuraminidase inhibitors [112,113]. Several systematic reviews have recently been published that have addressed this issue [39,114-118]. In individuals without risk factors, OS and ZA therapies were shown to reduce the duration of illness by approximately 0.5-1 day when started within 48 hrs. Both drugs were found to be effective as a post-exposure prophylaxis. The impact of therapy increases when initiated sooner after onset of symptoms [117]. Clinical study data with PER and LANO were comparable to those found for the traditional NAIs [60,62,119-121].

Although there is lack of randomized clinical trials studying patients at-risk, observational studies have shown improved clinical outcome, including reduced mortality with the use of neuraminidase inhibitors [122-126]. For those patients who eventually require intensive medical care treating their influenza infection remains a challenge [55,127,128], especially when extracorporeal membrane oxygenation (ECMO) is ultimately required to compensate for influenza associated lung failure. Given the high mortality and frequent emergence of antiviral resistance in these

patients there is a clear need for further improvement [108,129,130]. Little is known about the impact of antiviral resistance mutations on the effectiveness of antiviral therapy. Antiviral therapy was shown to be less effective for treatment of H274Y mutant H1N1 viruses [131-133]. A similar impact on the efficacy was seen for the adamantanes [134]. For many other mutations, especially those that do not cause a dramatic shift in IC_{50}, the impact of these mutations is unclear.

2 Outline of this thesis

In the last 10 years, three events in the field of influenza have increased the focus on antiviral therapy, antiviral resistance and therapeutic effectiveness. Sequentially, these were the increase of adamantane resistance since 2003, the emergence of an OSC-resistant influenza A/H1N1 virus during the 2007-2008 winters season and the outbreak of the 2009 influenza pandemic. These events stressed the importance of a better understanding of the underlying mechanisms of influenza antiviral resistance. They also uncovered the shortcomings of the current available influenza antiviral therapies.

To aid in the development of improved therapeutics and patient management in the clinic, rapid diagnosis is essential. Furthermore, knowing virus type and subtype, subtype as well as its antiviral resistance profile has become essential for decision making of a treating physician. Therefore, in the second chapter of this thesis (chapter 2.1), the development of a set of diagnostic real-time RT-PCR based assays are described. These assays detect all currently circulating influenza viruses in humans. In addition, similar assays are described that allow for screening of all major NAI resistance mutations, including those for 2009 pandemic influenza virus (chapter 2.2). Especially, the assay to detect the H274Y mutation in pandemic influenza A/H1N1 viruses has proven its value. It was implemented successfully in several diagnostic units in the Netherlands and other parts of Europe and the first OSC-resistant cases in hospitals in the Netherlands and in the United Kingdom were identified with this assay.

In particular, the addition of an internal control and virus quantification standards and the ability to monitor antiviral susceptibility has significantly improved influenza virus diagnostics in the virology unit of the Erasmus Medical Centre and in the Influenza Resistance Information Study (IRIS; NCT00884117). This post marketing natural and therapy induced antiviral resistance surveillance study, initiated by Hoffmann-La Roche Ltd in 2008, monitors global OSC resistance in clinical settings. Three cases, in which these assays have proven their value, are described in chapter 3. In chapter 3.1, a fatal infection is described of an immunocompromised patient with an H274Y OSC-resistant influenza A/H1N1 virus. This case has been illustrative for the unexpected pathogenicity of the H274Y mutant viruses that emerged in 2007/2008.

During the outbreak of the 2009 influenza A/H1N1 virus pandemic several non-approved antiviral therapies were administered on the basis of an investigational emergency compassionate use agreement. In chapter 3.2, the use of ZA therapy given

intravenously (IV) was evaluated for the treatment of critically-ill patients in the light of the 2009 influenza pandemic. In one of these patients, an immunocompromised child with acute lymphoblastic leukaemia (ALL), a novel neuraminidase inhibitor resistance mutation was identified (chapter 3.3). This mutation, an isoleucine to arginine change at position 222 (I222R) of the pandemic neuraminidase, was identified subsequently after OSC and IV ZA therapy failure.

Following up on the identification of this I222R NAI resistance mutation the mechanism by which this mutation causes antiviral resistance is described in chapter 4. To unravel the mechanism, X-ray crystal structures were solved of the I222R mutant neuraminidase and were compared to those obtained for the wild type and H274Y mutant neuraminidases (chapter 4.1). The impact of the I222R mutation on the pathogenicity of the 2009 pandemic virus was addressed using the ferret model (chapter 5.1).

The clinical cases presented in this thesis are clear examples of the shortcomings of the current available antiviral therapies. Treatments of significant numbers of Influenza infected individuals in numerous randomized clinical trials have proven NAI efficacy for non-risk patients for whom influenza usually is a self-limiting disease. In contrast, antiviral strategies for those patients who could clearly benefit from antiviral compound use, such as immunocompromised patients, are less well defined. To aid in the development of future antiviral therapies, tailored for the immunocompromised, an immunocompromised ferret model is presented in chapter 5.2.

Finally, the main findings of this thesis, the potential of this new immunocompromised ferret model, and a hypothesis on the emergence of the 2007/2008 OSC resistant H1N1 virus are summarized and discussed in chapter 6 and 7.

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Chapter 2.1

Molecular assays for quantitative and qualitative detection of influenza virus and oseltamivir resistance mutations: quantitative influenza virus diagnostics

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Abstract

Sensitive and reproducible molecular assays are essential for influenza virus diagnostics. This manuscript describes the design, validation and evaluation of a set of real-time RT-PCR assays for quantification and subtyping of human influenza viruses from patient respiratory material. Four assays are included for detection of oseltamivir resistance mutations H275Y in pre-pandemic and pandemic influenza A/H1N1 and E119V and R292K in influenza A/H3N2 neuraminidase. The lower limits of detection of the quantification assay were determined to be 1.7$\log_{10}$ virus particles per millilitre (vp/ml) for influenza A and 2.2$\log_{10}$vp/ml for influenza B virus. The lower limits of quantification were 2.1 and 2.3$\log_{10}$vp/ml respectively. The RT-PCR efficiencies and lower limits of detection of the quantification assays were only marginally affected when tested on the most dissimilar target sequences found in the NCBI database. Finally, the resistance RT-PCR assays detected at least 5% mutant viruses present in mixtures containing both wild type and mutant viruses with approximated limits of detection of 2.4$\log_{10}$vp/ml. Overall, this set of RT-PCR assays is a powerful tool for enhanced influenza virus surveillance.

1 Introduction

Since the outbreak of the 2009 influenza pandemic [1], the influenza A/H3N2 and A/H1N1 subtypes are the two influenza viruses, together with influenza B virus, causing disease in humans. Another influenza A virus of the H1N1 subtype, which had circulated in the population between 1977 and 2009, seems to be replaced by the 2009 pandemic virus. However, re-emergence of this subtype can not be ruled out.

Strategies to combat influenza virus induced disease rely on vaccination as preventive measure [2]. In cases when vaccine efficacy is low, antiviral drugs are useful as prophylaxis especially during future influenza outbreaks. Moreover, for individual patients, especially when their immune system is compromised, antiviral therapy may be life saving [3]. To be effective against influenza, antiviral therapy should preferably be initiated within 48 hours after onset of symptoms [4]. A rapid diagnosis and subsequent initiation of therapy is, therefore, crucial. In addition, intensified monitoring of high-risk hospitalized patients infected with influenza, may give valuable information regarding therapy efficacy and emergence of antiviral resistance.

Traditionally, both the adamantane and neuraminidase inhibitor class of drugs have been available for prophylaxis and therapy [5]. However, the majority of recently circulating influenza viruses is resistant to the adamantanes. Besides the emergence of these adamantane resistant viruses, pre-pandemic A/H1N1 virus viruses emerged by the end of 2007, which were naturally resistant to the neuraminidase inhibitor oseltamivir [6]. These viruses harboured an amino acid substitution in the neuraminidase at residue 275, where a tyrosine was replaced by a histidine (H275Y, N1 numbering). Also in the 2009 pandemic influenza A/H1N1 virus background, the H275Y substitution is the most frequently detected antiviral resistance mutation. The two major mutations conferring oseltamivir resistance in influenza A/H3N2 viruses are neuraminidase substitutions E119V and R292K (N2 numbering). These two changes, as well as the H275Y in the 2009 pandemic virus, are predominantly found in viruses isolated from oseltamivir treated immune compromised influenza virus infected patients [7-11]. Notably, these viruses remain susceptible to zanamivir. Since its introduction in 1999, resistance to zanamivir has been reported in only a limited number of treated patients [12-15].

In the present manuscript, we set out to study the quantitative and qualitative aspects of RT-PCR assays for detection of influenza A and B virus and an RT-PCR assay for detection of the H275Y oseltamivir resistance mutation in 2009 pandemic neuraminidase. This includes determination of the lower limits of detection, lower

limit of quantification and the robustness of these assays. In addition, two newly developed RT-PCR assays detecting oseltamivir resistance mutations E119V and R292K in the influenza A/H3N2 virus subtype are described which complement the set of antiviral resistance detection assays in currently circulating influenza A viruses [16,17].

2 Materials and methods

2.1 Viruses, quantification standards and assay controls

Electron microscopy (EM) counted influenza virus strains A/PR/8/34 (11.48log$_{10}$ vp/ml) and B/Lee/40 (10.93log$_{10}$ vp/ml) (cat. numbers 10-210-500 and 10-220-500, Advanced Biotechnologies INC, USA) were used to determine the lower limits of detection and quantification (LLOD/Q) of the quantitative influenza A/B real time RT-PCR assay (quantification assay). Phocine distemper virus type 1 (PDV) was added as a universal internal control [18]. Influenza viruses used as positive controls are summarized in table 1. All influenza viruses used for assay validation were collected

Table 1 Panel of influenza viruses used as positive control samples

(Sub)type	Mutation	Virus	Genbank ID*	IC_{50} value (nM)†	
				OS (SD)	ZA (SD)
Influenza A/B quantitative RT-PCR assay					
A/H1N1		A/PR/8/34	(EF467823)	0.4 (0.1)	0.3 (0.1)
B		B/Lee/40	(JO2095)	1.7 (0.3)	1.9 (0.6)
Oseltamivir resistance RT-PCR assays					
A/H3N2	R292K	A/Tilburg/43659/2005	(AB462529)	>1000	4.8 (2.0)
A/H3N2	E119V	A/Leiden/01272/06	(AB462546)	4.3 (0.5)	1.1 (0.1)
A/H1N1	H275H	A/Netherlands/059/2008	-	0.3 (0.0)	0.2 (0.0)
A/H1N1	H275Y	A/Netherlands/026/2008	-	88 (2.8)	0.4 (0.1)
A/H1N1	H275H	A/Netherlands/2631b/2010	(JF906185)‡	9.1 (1.2)	2.2 (0.1)
A/H1N1	H275Y	A/Netherlands/2631d/2009	-	110 (12)	0.5 (0.0)

*Genbank accession number of the neuraminidase genes. †Virus susceptibility to oseltamivir (OS) and zanamivir (ZA) were determined *in duplo* using the NA-star neuraminidase inhibitor resistance detection kit (Live Technologies, Nieuwerkerk a/d IJsel, The Netherlands). 50% inhibitor constants (IC50) were calculated using Graphpad Prism software (version 4.0) and sigmoid dose response curve-fitting. ‡Neuraminidase harbours a neuraminidase inhibitor resistance mutation at position 223.

in the Netherlands between 1999 and 2010. These viruses were propagated on Madin-Darby Canine kidney (MDCK) cells as described previously [19].

2.2 Patient materials

In total, 245 respiratory specimens were used for assay evaluation, which were collected from 87 patients between January and April 2009. Patients, living in Asia, Europe and USA, were included in an observational research trial evaluating influenza antiviral resistance (IRIS, NV20237).

2.3 Assay design

Primers and probes

The quantification assay consisted of two previously described primer and probe sets combined here in a duplex assay [16,20]. Primers and probes for the influenza A/H1N1 and A/H3N2 subtyping assay were designed by alignment of human influenza hemagglutinin gene sequences deposited in the NCBI public sequence database between 2000 and 2009. The primer pairs and probes for the (pre-) pandemic H275Y, E119V and R292K real time RT-PCR assays (resistance assays) were designed around the single nucleotide polymorphisms (SNP) conferring antiviral resistance to oseltamivir. Each resistance assay contained two probes with locked nucleic acids (LNA) binding either the wild type or mutant sequence. A minor groove binding (MGB) probe was used in the R292K resistance assay (Live Technologies, Warrington, UK). All other primers and probes were purchased from Eurogentec (Maastricht, the Netherlands) and are listed in table 2 or described elsewhere [17].

Robustness of the quantification assay

The most dissimilar influenza A and B sequence were selected from the NCBI influenza sequence database (accessed May 2011) using the following 4 selection criteria: Only sequences derived from human virus isolates containing the full target sequence (1). For influenza A only currently circulating virus were included (between 2005 and 2011, total 11.548 sequences) and due to more limited availability of influenza B sequences, all available sequences were included, which were isolated from humans (total 758 sequences) (2). Sequences were included only if these were present at least twice to exclude targets with sequencing errors or targets from evolutionary dead-end virus lineages (3). Priority was given to targets with multiple mutations in the probe region or mutations in the primer region if positioned within the first

five nucleotides counted from the 3'end [21] (4). Three RNA run-off transcripts were generated as previously described [17]. One consisted of the matching influenza A and B targets placed in tandem. For the other two RNA transcripts, one of the targets was substituted for its most dissimilar counterpart.

Detection limits of the quantification and resistance assays

The lower limits of detection (LLOD) of the quantification assay were determined by testing virus dilutions of the EM stocks in 48-fold containing 1000, 500, 250, 150, 100, 75, 25, 10 or 0 vp/ml. The LLODs, defined as the 95% hit-rate, were determined by probit analysis using SPSS PSAW software version 17.0.2. The LLOQ was defined as the particle count at which the coefficient of variation (%CV) reached 10%. Approximated limits of detection of the resistance assays were determined in 8-fold using low-titre viral RNA dilutions containing 1000, 500, 250, 100, 50, 25, 10, 5 and 1 RNA copies per millilitre, as calculated on basis of the quantification assay.

2.4 Protocols

Isolation of nucleic acids

Nucleic acids were extracted from 190µl of respiratory specimen and eluted to a final volume of 110µl using the pathogen-complex-200 protocol and the QiaSymphony (QS) machine (Qiagen, Venlo, the Netherlands). Before nucleic acid isolation, 10µl PDV was added automatically to each respiratory specimen by the QS machine.

RT-PCR reactions

The rtTH- based EZ kit, the Gold and the Fast Viral Master Mixes (FVMM) (Live Technologies, the Netherlands) were used for the RT-PCR assays (Table 2). The cycling conditions for all RT-PCR procedures were similar. The EZ kit reactions were performed using the following amplification conditions: UNG reaction at 50°C for 2 min, cDNA reaction at 60°C for 30 min and PCR amplification at 95°C for 5 min, followed by 45 cycles at 95°C for 20s, 62°C (60°C for the R292K assay) for 1min. Gold or FVMM reactions were performed using following the amplification conditions: cDNA reaction at 50°C for 5min and PCR amplification at 95°C for 20s, followed by 45 cycles at 95°C for 3s and 60°C for 30s. Final primer and probe concentrations in the PCR reactions are listed in table 2 or described elsewhere [17]. All reactions were performed in a total reaction volume of 50µl, including 20µl of nucleic acid extract. Amplification and detection was performed on a LightCycler (LC) 480 instrument

using the FAM (465-510nm), Dragonfly (Dr1) (533-580nm) and Cy5 (618-660nm) filters (Roche, Almere, the Netherlands). To allow simultaneous data acquisition, colour compensation runs were performed during data analysis to compensate for overlapping emission spectra of the fluorescent dyes.

Internal and external assay standards

Aliquots of external quantification standards contained 7.48, 5.48, 3.48 $\log_{10}$ vp/ml (influenza A virus) and 7.93, 5.93, 3.93 $\log_{10}$ vp/ml (influenza B virus) and were stored at -80°C until use. These were processed in parallel each time respiratory specimens were analyzed. In addition, for each sample the complete process from nucleic acid isolation to end result was monitored using a PDV RT-PCR assay (primers and probes are listed in table 2). A fixed amount of PDV was added to each sample equivalent to an approximate cycle threshold (Ct)-value of 29. Ct-value fluctuations of PDV and external quantification standards were monitored using MedLabQC software version 3.25 (Biologiste des Hopitaux, Metz, France). A sample was isolated again if the Ct-value of PDV was below or above two times the standard deviation (2xSD) of a longitudinal stored data set. Only a viral load was assigned to a sample if the Ct-value of at least two external standards were within the 2xSD range from a longitudinal stored data set.

Table 2 Primers and probes used for the influenza and PDV RT-PCR assays

Name	Sequence (5'-3') (5'/3'Fluorescent label)	pmol/reaction	Positions[§]
Influenza A/B real time RT-PCR quantification assay (EZ assay kit)			
InfA-forward	AAGACCAATCCTGTCACCTCTGA	40	144-166
InfA-reverse	CAAAGCGTCTACGCTGCAGTCC	30	217-238
InfA-probe	TTTGTGTTCACGCTCACCGTGCC (FAM/BHQ-1)	5	184-206
InfB-forward	GAGACACAATTGCCTACCTGCTT	10	14-36
InfB-reverse	TTCTTTCCCACCGAACCAAC	40	89-108
InfB-probe	AGAAGATGGAGAAGGCAAAGCAGAACTAGC (Dr1/BHQ-2)	10	45-79
Influenza A H1/H3 Subtyping RT-PCR assay (EZ assay kit)			
H1-forward	ATCAGATTCTGGCGATCTACTCAA	20	1583-1606
H1-reverse	GAACACATCCAGAAGCTGATTGC	20	1645-1667
H1-probe	TCGCCAGTTCTCTGGTTCTTTTGGTCTC (FAM/BHQ-1)	5	1610-1637
H3-forward	GGGAAAAGCTCAATAATGAGATCAG	20	835-859
H3-reverse	TTGGGAATGCTTCCATTTGG	30	898-917
H3-probe	TGCACCCATTGGCAAATGCAATTC (Dr1/BHQ-2)	5	861-884
Influenza A/H3N2 E119V resistance assay (Gold or FVMM assay kit)			
E119V forward	AGGACAATTCGATTAGGCTTTCC	20	305-327
E119V reverse	CTGTCCAAGGGCAAATTGGTA	20	388-408
E119E-probe	ACaAGaG<u>a</u>AcCTTaTG[†] (FAM/BHQ-1)	5	349-364
E119V-probe	ACaAGAG<u>t</u>AcCTTaTG[†] (Dr1/BHQ-2)	5	349-364
Influenza A/H3N2 R292K resistance assay (EZ or FVMM assay kit)			
R292K forward	GTGCTCCTGCTATCCTCGATATC	20	831-853
R292K reverse	TTTATATCTACGATGGGCCTATTGG	20	893-917
R292R-probe	CCaGttGtCt<u>c</u>TGC[†] (FAM/BHQ-1)	5	872-885
R292K-probe	CTGCA<u>A</u>AGACAACTGG[‡] (VIC/NFQ)	5	870-885
Influenza A/H1N1 H275Y resistance assay (Gold or FVMM assay kit)			
275Y forward	AAAAGGGGAAGGTTACTAAATCAATAGAGT	10	776-805
H275Y reverse	CAGTGTCTGGGTAACAGGAGCATT	30	833-856
H274H-probe	CAcCcAATtTT<u>c</u>ATtA[†] (FAM/BHQ-1)	5	812-827
H275Y-probe	CAcCcAATtTT<u>t</u>ATtA[†] (Dr1/BHQ-2)	5	812-827
PDV Internal assay control (EZ assay kit)			
PDV-forward	CGGGTGCCTTTTACAAGAAC	30	
PDV-reverse	TTCTTTCCTCAACCTCGTCC	40	
PDV-probe	ATGCAAGGGCCAATTCTTCCAAGTT (Cy5/DDQ)	5	

[†]DNA nucleotides are denoted in upper case, LNA nucleotides are denoted in lower case and LNA nucleotides complementary to the single nucleotide polymorphism (SNP) are underlined. [‡]The R292K-probe is a minor groove binding (MGB) probe. [§]Primer and probe position numberings are counted starting from the first nucleotides of the start codons of the influenza hemagglutinin, neuraminidase and matrix genes. Either the rtTH-based EZ RT-PCR kit (EZ-kit), GOLD or the FVMM mix was used (Live Technologies, Nieuwerkerk a/d IJssel, the Netherlands).

3 Results

3.1 Quantitative influenza A/B real time RT-PCR assay

Assay linearity

First, the linearity of the quantitative influenza A/B real time RT-PCR assay (quantification assay) was studied. This was done by detection of viral RNA in two 10-fold virus dilution series derived from electron microscopy (EM) counted stocks of influenza viruses A/PR8/34 and B/Lee/40. Viral RNA was isolated first from 48 replicates of each dilution and cycle-threshold (Ct) values were then determined. These values were plotted as function of the expected viral particle count to obtain standard curves with slopes of -3.21 (r^2=0.99) for the influenza A and -3.10 (r^2=0.99) for the influenza B standard curve (Figure1). The quantification assay was linear for both virus types if virus concentrations in the analyzed dilutions ranged from 1.9 to 8.5log_{10} virus particles per millilitre (vp/ml).

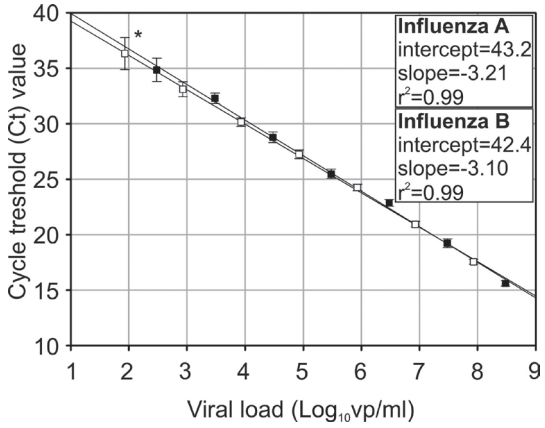

Figure 1 Linearity of the quantification assay
The linearity of the quantification assay was addressed by analyzing 10 fold serial dilutions of electron microscopic counted influenza A (A/PR/8/34, white squares) and B (B/Lee/40, black squares) virus stocks. Cycle threshold values were plotted as function of the viral load, as determined by the manufacturer. Standard curves for influenza A and B virus (black lines) were obtained by linear curve fitting. The asterisk marks an influenza A virus dilution below the lower limit of quantification and was excluded from the standard curve.

Assay sensitivity

Second, the sensitivity of the quantification assay was addressed by testing assay performance when analyzing a set of low-titer virus dilutions. By 95% hit-rate analysis,

a lower limit of detection of 1.7 (1.6-2.2)log_{10}vp/ml for influenza A virus and 2.2 (2.1-2.7)log_{10}vp/ml for influenza B virus was determined (Figure2A). Within the same dataset, the lower limit of quantification, defined as the concentration where the coefficient of variation (%CV) reached 10%, was found to be 2.1 log_{10}vp/ml and 2.3log_{10} vp/ml respectively (Figure 2B).

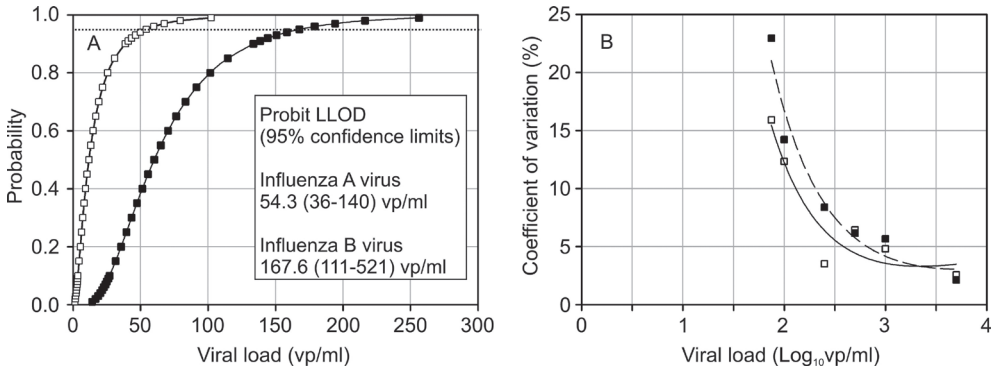

Figure 2 Lower limits of detection and quantification of the quantification assay
In panel A, the lower limits of detection (LLOD) for influenza A and B virus were obtained by testing replicates (n=48) of low-titer virus dilutions and 95% hit rate probit analysis. The dotted line indicates the 95% confidence detection limit. A lower limit of detection of 1.7 (1.6-2.2)log10vp/ml for influenza A virus and 2.2 (2.1-2.7)log10vp/ml for influenza B virus was determined. In panel B, the coefficient of variation (%CV) was plotted as function of the viral load. The LLOQ was defined as the particle count at which the %CV reached 10%. By non-linear curve fitting, the lower limits of quantification were determined to be 2.1log10vp/ml for influenza A virus (solid line and white squares) and 2.3log10vp/ml for influenza B virus (dashed line and black squares).

Assay robustness

Finally, to evaluate if potential mismatches in the target sequences of the quantification assay in circulating Influenza strains would affect assay performance, the most dissimilar target sequences were selected from the NCBI public sequence database. Subsequently, RT-PCR efficiencies and hit rates were compared, which were obtained using 10-fold serial dilutions of either matched or dissimilar target RNA. The most dissimilar influenza A target found was A/Canada-PQ/RV3189/2009 (HQ239826). This target contained two mutations in the forward primer at positions 2 (A to G) and 13 (T to C) and a single mutation in the reverse primer at position 21 (C to T) all counted from the 3' end of each primer (Table 2). B/Connecticut/07/1993 (CY018806) was found to be the most dissimilar influenza B virus. This virus

contained a single mutation in the probe at nucleotide position 6 (A to G) and in the reverse primer at position 20 (A to G). As compared to data obtained when matched RNA was targeted, RT-PCR efficiencies changed to 105% for influenza A and to 93% for influenza B. For influenza A the approximated hit-rate at the 95% confidence limit, decreased 2-fold from 1.4 to 1.7log_{10}vp/ml (Figure3). For influenza B, the hit rate dropped approximately 4-fold from 2.3 to 2.9log_{10}vp/ml. Fluorescence signals dropped approximately 40% when dissimilar influenza B RNA was targeted relative to the fluorescence signal obtained when matching RNA was targeted.

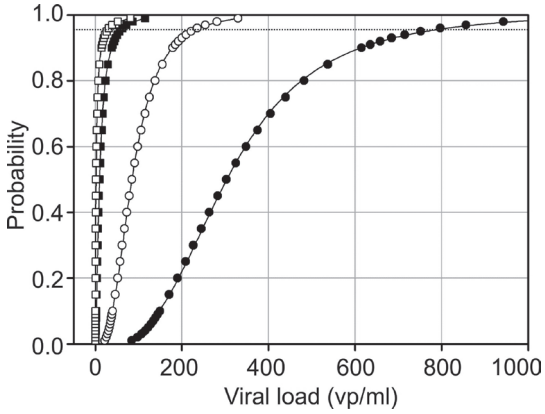

Figure 3 Impact mismatches on performance of quantification assay
Approximated lower limits of detection of the quantification assay if matched (white) or most dissimilar RNA was targeted (black). Probability of a positive result, as determined by 95% hit rate analysis, was plotted as function of the viral particle count per millilitre for influenza A (squares) and B (circles).

Assay sensitivity

Low virus titer dilutions of wild type and mutant virus clones were analyzed using the pandemic H275Y and E119V and R292K resistance assays. By hit rate analysis using low-titer viral RNA dilutions, the approximated limits of detection for each assay were determined to be approximately 2.4log_{10}vp/ml (Table3). When analyzing mixtures of wild type and mutant RNA containing approximately 6.0log_{10} copies/ml, the 275Y, 119V, 292K assays detected 100% (8/8) of the minority resistance mutants in mixtures containing 5% mutant species. When only 2% of the mutant genotype was present, the signal to noise ratio became too small to score for positivity for all assays.

Table 3 Hit rate of the resistance assays using low titer virus dilutions

Viral load	H275Y assay		E119V assay		R292K assay	
(log_{10}vp/ml)	H275	Y275	E119	V119	R292	K292
3.0	8	8	8	8	8	8
2.7	8	8	8	8	8	8
2.4	8	8	8	8	8	8
2.0	5	6	8	8	8	7
1.7	2	3	4	7	7	5
1.4	1	3	2	5	6	2
1.0	0	2	1	1	1	1
0.7	0	0	0	1	2	0
0	0	1	0	1	1	1

*For each low titre viral RNA dilution 8 replicates were tested

3.2 The H275Y, E119V and R292K resistance RT-PCR assays

Assay robustness

In total, 96 pre-pandemic influenza A/H1N1 viruses from the epidemic of 2007-2008 were analyzed by the H275Y assay to check the robustness of the assay. Thirty of these viruses were found to be wild type (H275) and 66 of these viruses were mutant (Y275). No virus mixtures were detected and results were confirmed using Sanger sequencing. To test the performance of the E119V and R292K assay, 77 influenza A/H3N2 viruses isolated in epidemics between 1999 and 2007 were analyzed. For the E119V assay, 76 of these viruses were found to be wild type. From one sample, no wild type or mutant signal was detected due to a mismatch in the probe at a position where an LNA base was incorporated. The R292K assay gave double negative result in the wild-type (R292) and mutant detection channel (292K) for 8 out of 77 samples, all because of mismatches in the probe region.

Assay evaluation

In total, 245 respiratory specimens were analyzed to evaluate the complete set of presented RT-PCR assays. First, the influenza quantification assay was used to check for virus positivity and to obtain virus particle counts for all analyzed samples. Subsequently, influenza A viruses were subtyped and tested for presence of

oseltamivir resistance mutations H275Y (A/H1N1) or E119V and R292K (A/H3N2) using the resistance RT-PCR assays.

In total, 129 respiratory specimens were tested positive for influenza A and 60 for influenza B virus. One sample was tested positive for both virus types. All pre-pandemic influenza A/H1N1 virus positive samples (n=81), from which a positive signal could be obtained, carried the H275Y mutation (n=73). Double negative results were obtained for 8 (10%) samples. For 6 of these samples, the viral load was less than 3.4$\log_{10}$vp/ml. The two remaining double negative samples, which contained 4.4 and 4.6$\log_{10}$vp/ml, were positive for the H275Y mutation when retested. Analysis of the influenza A/H3N2 viruses (n=48) using the E119V and R292K resistance assays did not reveal any viruses that carried an oseltamivir resistance mutation. In total, 8 (10%) double negative results were obtained using the E119V resistance assay. The viral loads of 5/8 these samples were below 3.9$\log_{10}$vp/ml. The R292K assay showed a 100% score when analyzing the same samples. No virus mixtures were found using the pre-pandemic H275Y, E119V or R292K resistance assay.

4 Discussion

In the present manuscript we describe the sensitivity and robustness of previously described RT-PCRs for the detection of influenza A and B virus. In addition, newly developed assays for the detection of wild type and mutant viruses at neuraminidase positions where the most commonly found oseltamivir resistance mutations occur in the H3N2 influenza subtype are described. Combined with previously described assays detecting antiviral resistance associated mutations in 2009 pandemic H1N1 virus, these assays are a powerful tool for the clinical management of influenza virus infected patients. Inconsistencies when taking a nose or throat swab may result in false negative results during screening and follow-up sampling of influenza virus infected patients. Quality of a swab procedure may be monitored by quantification of the number of human cells in a sample. Particular for individual patient management, it is therefore recommended to add an additional quantitative RT-PCR assay control, which targets a human housekeeping gene. This will allow quality confirmation of the tested sample. Given the robustness of these assays, they may also be used to analyze virological parameters in large patient populations in global clinical trials aimed at analyzing efficacy of antiviral compounds.

Real time RT-PCR, has proven to be sensitive and a good alternative for conventional diagnostic methods [22]. Comparing quantitative influenza RT-PCR data

between laboratories and studies is hampered by the lack of an international accepted standard [23]. Here we make use of two commercial available electron microscopy counted influenza virus stocks for calculation of a viral particle count instead of in-house quantified RNA or armed particle stocks. This would allow comparison of data generated in different laboratories. In addition, phocine distemper virus was used as a universal internal standard. By addition of PDV, the complete process from virus isolation to RT-PCR assays was monitored for each sample. By monitoring of Ct-values of internal and external quantifications standards for extended periods of time, longitudinal assay performance measures were obtained (Figure 4). This allows monitoring of potential assay drift by, for example degradation of primers, probes or standards, reduced fluorescence signals of the dyes or suboptimal assay kits.

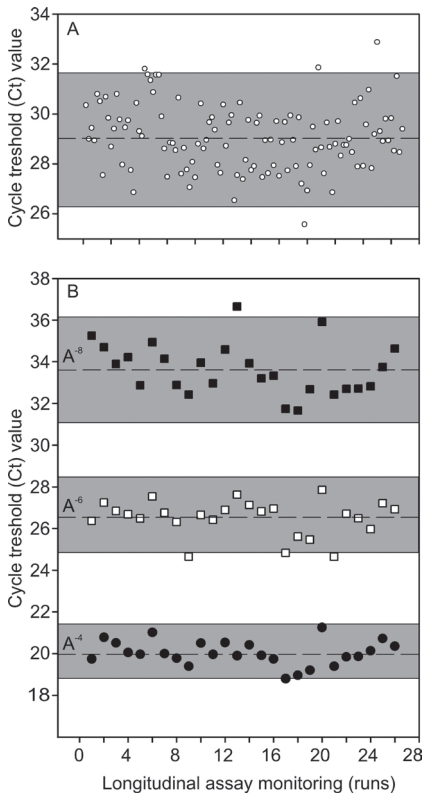

Figure 4 Longitudinal internal and external assay controls
In panel A, Ct-values of the universal internal standard (PDV) are displayed (white dots) obtained when analyzing 114 respiratory specimens isolated in 26 consecutive runs. In panel B, Ct-values of the high (A-4), mid range (A-6) and low (A-8) viral load external standards for influenza A virus are displayed. The grey bars indicate the 2 times the standard deviation range.

We determined the LLOQ of the assay to be 2.1 and 2.3log_{10}vp/ml for influenza A and B virus respectively (Figure2B). The difference between the LLOD of both targets is small (<0.5log_{10}), but may be explained by slightly lower RT-PCR efficiency of the influenza B RT-PCR reaction. Lower influenza B RT-PCR reaction performances were also observed when RNA run-off transcripts were targeted, which contained the influenza A and B target sequence placed in tandem on a single RNA copy. However, these small differences may also be explained by discrepancies between the expected and observed viral particle counts in the (diluted) EM stocks used as standards in this study.

Guidelines for determining the theoretical impact of mutations in the primers of real-time RT-PCRs are relatively well defined, in contrast to mutations in the probe region [21]. When testing the robustness of the quantification assay, primer mutations in the influenza A target sequence did not have a major impact on the RT-PCR efficiency and the LLOD. The hit-rate on virus dilutions with a low virus particle count was reduced when influenza B RNA was targeted. This may be explained by the reduced fluorescence signals, probably due a mismatch between the probe and the influenza B target sequence. Due to significant sequence variation in influenza A and B viruses, performance of virus detection and quantification assays need to be re-evaluated on a yearly basis. However, only 2 out of 11548 influenza A and 6 out of 758 selected influenza B sequences contained the profiles similar to the most dissimilar genotypes studied here.

Recently, several methods have been described for rapid detection of influenza viruses carrying antiviral resistance mutations. These methods include pyrosequencing [24], micro-array based techniques and several RT-PCR based methods [25-28]. The RT-PCR based assays described here showed comparable sensitivity and can be performed without the need of buying additional equipment.

For the evaluation of these assays, respiratory specimens were analyzed from patients (n=87) that were enrolled in a prospective study of influenza illness, including assessment of NAI resistance. All pre-pandemic influenza A/H1N1 viruses carried the H275Y oseltamivir resistance mutation. No influenza A/H3N2 viruses were found with an E119V or R292K oseltamivir resistance mutation and no virus mixtures were detected during the evaluation of the three resistance RT-PCR assays. In approximately 10% of the samples tested, the resistance RT-PCR assays obtained double negative results. This can be explained by the presence of mismatches in the primer and probe regions (a general limitation of genotypic screening for antiviral resistance mutations by RT-PCR) or by a higher LLOD of the H275Y and E119V resistance assays relative to the quantification assay. We could not determine whether these

negative results could be explained by nucleotide mismatches, because viral loads in these samples were too low to be propagated on MDCK cells (Ct-values higher than 32.0). However, up to 97% of the viruses could be genotyped at position H275Y, E119V or R292K by the resistance assays of the samples that were collected from patients early after onset of symptoms. The majority of these samples contain viral loads substantially higher above the LLOD. This suggests that an increased sensitivity of the resistance RT-PCR assays, would further improve performance of the resistance assays. In addition, for the H275Y and E119V resistance assay, the Gold assay kit was used. The EZ assay kit was used for the R292K resistance assay, which scored 100%. After the evaluation of the assays, all resistance assays were performed using the FVMM kit, which did not affect the specificity of the assays (data not shown). This resulted in a substantially decrease of the LLODs from 3.6 to 2.4$\log_{10}$vp/ml. In recent years, real-time RT-PCR based assays have become the standard in most diagnostic laboratories worldwide. The assays described here cover all currently circulating human influenza viruses and can detect major oseltamivir resistance mutations. By introducing external quantification and internal standards longitudinal assay performance can be monitored carefully and a virus particle count be assigned to an analyzed sample. This algorithm can generate useful data to assist in the management of individual influenza virus infected patients[15], and to evaluate clinical trials. Within one working day, information regarding influenza virus (sub) type, viral load and antiviral susceptibility can be obtained in parallel.

4.1 Acknowledgments

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Chapter 2.2

Evaluation of a rapid molecular algorithm for detection of pandemic influenza A (H1N1) 2009 virus and screening for a key oseltamivir resistance (H274Y) substitution in neuraminidase

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Abstract

Rapid and specific molecular tests for identification of the recently identified pandemic influenza A/H1N1 2009 virus as well as rapid molecular tests to identify antiviral resistant strains are urgently needed. We have evaluated the performance of two novel reverse transcriptase polymerase chain reactions (RT-PCRs) targeting specifically hemagglutinin and neuraminidase of pandemic influenza A/H1N1 virus in combination with a conserved matrix PCR. In addition, we investigated the performance of a novel discrimination RT-PCR for detection of the H274Y resistance mutation in the neuraminidase gene. Clinical performance of both subtype specific RT-PCR assays was evaluated through analysis of 684 throat swaps collected from individuals meeting the WHO case definition for the novel pandemic influenza virus. Analytical performance was analysed through testing of 10-fold serial dilutions of RNA derived from the first Dutch sequenced and cultured confirmed case of novel pandemic influenza infection. Specificity and discriminative capacities of the H274Y discrimination assay was performed by testing wild type and recombinant H274Y pandemic influenza. 121 throat swaps collected from April 2009 to July 2009 were positive by at least two out of three RT-PCRs, and negative for the seasonal H3/H1 subtype specific RT-PCR assays. 117 of these were tested positive for all three (Ct-values from 15.1 to 36.8). No oseltamivir resistance was detected. We present a sensitive and specific approach for detection of pandemic influenza A/H1N1 2009 and a rapid RT-PCR assay detecting a primary oseltamivir resistance mutation which can be incorporated easily into clinical virology algorithms.

1 Introduction

The early identified outbreak of the new variant influenza A/H1N1 virus or 'Mexican flu' in April 2009 has recently been declared the new influenza pandemic [1]. Many countries around the world have been stockpiling antiviral drugs for the treatment of infected patients and to minimize further spread of the virus during the first pandemic phase, the period when no appropriate vaccine against the pandemic influenza strain is available [2].

Rapid detection of the pandemic influenza A/H1N1 variants, distinguishing these strains from circulating epidemic strains and identification of antiviral resistant variants is important for pandemic surveillance and patient management. Currently, the pandemic influenza variant is naturally resistant to amantadine and rimantidine, but is still susceptible to the neuraminidase inhibitors (NAIs) oseltamivir (Tamiflu) and zanamivir (Relenza) [3].

Since the beginning of the seasonal influenza epidemic of 2007-2008, resistance of influenza A/H1N1 viruses to the antiviral drug oseltamivir has been a cause of concern. Of the circulating viruses that year, 16% were found to be oseltamivir resistant and early data from the southern hemisphere suggested up to 100% oseltamivir resistance for the 2008-2009 epidemic [4]. All of these viruses were found to be resistant by a histidine to tyrosine substitution in the viral neuraminidase at position 274 (residue 275 in the N1 gene). The same resistance mutation was found in viruses isolated from influenza A/H5N1 infected patients treated with oseltamivir [5]. As a consequence of massive use of NAIs during the pandemic, appearances of influenza viruses resistant to these drugs are to be expected [6,7].

The gold standards for antiviral resistance screening are phenotypic resistance assays [8], but these are time consuming and would include viral culturing of a pandemic strain. To date, NAIs resistance patterns of the pandemic influenza A/H1N1 virus are unknown. However, potential resistance patterns may occur in a similar way, since a structural comparison between the neuraminidase of the epidemic and pandemic influenza A/H1N1 show great similarities between the active sites of both enzymes [3].

A single nucleotide mutation (cytosine to thymine) at position 823 of the pandemic neuraminidase gene can result in a histidine to tyrosine substitution at position 274. Pyrosequencing, a method to detect single nucleotide polymorphisms (SNP), has become a well accepted method for sensitive screening of SNP resistant mutations as such [9]. However, as a result of extensive washing procedures with reverse transcriptase polymerase chain reaction (RT-PCR) generated amplicons

this technique may lead to false positive results. RT-PCR can be used for SNP analysis as well, by designing primers and probes that discrimination between wild type and mutant sequences. Excellent discrimination properties are achieved by incorporation of locked-nucleic acid (LNA) bases in these probes that increase melting temperatures, thus allowing the probe to be shorter with increased discriminative capacities [10]. We have developed and evaluated the performance of a novel RT-PCR assay targeting both the hemagglutinin and neuraminidase genes of the pandemic influenza A/H1N1 virus and a discrimination assay for detection of the H274Y oseltamivir resistance mutation in neuraminidase.

2 Materials and methods

2.1 Laboratory strains

Laboratory cultured influenza H1N1 virus A/NL/602/2009(v), propagated on Madin-Darby canine kidney (MDCK) cells to a titer of 6.31x107 TCID50/ml, was 10-fold serial diluted for positive control samples. For conversion of RT-PCR threshold cycle (Ct) values into a semi-quantitative viral particles count, dilutions of an electron microscopic counted influenza virus A/PR/8/34 stock (Advanced biosciences, Columbia, USA) were run in parallel. To obtain a pandemic influenza A/H1N1 neuraminidase target with a H274Y substitution in the neuraminidase, the influenza virus A/NL/602/2009 (Genbank accession number CY039528 for neuraminidase gene) was rescued as described previously [11]. In the neuraminidase gene of this clone a cytosine to thymine mutation was introduced at position 823 by site-directed mutagenesis (Quick-change, Stratagene, The Netherlands) and primer c823t_sense 5'-tcagtcgaaatgaatgcccctaattattactatgaggaatgc-3'.

2.2 Specimens

For the validation of both subtype specific RT-PCR assays, a total of 684 samples were used that were collected from suspected pandemic influenza infected individuals, according to the WHO case definition of a suspected novel pandemic influenza cases [12]. All samples were tested for influenza A with a slightly modified version of a previously described RT-PCR targeting a conserved region in the influenza matrix gene [13,14]. The neuraminidase genes of clinical isolates with viral loads higher than 1.0x103 viral particles per milliliter (vp/ml) were sequenced by conventional Sanger sequencing. Cross-reactivity of the RT-PCR assays were checked by analyzing 53 throat swaps that were tested positive for seasonal influenza A/H1N1 (35) or A/H3N2

(18) virus (season 2008-2009), 87 supernatants of cultured seasonal influenza A/H1N1 virus (season 2007-2008) and 19 respiratory samples tested negative for influenza A virus but positive for rhino- (13), adeno- (3) or coronavirus 229E (3). The samples were collected in The Netherlands, Norway, France, Poland and in the United States.

2.3 Assay design

To design primers and probes for the pandemic influenza A/H1N1 RT-PCR assays reference sequences of the hemagglutinin (HA) and neuraminidase (NA) that were deposited in the NCBI (National Center of Biotechnology Information: accessed May 2009) were aligned by clustalW. Primer Express 3.0 software (Applied biosystems, Nieuwerkerk a/d Ijssel, The Netherlands) was used to design primers and probes. The primers and probes for the H274Y discrimination assay were designed around nucleotide position 823 of the neuraminidase gene. Five LNA bases were incorporated in the probes allowing them to be shorter with increased discriminative capacities. The primers and probes are listed in table 1 (Eurogentec, Maastricht, The Netherlands).

2.4 H274Y discrimination RT-PCR

For the evaluation of the primers and probes for the H274Y discrimination assay, two plasmids pJET1.2-NA-H274H and pJET1.2-NA-H274Y were constructed containing neuraminidase regions 797-836 of influenza virus strain A/NL/602/2009(v). At position 823 of pJET1.2-NA-H274Y, the cytosine was replaced by a thymine, resulting in the mutant sequence. Run-off RNA transcripts were produced by XbaI (New England Biolabs, Westburg, Leusden, The Netherlands) linearization of the constructs and the T7 Ribomax express large scale RNA production system (Promega, Leiden, The Netherlands) according to the manufactures protocol.

2.5 Protocol

Nucleic acids were extracted from 350 µl of respiratory specimen. Nucleic acids were eluted to a volume of 110µl using the pathogen complex 200 protocol and the QiaSymphony machine (Qiagen, Venlo, The Netherlands) according to the instructions of the manufacturer. To monitor the whole process from isolation of nucleic acids up to RT-PCR, a universal internal control consisting of a seal distemper virus (PDV) preparation, was added to the original clinical sample [15]. The rtTH-based EZ RT-PCR kit (Applied biosystems, Nieuwerkerk a/d Ijssel, The Netherlands) was used for all RT-PCR assays, but with different cycle conditions. For the influenza

Table 1 Primers and probes used for the RT-PCR assays

Primers and probes	Sequence (5'-3')	Label (5'-/-3')	Position	References
Influenza A matrix RT-PCR				
InfA-sense-TM	aagaccaatcctgtcacctctga		144-166	[14]
InfA-antisense-TM	caaagcgtctacgctgcagtcc		217-238	[14]
InfA-probe-2	tttgtgttcacgctcaccgtgcc	6-FAM/BHQ-1	184-206	[13]
Pandemic influenza A/H1N1-H1 and A/H1N1-N1 2009 duplex RT-PCR				
panH1-forward	ggaaagaaatgctggatctggta		822-844	This study
panH1-reverse	atgggaggctggtgtttatagc		904-926	This study
panH1-probe	tgcaatacaacttgtcagacacccaaggg	Dragonfly/BHQ-2	874-902	This study
panN1-sense	acatgtgtgtgcagggataactg		865-887	This study
panN1-antisense	tccgaaaatcccactgcatat		949-969	This study
panN1-probe	atcgaccgtgggtgtctttcaacca	6-FAM/BHQ-1	899-923	This study
Pandemic influenza A/H1N1-N1 H274Y				
panN1-H275-sense	cagtcgaaatgaatgcccctaa		797-818	This study
panN1-H275-antisense	tgcacacacatgtgatttcactag		854-877	This study
panN1-275H-probe	ttaT<u>C</u>ActAtgAggaatg*	6-FAM/BHQ-1	819-837	This study
panN1-275Y-probe	ttaT<u>T</u>ActAtgAggaatg*	Dragonfly/BHQ-2	819-837	This study

* LNA nucleotides are denoted in upper case, DNA nucleotides are denoted in lower case and LNA nucleotide complementary to the predicted single nucleotide polymorphism (SNP) is underlined.

A matrix, pandemic H1 and N1 RT-PCRs the following amplification conditions were used: UNG reaction at 50°C for 2 min, cDNA reaction at 60°C for 30 min and PCR amplification at 95°C for 5 min, followed by 45 cycles at 95°C for 20s, 62°C for 1 min. The matrix RT-PCR included 40pmol forward, 30pmol reverse primer and 5pmol of the probe. For the pandemic H1 and N1 RT-PCR, 30pmol of the primers and 10pmol probes was used. The H274Y discrimination assay compromises 20pmol for forward and reverse primer and 5pmol for both probes and the thermal cycling profile was similar except for the elongation temperature which was 60°C instead of 62°C. All reactions were done in a total reaction volume of 50 µl, including 20 µl of nucleic acid. Amplification and detection was performed on a LightCycler 480 instrument using the FAM (465-510nm) and Dragonfly (533-580nm) filter (Roche, Almere, The Netherlands).

3 Results

3.1 Assay performance of subtype specific RT-PCR assay

Analytical sensitivity

Serial ten-fold dilutions of RNA from the pandemic influenza A/NL/602/2009 strain in RNAse-free water were used for determination of the linear range of the RT-PCR assays targeting hemagglutinin and neuraminidase genes. The approximated sensitivity of RT-PCR assays was determined to be 1.6x103 viral particles per milliliter (vp/ml) for the H1 (4/4 reactions detected) and 1.6x102 vp/ml for the N1 (4/4 reactions detected). Both assays were linear over a wide and clinical significant range with slopes of -3.54 (R2=0.99) and -3.28 (R2=0.99) for the H1 and N1 RT-PCR respectively (Figure 1).

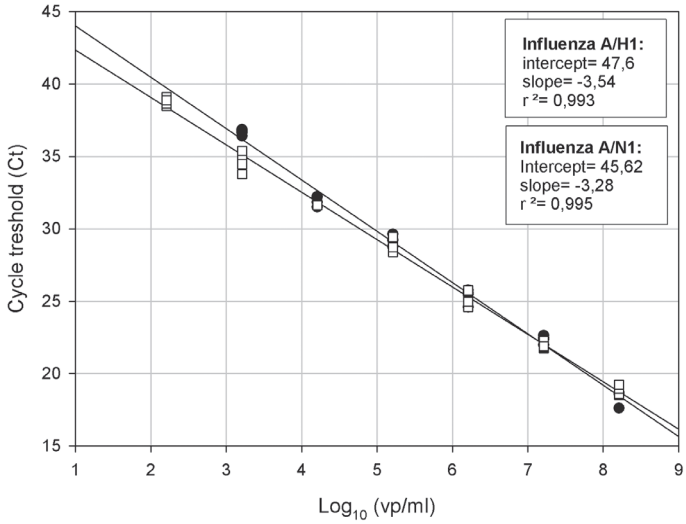

Figure 1. Linearity of the H1 and N1 subtype specific RT-PCRs for detection of pandemic influenza A/H1N1 (2009)

10-Fold serial dilutions of target RNA obtained from A/NL/602/2009(v) were analyzed by both sub type RT-PCRs in fourplex and plotted as Ct value versus the log of the calculated viral particles per milliliter in each dilution.

Clinical sensitivity

Analysis of 684 throat swaps collected from individuals suspected for infection with pandemic influenza revealed 121 pandemic influenza samples tested positive by at

Table 2 Performances of the influenza A matrix, pandemic influenza A/H1 and A/N1 RT-PCR assay

RT-PCR target(s)	Positive	Mean (Ct) [a]	Range (Ct)
Scores when Influenza A matrix positive			
H1 and N1	117	27.1	(15.1-36.8)
H1 (N1 negative)	1	37.3	
N1 (H1 negative)	1	37.7	
Scores when Influenza A matrix negative			
H1 and N1	2	37.5	(34.0-37.4)
N1 (H1 negative)	7	37.1	(34.5-39.0)
H1 (N1 negative)	5	35.9	(34.6-37.0)

[a] Ct-values from the influenza A matrix RT-PCR were taken when influenza A was positive. Samples were determined positive for novel pandemic influenza A/H1N1 2009 virus when either all three or two out of three RT-PCRs were positive.

least two out of three RT-PCR assays (Table 2). 14% of these samples were follow-up samples. In total, 117 samples were tested positive by the influenza A matrix (Ct-values from 15.1 - 36.8) and both sub-typing RT-PCR assays (96.7%). Samples that were not detected by all RT-PCRs all had high Ct-values, indicating low viral loads in these samples.

Specificity

A total of 159 pandemic virus negative samples were tested for cross-reactivity. These samples were tested positive for seasonal influenza A virus H1N1 (122), H3N2 (18) or were confirmed to contain at least one other respiratory virus (19). No cross-reactivity was observed when testing these samples.

3.2 Performance of pandemic influenza H274Y discrimination RT-PCR

Sensitivity

The assay sensitivity was tested using both RNA obtained from influenza A/NL/602/2009 virus as well as the H274Y recombinant. For both targets, the cut-off value was determined to be approximately 5.0x103 vp/ml (Ct~34).

Discrimination capacity

Serial ten-fold dilutions of in vitro transcribed RNA containing wild type or mutant sequence were analyzed using the H274Y discrimination RT-PCR assay. Two

differently labeled LNA probes are used in this multiplex assay which allows data acquisition in both FAM (465-510 nm) and Dragonfly (533-580 nm) filter. Only when wild type RNA was present fluorescence was detected in the FAM-filter and, visa versa, when mutant RNA was present only fluorescence emission was detected in the Dragonfly filter. No wild type or mutant fluorescence was emitted in the negative controls and the samples that were used to check for cross-reactivity. In mixtures of wild type H274 and mutant H274Y RNA, up to 5% of mutant virus could be detected in a reaction with a total input of 1.0x105 vp/ml. In addition, when analyzing 61 clinical samples from oseltamivir naïve pandemic influenza A/H1N1 infected patients, only the wild type genotype was found (Figure 2).

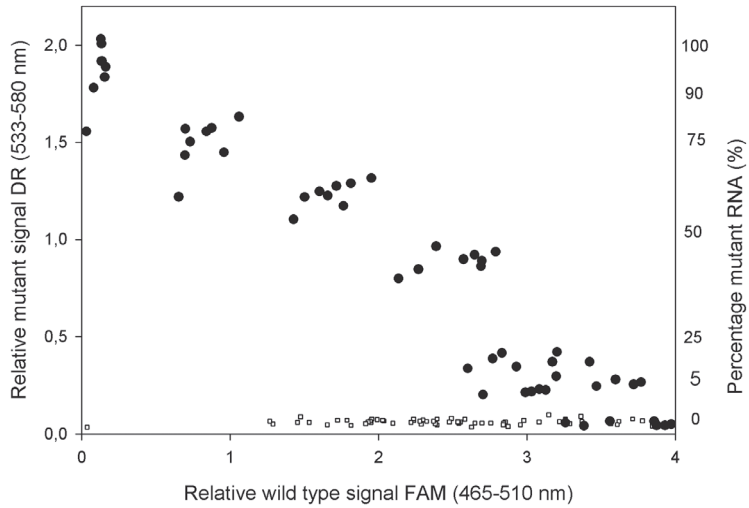

Figure 2. H275Y endpoint fluorescence scatter plot

Mixtures of in vitro transcribed wild type and mutant RNA (total input 1.0×105 vp/ml) were analyzed using the H275Y discrimination assay (black dots). Relative H275 wild type (465–533 nm) and 275Y mutant (533–580 nm) fluorescence emissions were plotted on the x-axis and y-axis, respectively. In addition, 61 clinical isolates from naïve pandemic influenza A/H1N1 (2009) infected patients were analyzed (white squares) to determine a threshold for detecting mutant genotypes in mixed virus populations (5%).

4 Discussion

Within the first months of the novel pandemic influenza outbreak antiviral drugs are widely used for the treatment of infected patients and to temper viral transmission. Currently, no vaccines are available and antiviral drugs are the only line of defense. Recently, a pandemic influenza sequence was uploaded to the influenza sequence database that encoded for a H274Y variant (Genbank accession no. GQ365445). It is still unclear whether this virus is oseltamivir resistant and can be transmitted, but since the global spread of oseltamivir resistance in the seasonal influenza A/H1N1 virus population, focus on this mutation is needed.

We have evaluated a novel RT-PCR assay for sub-typing the pandemic influenza A/H1N1 virus. Both RT-PCRs are sensitive, specific and can be incorporated directly into existing influenza detection and sub-typing algorithms. For validation of the H274Y discrimination assay, virus isolates were chosen that were derived from pandemic influenza A/H1N1 2009 infected patients before oseltamivir treatment. These patients respond well to oseltamivir treatment in general [16], therefore we assume that no H274Y minor variants were present in these samples. Taken these samples as a baseline, up to 5% for the H274Y oseltamivir resistant mutants could be detected in mixed virus populations using the H274Y discrimination assay (Figure 2).

A drawback of genotypic screening for antiviral resistance mutations by RT-PCR is the potential appearances of unwanted mismatches in the primer and probe regions, due to antigenic drift. For diagnostic purposes, the discrimination RT-PCR assays should therefore be performed in parallel with influenza RT-PCR assays targeting more conserved regions of the influenza viral genome.

In conclusion, the sub-typing RT-PCR assays described appear sensitive and specific in this validation pilot. Using the H274Y discrimination RT-PCR, pandemic influenza A/H1N1 viruses can be screened for oseltamivir susceptibility within 4-6 hours time, which makes it useful for surveillance and valuable in clinical patient management.

4.1 Acknowledgments

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Chapter 3.1

Fatal oseltamivir-resistant influenza virus infection

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1 Case report

The incidence of influenza A (H1N1) viruses that carry the neuraminidase H274Y mutation has increased by 30% this year in the Netherlands [1]. Influenza A (H1N1) viruses that carry this mutation are resistant to oseltamivir but remain sensitive to zanamivir [2]. However, these mutant viruses are considered to have attenuated pathogenicity [3,4].

A 67-year-old man who had received a diagnosis of chronic lymphocytic leukemia 3 years earlier was admitted to the hospital because of dyspnea, dry cough, and fever. One week before admission, he had received a course of cyclophosphamide, vincristine, and prednisolone chemotherapy. At admission, his white-cell count was 137,000 per cubic millimeter, with 99% lymphocytes and no neutrophils. Because of acute respiratory failure, empirical antibacterial therapy was initiated, and mechanical ventilation was required by the second hospital day. Computed tomography (CT) revealed patchy infiltrates in both lungs, and influenza A (H1N1) virus was detected in respiratory secretions. During the entire hospital course, no other respiratory pathogens were detected in bronchoalveolar-lavage specimens. The only other pathogens identified in blood cultures were *Candida albicans* and *Enterococcus faecium,* for which fluconazole and vancomycin were given.

Oseltamivir was administered for the influenza virus infection, beginning on the sixth hospital day, but it was discontinued on day 13 because sequence analysis revealed the H274Y mutation, and no decrease in the viral load was observed. In retrospect, the H274Y mutation was present in the specimen obtained before oseltamivir therapy was initiated. The patient's hospital record and his family indicated that he had had no contact with patients who had received oseltamivir. On day 15, amantadine was added to the patient's treatment regimen. Four days later, the neutrophil count increased, indicating bone marrow recovery. Mechanical ventilation was discontinued on day 20, and zanamivir by inhalation was initiated. However, respiratory failure occurred on day 22, mechanical ventilation was reinstituted, and therapy with zanamivir was discontinued. On day 26, the influenza virus was no longer detectable. Because sequence analyses showed an amantadine-resistance mutation in the viral M2 protein (L26F) and zanamivir therapy had been limited to three doses, clearance of the virus was probably due to recovery of the immune system. A second CT scan, obtained on day 28, revealed progression of the pulmonary infiltrates. Because of the poor prognosis, mechanical ventilation was discontinued on day 34. The patient died 3 days later.

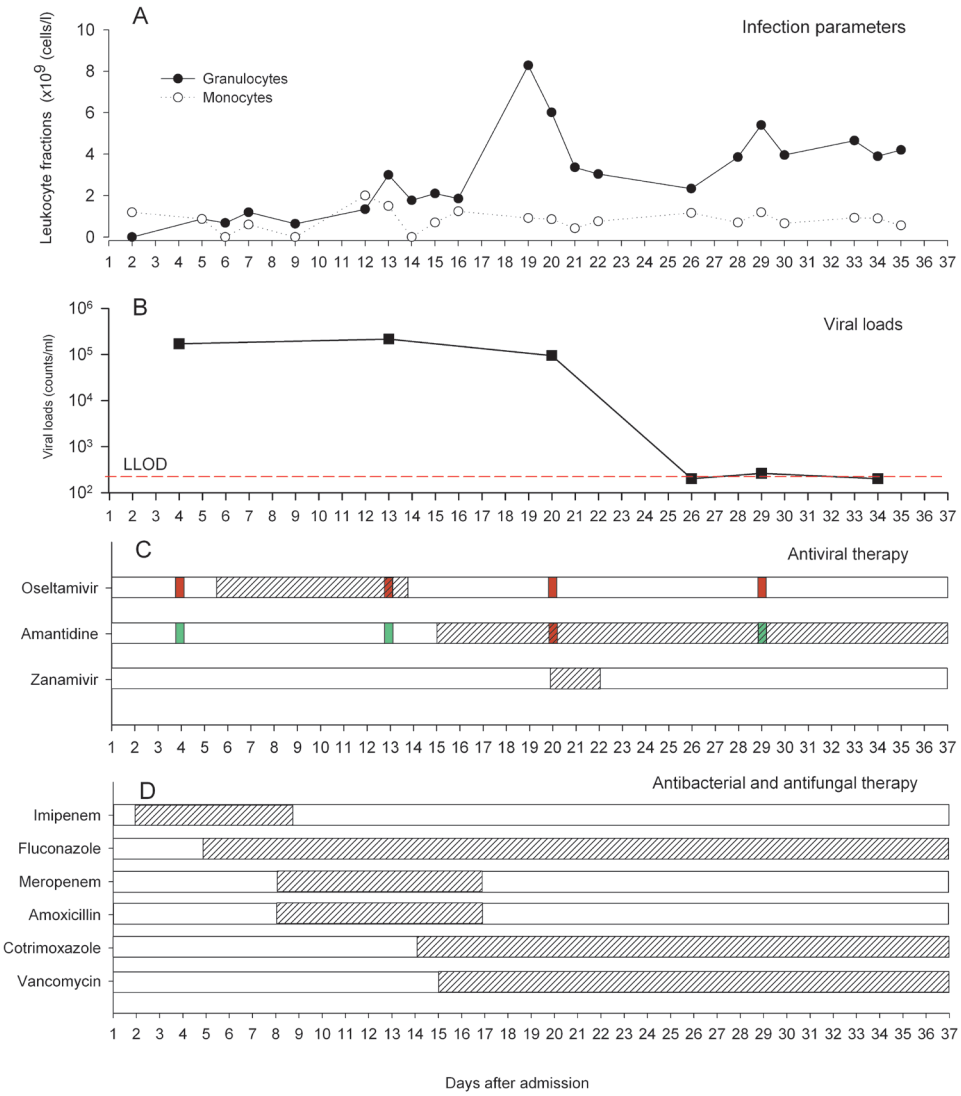

Figure 1 Leukocyte Counts, Viral Loads, and Treatment during the Hospital Course in a Patient Infected with Influenza A (H1N1) Virus with the H274Y Mutation

Panel A shows the patient's granulocyte and monocyte counts. The gradual increase in the granulocyte count was consistent with bone marrow recovery. Panel B shows the viral load in the respiratory specimens. The dashed red line indicates the lower limit of detection. Various therapeutic and empirical antiviral therapies, shown in Panel C, and antibacterial and antifungal therapies, shown in Panel D, were given to the patient at different intervals (shaded bars). The red portions of the bars in Panel C indicate detection of resistance mutations for either oseltamivir (neuraminidase H274Y) or amantidine (M2-channel L26F), and the blue boxes indicate detection of the wild-type genotype. The L26F resistance mutation in the M2 protein was detected only on day 20, whereas the H274Y mutation was present before and after oseltamivir was administered.

It has been suggested that the H274Y mutation, which confers resistance to oseltamivir, leaves the influenza A (H1N1) virus severely compromised [REFS 3,4]. However, the case we describe suggests that this oseltamivir-resistant virus can be pathogenic, at least in an immunocompromised patient.

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Chapter 3.2

Evaluation of the Antiviral Response to Zanamivir Administered Intravenously for Treatment of Critically Ill Patients With Pandemic Influenza A (H1N1) Infection

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Abstract

A retrospective nationwide study on the use of intravenous (IV) zanamivir in patients receiving intensive care who were pretreated with oseltamivir in the Netherlands was performed. In 6 of 13 patients with a sustained reduction of the viral load, the median time to start IV zanamivir was 9 days (range, 4–11 days) compared with 14 days (range, 6–21 days) in 7 patients without viral load reduction (P = .052). Viral load response did not influence mortality. We conclude that IV zanamivir as late add-on therapy has limited effectiveness. The effect of an immediate start with IV zanamivir monotherapy or in combination with other drugs need to be evaluated.

1 Introduction

During last year's influenza pandemic, life-threatening disease was encountered in a proportion of patients [1-4]. Despite extensive treatment, the mortality rate among these patients remained high [1]. One of the problems encountered was the development of resistance to oseltamivir. In addition, in isolated cases, oseltamivir use was complicated due to absent gastric mobility [5]. Currently, zanamivir is the alternative drug of choice to use when oseltamivir-resistant viruses develop. However, at this moment it is only approved for use as an inhaled formulation. Respiratory problems following inhalation, dysfunction of ventilators due to blockade of the filters by the lactose carrier, and suboptimal drug disposition are issues associated with its formulation [6]. The use of intravenous (IV) zanamivir may be an option to overcome these problems. Currently, only limited peer-reviewed data in humans and one study in primates have been published [7-10]. We here report a retrospective observational nationwide study in the Netherlands on the antiviral efficacy of IV zanamivir.

2 Methods

Patients included in this study were identified, retrospectively, through a request to all registered medical microbiologists in the Netherlands. A standardized form was provided to collect relevant clinical and virological data. All patient data were reported anonymously. The decision to start IV zanamivir and the duration of therapy were not standardized and depended on the clinical judgment of the treating physician. Initially, compassionate use was granted by GlaxoSmithKline; from January 2010 onward, this was continued by the National Institute for Public Health and the Environment. Intravenous zanamivir was provided for patients unable to use oseltamivir or in whom treatment with IV zanamivir was considered desirable for other reasons. Nasopharyngeal swabs, nose washes (upper respiratory tract [URT] samples), tracheal aspirates, and broncheo-alveolar lavages (lower respiratory tract [LRT] samples) were collected during routine care.

Inclusion criteria for patients in this study were as follows: laboratory-confirmed pandemic influenza A (H1N1) infection by real-time reverse-transcription polymerase chain reaction (RT-PCR); admission to an intensive care unit (ICU) in a Dutch hospital; use of IV zanamivir for >48 hours; and availability of follow-up virological samples. Patients with proven resistance to zanamivir at baseline or at ICU admittance were excluded from the study.

Detection and quantification of viral RNA and screening for the neuraminidase (NA) H275Y oseltamivir resistance mutation was performed in all centers by means of standardized RT-PCR–based methods. To obtain sequences of NA genes, viral RNA was reverse transcribed and subsequently amplified using gene-specific primers (sequences are available upon request). Attempts to culture virus were made at sample viral loads of >4 × 103 virus particles (vp) per milliliter. Phenotypic sensitivity to oseltamivir and zanamivir was measured using the NA-star neuraminidase inhibitor resistance detection kit (Applied Biosystems, Nieuwerkerk aan den IJsel, the Netherlands). Clinical data were extracted from the medical records. Immunosuppression was defined as any of the following: receipt of treatment for any cancer within 6 months before influenza infection, the use of any immunosuppressive medication to prevent transplant rejection or for management of pulmonary or autoimmune conditions, or a diagnosis of AIDS. Acquired respiratory distress syndrome (ARDS) was defined as bilateral infiltrates on chest radiographs, with a Pa O2/F IO2 ratio of <200 mm Hg in the absence of cardiogenic pulmonary edema.

The viral response to treatment with IV zanamivir was determined by the differences in viral load between a sample collected closest to the day of the start of IV zanamivir (baseline sample) and those obtained at follow-up. Sustained reduction of the viral load was defined as a decrease of ≥1 log10 vp/mL for at least 10 days. In case of death or cessation of IV zanamivir treatment, the last sample collected while the patient was still receiving therapy was considered for the endpoint of analysis. If the reduction of the viral load took >7 days of treatment to occur, it was considered not to be treatment related. For statistical analysis between groups, the Mann-Whitney U test was used. P values of <.05 were considered to be significant. The study protocol was reviewed and approved by the medical ethics board of the Erasmus University Medical Center (study no. 2010-162). Informed consent was waived because patient inclusion was performed retrospectively and anonymously. There was no sponsor involvement in the design, data collection, and analysis of this study.

3 Results

Based on data provided by GlaxoSmithKline during the influenza season in the Netherlands, 26 ICU patients were treated with IV zanamivir. Overall, we obtained clinical data of 19 patients from 6 hospitals. Of these 19 patients, 6 did not meet the inclusion criteria. Reasons for exclusion from the study were death (n = 2), recovery and treatment interruption within 48 hours after start of IV zanamivir (n = 2), absence of samples for virological evaluation (n = 1), and proven resistance to zanamivir before admittance to the ICU (n = 1). The patient characteristics are summarized in table 1.

Table 1

Patient	Underlying disease	Age (years)	Immune compromised	ARDS	Oseltamivir Pretreatment (days)	Viral resistance to oseltamivir 275	Viral resistance to oseltamivir IC_{50} (nM)	Baseline viral load URT (vp/ml)	Baseline viral load LRT (vp/ml)	Days to zanamivir (days)	Susceptibility for ZA IC_{50} (nM)	Zanamivir One log Response	Outcome
1.	None	46	No	Yes	4	H	0.4 (S)	ND	4.9E+3	12	0.1 (S)	No	Survived
2.	Cerebral vasculitis, pulmonary embolisms long term high dosage prednisone use	41	Yes	Yes	14	H	NR	1.0E+05	-	14	NR	No	Died
3.	Chronic myeloid leukaemia, bone marrow transplantation, graft vs. host disease, clinical significant Aortic valve stenosis	58	Yes	Yes	18 + ZA inh (9d)	H	132.8(R)	4.0 E+4	3.76E+5	19	0.2 (S)	No	Died
4.	None	14	No	Yes	1	H	0.2 (S)	2.0E+05	9,8E+06	4	0.2 (S)	Yes	Died
5.	Down syndrome, acute lymphoblastic leukaemia	5	Yes	Yes	5	H	0.4 (S)	1.0E+04	ND	14	0.1 (S)	No	Survived
6.	Follicular lymphoma	59	Yes	Yes	7	H/Y*	7.1 (R)*	1.2E+08	-	11	0.4 (S)	Yes	Died
7.	Minimal change nephropathy, prednisone use	39	Yes	Yes	7	Y	NR	-	9.0E+03	9	NR	Yes	Survived
8.	Obesity	42	No	Yes	4	H	0.4 (S)	4.3E+04	6.5 E+05	6	0.1 (S)	No	Survived
9.	Obesity, ovarian adenoma (identified during hospital admission)	30	No	Yes	6	NR	NR	4.5E+2	-	9	NR	Yes	Survived
10.	Systemic lupus erythematosis	40	Yes	Yes	3	H	0.3 (S)	-	3.5E+6	10	0.2 (S)	Yes	Died
11.	Acute myeloid leukaemia	49	Yes	Yes	6	H	0.3 (S)	3E+04	-	10	0.3 (S)	No	Died
12.	Alcohol abuse	52	No	Yes	1	NR	NR	ND	9.1E+05	21	NR	No	Survived
13.	Biliary atresia, hepatocellular carcinoma, liver transplantation	1	Yes	No	9	Y	NR	3.0E+4	-	11	0.2 (S)	Yes	Survived

NOTE. Reference median inhibitory concentration (IC_{50}) values (n = 60) for oseltamivir are 0.20 ± 0.08 nmol/L (median +/– 95% CI) and for zanamivir are 0.22 ± 0.12 nmol/L. ARDS, acquired respiratory distress syndrome; H, histidine; IC_{50}, median inhibitory concentration; LRT, lower respiratory tract; NA, neuraminidase; ND, not detectable; NR, no result could be obtained; R, resistant; S, susceptible; URT, upper respiratory tract; vp, virus particles; Y, tyrosine.

[a]Plus inhaled zanamivir therapy for 9 days.

[b]Virus mixture of wild type and mutants with an H275Y mutation.

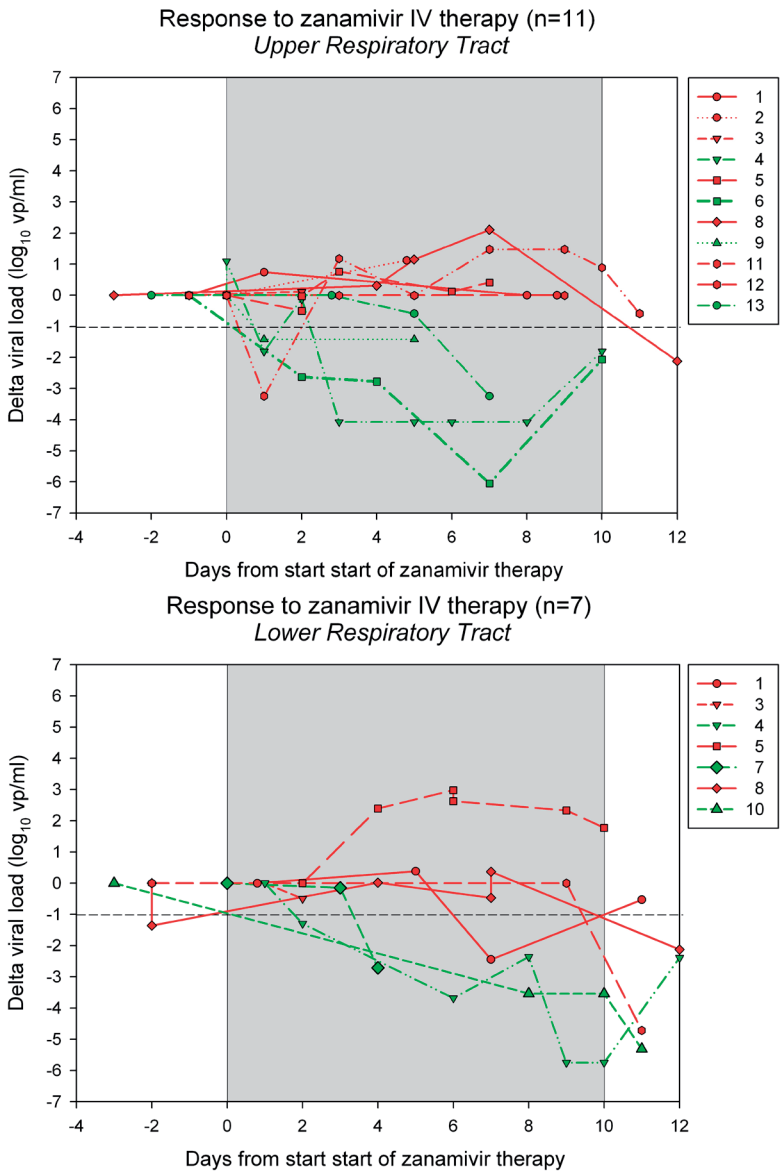

Figure 1 Virological response to intravenous (IV) zanamivir in 13 patients

A. Change in baseline viral load detected in the upper respiratory tract (URT); B. Change in baseline viral load detected in the lower respiratory tract (LRT). Eight patients were treated for >10 days, and 5 patients were treated for <10 days. Of those 5 patients, 2 died and 3 clinically recovered, and zanamivir IV medication was stopped (patients 7, 9, and 13). Because patient 12 had undetectable viral loads in the URT sample, these data are not included in the figure for change in baseline URT viral load. Each line represents a single patient. Data obtained from patients with a sustained viral load reduction are depicted in green, and data for those without a viral response are depicted in red; the black dashed line represents the delta viral load of –1 $\log_{10}$ virus particles (vp) per milliliter.

Most patients had a pre-existing medical condition and were immunocompromised. All patients were pretreated with oral oseltamivir for a median period of 5 days (range, 1–18 days). Oseltamivir was dosed at 75 mg every 12 hours or its pediatric equivalent, except in 3 patients (patients 4, 5, and 8) in whom 150 mg every 12 hours was used. Three patients received IV zanamivir monotherapy (patients 7, 9, and 13), whereas the remaining 10 patients received IV zanamivir combined with continued use of oseltamivir. No other antiviral therapy was prescribed.

The median baseline viral load was 3 × 104 vp/mL, (range, <17–1.2 × 108 vp/mL) in the URT samples and 2.3 × 105 vp/mL (range, <17–9.8 × 106 vp/mL) in the LRT samples. Two patients (patients 1 and 12) had an undetectable viral load in the URT sample but a high viral load in the LRT sample at baseline. One patient (patient 5) had an undetectable viral load in the LRT sample but a high viral load in the URT sample at baseline. The response analysis was complicated for 2 patients because their baseline samples were obtained 3 days before IV zanamivir initiation (patients 8 and 10). Overall, sustained reduction of the viral load was observed in only 6 patients (Figure 1).

Three patients had persistent undetectable viral loads after 7 days of treatment (patients 7, 9, and 13). There were no significant differences between baseline viral loads in the URT and LRT samples of patients with and without viral load reduction (P = .315 and P = .250, respectively). Overall, the median interval between first onset of symptoms and start of IV zanamivir was 11 days (range, 4–21 days). However, among patients who had a sustained reduction of the viral load, the median time to start of IV zanamivir was 9 days (range, 4–11 days), compared with 14 days (range, 6–21 days) among patients in whom treatment had no effect (P = .05). All 3 patients receiving monotherapy with IV zanamivir showed a sustained viral load response.

A sequence could be obtained from viruses isolated from 10 patients before start of IV zanamivir, but sequences could not be obtained from viruses isolated from patients 7, 9, and 12. Viruses isolated from patients 3, 6, 7, and 13 carried the H275Y oseltamivir resistance mutation in NA as determined by sequencing and/or RT-PCR specific for the H275Y mutation. No other mutations associated with drug resistance were found in the NA genes. For 9 patients, phenotypic resistance data could be measured in isolates obtained before start of IV zanamivir. All 9 isolates were susceptible to zanamivir (Table 1). Virus could not be cultured from all 19 samples eligible for culture that were obtained after the start of IV zanamivir, despite relatively high loads with a median of 4.0 × 105 vp/mL (range, 4.9 × 103–14 × 105 vp/mL).

Twelve patients were admitted to the ICU because of respiratory failure; 1 patient was already admitted. All patients needed supplemental oxygen: 12 patients were mechanically ventilated and 4 patients required additional extracorporeal membrane oxygenation support. In all mechanically ventilated patients, ARDS was diagnosed. The overall mortality was significant, with a total number of 6 deaths. There was no relation between viral response and survival, with death occurring in 3 of 6 patients in the group with sustained viral load reduction and in 3 of 7 patients in the non-responder group. Most deaths (5 of 6) occurred in patients with an impaired immune system. The remaining 7 surviving patients were discharged after a median hospital stay of 52 days (range, 11–62 days), of which 37 days (range, 39–77 days) were spent in the ICU. All patients received broad-spectrum antibiotics at least once. In none of the patients was IV zanamivir stopped because of serious adverse events.

4 Discussion

In this study, which is to our knowledge the largest published peer-reviewed case series to date, we have retrospectively analyzed the virological response to IV zanamivir treatment in patients pretreated with oral oseltamivir for severe influenza infection. A consistent virological response to add-on IV therapy was seen in only 6 of 13 patients. There was a trend toward an increased likelihood of response for those who started IV zanamivir at an earlier stage of disease.

This study has several limitations. Due to the retrospective nature, the absence of a control group, and the limited number of patients enrolled, we cannot make a definitive conclusion on the clinical efficacy of IV zanamivir. Still, we feel that our observations are important, because they are not in line with previously published reports on individual patients with encouraging results [7-9,11]. Several factors may explain the limited effectiveness of IV zanamivir seen in 7 of 13 patients. First, the patients included were severely ill as reflected by the high number of immunocompromised patients, poor response to oral oseltamivir, and extended duration of hospitalization. A potential explanation for the limited virological efficacy may have been the development of viruses resistant to zanamivir [12]. Unfortunately, because we were unable to obtain viral isolates or relevant viral sequences after start of zanamivir therapy, it remains unknown to what extent resistance contributed to the absence of virological response. Another explanation for the absence of response could be that insufficient zanamivir levels were achieved at some sites of active viral replication. Further detailed pharmacological studies are

warranted to investigate this possibility. Of interest is a recent finding in patients with influenza A (H3N2) infection that the combination of oseltamivir and zanamivir may be associated with a decreased inhibition of viral replication. However, in that study all patients did clear the virus rapidly [13]. In this light, it is important to note that all patients described in our study had been pretreated with oseltamivir monotherapy. Resistance to oseltamivir was found in only 4 of them. Therefore, we hypothesize that the same mechanism accounts for the limited efficacy of both IV zanamivir and oral oseltamivir.

Because of the prolonged viral replication before the start of IV zanamivir, irreversible lung injury may already have occurred. Thus, the relatively late start with IV zanamivir in the course of disease may have compromised its potential efficacy. This is suggested by the shorter intervals between illness onset and initiation of IV zanamivir in patients with a sustained suppression of the viral load. The extensive damage to the lungs may have resulted in viral salvage sites inaccessible to the immune system and antiviral medication. Earlier start of IV zanamivir may help to prevent formation of these sites. Indeed, previously published data show that early start with neuraminidase inhibitors results in a better outcome in hospitalized patients [14]. Finally, it is well recognized that bacterial superinfections may also contribute to clinical deterioration; we cannot rule out the possibility that this has also occurred in our population, despite the fact that all of our patients received antibiotic therapy.

In conclusion, the use IV zanamivir as late add-on therapy for H1N1 virus–infected patients in the ICU showed limited antiviral effectiveness. In our selected population, reduction of viral load did not affect mortality. Further studies evaluating earlier start with IV zanamivir monotherapy therapy alone or in combination with other treatment strategies are needed.

4.1 Acknowledgments

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4.2 Footnotes

Potential conflicts of interest: P. F., A. O., and C. B. participate in the Influenza Resistance Information Study (IRIS) trial sponsored by Hoffmann–La Roche. A. O. is a part-time employee of Viroclinics Biosciences Besloten Vennootschap and performs

contact research for pharmaceutical companies. All other authors: no conflicts. Presented in part: Pandemic Influenza in the Netherlands evaluation meeting at the RIVM, Bilthoven, the Netherlands, 9 December 2010.

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Chapter 3.3

Emergence of a Multidrug-Resistant Pandemic Influenza A (H1N1) Virus

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1 Case Report

Since the outbreak of influenza A (H1N1) virus pandemic, almost 300 cases of infection with an oseltamivir-resistant influenza virus have been reported to the World Health Organization as of June 2010 [1]. These strains typically contain a single histidine-to-tyrosine substitution at position 275 (H275Y) of the viral neuraminidase, but they remain susceptible to zanamivir. Successful clearance of an oseltamivir-resistant virus with zanamivir, the other approved neuraminidase inhibitor, has been reported [2]. We report the emergence of a pandemic influenza virus in a patient treated with neuraminidase inhibitors, with a novel resistance pattern that conferred resistance to oseltamivir, zanamivir, and peramivir.

On November 11, 2009, a 5-year-old boy with high-risk acute lymphoblastic leukemia (undergoing preparation for hematologic stem-cell transplantation) was admitted to the hospital because of influenza-like symptoms. Pandemic influenza A (H1N1) virus was detected in a respiratory specimen by real-time–polymerase-chain-reaction assay [3]. Treatment with broad-spectrum antibiotics and oseltamivir (45 mg twice daily [pediatric dose]) was initiated and continued during conditioning for transplantation. Consequently, influenza virus was not detected in two consecutive respiratory specimens, and allogeneic transplantation was performed on November 23. On December 2, however, an increased influenza viral load was detected, and since the H275Y mutation was present in a specimen from November 23 [3], intravenous zanamivir therapy (20 mg per kilogram of body weight twice daily) was started on December 3 under an emergency investigational-new-drug protocol. The viral load decreased (to the lower limit of assay detection), and the patient recovered clinically; he was discharged home on December 23. On January 11, 2010, he was readmitted with signs of a lower respiratory tract infection, and intravenous zanamivir therapy was reinitiated. In addition to influenza virus, cytomegalovirus DNA was detected in the blood (for which ganciclovir was administered), and rhinovirus was detected in respiratory specimens. Unfortunately, the patient's clinical situation worsened. The acute respiratory distress syndrome developed, and he ultimately died.

Because influenza virus persisted, further resistance analyses were performed retrospectively. Sequencing of the neuraminidase gene of a virus isolated on January 4 revealed a novel isoleucine-to-arginine amino acid substitution at residue 223 (I223R). This residue has been linked to oseltamivir resistance for another influenza subtype (H3N2) [4]. Subsequent in vitro phenotypic testing of the virus showed that the 50% inhibitory concentration for the drugs taken by this patient (oseltamivir and zanamivir) was increased by a factor of 45 and 10, respectively. Moreover, when

we tested the sensitivity to peramivir, a novel neuraminidase inhibitor in clinical development, we observed an increase in the 50% inhibitory concentration by a factor of seven.

The absence of an adequate immune response and possibly suboptimal drug levels may have contributed to the emergence of the I223R mutant. Further research is warranted to determine the clinical and epidemiologic consequences of mutants with reduced sensitivity to neuraminidase inhibitors, since they leave physicians with limited treatment options.

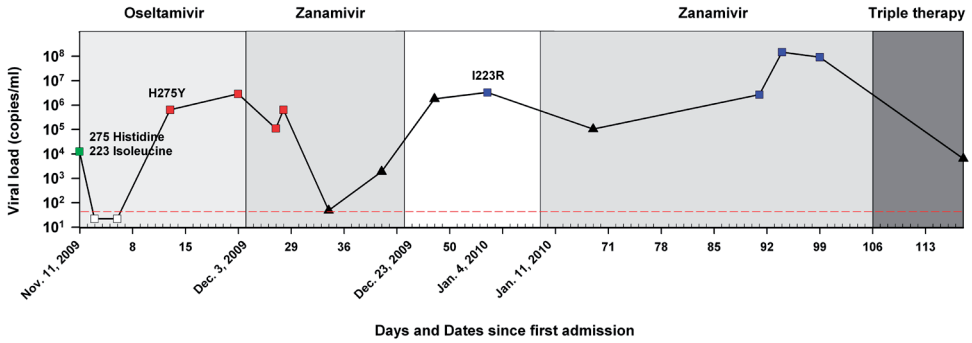

Figure 1 Viral Load, Antiviral Therapy, and Resistance Detection during Hospitalization of a Patient Infected with Pandemic Influenza A (H1N1) Virus

The viral load in respiratory specimens obtained during the course of the illness is shown. Colored squares indicate the absence of resistance mutations (green) or the H275Y (red) or I223R (blue) mutation, as detected by either real-time–polymerase-chain-reaction (RT-PCR) assay3 (H275Y) or conventional Sanger sequencing (I223R). White squares indicate virus-negative samples. Triangles indicate samples from which no neuraminidase sequence could be obtained. The dashed red line indicates the lower limit of detection of the influenza A semiquantitative RT-PCR assay. The duration of oseltamivir monotherapy and zanamivir monotherapy is indicated by light-gray and dark-gray shading, respectively, and the duration of triple therapy, which also included ribavirin, is indicated by hatching. Phenotypic resistance analysis (NA-Star detection kit, Applied Biosystems) revealed that the mean (±SD) 50% inhibitory concentrations (IC50) for oseltamivir, zanamivir, and peramivir were 108±12, 0.5±0.04, and 8.4±0.9 nM, respectively, for the H275Y mutation and 9.1±1.2, 2.2±0.1, and 0.7±0.02 nM, respectively, for the I223R mutation. The degree of resistance was determined by comparing the IC50 values for the mutants with those for 60 wild-type viral isolates (IC50 for oseltamivir, zanamivir, and peramivir, 0.20±0.08, 0.22±0.12, and 0.10±0.02 nM, respectively) from patients infected with pandemic influenza A virus who were not treated with neuraminidase inhibitors.

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Chapter 4

H1N1 2009 Pandemic Influenza Virus: Resistance of the I223R Neuraminidase Mutant Explained by Kinetic and Structural Analysis

Resistance mechanism of the I223R change

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Abstract

Two classes of antiviral drugs, neuraminidase inhibitors and adamantanes, are approved for prophylaxis and therapy against influenza virus infections. A major concern is that antiviral resistant viruses emerge and spread in the human population. The 2009 pandemic H1N1 virus is already resistant to adamantanes. Recently, a novel neuraminidase inhibitor resistance mutation I223R was identified in the neuraminidase of this subtype. To understand the resistance mechanism of this mutation, the enzymatic properties of the I223R mutant, together with the most frequently observed resistance mutation, H275Y, and the double mutant I223R/H275Y were compared. Relative to wild type, K_M values for MUNANA increased only 2-fold for the single I223R mutant and up to 8-fold for the double mutant. Oseltamivir inhibition constants (K_I) increased 48-fold in the single I223R mutant and 7500-fold in the double mutant. In both cases the change was largely accounted for by an increased dissociation rate constant for oseltamivir, but the inhibition constants for zanamivir were less increased. We have used X-ray crystallography to better understand the effect of mutation I223R on drug binding. We find that there is shrinkage of a hydrophobic pocket in the active site as a result of the I223R change. Furthermore, R223 interacts with S247 which changes the rotamer it adopts and, consequently, binding of the pentoxyl substituent of oseltamivir is not as favorable as in the wild type. However, the polar glycerol substituent present in zanamivir, which mimics the natural substrate, is accommodated in the I223R mutant structure in a similar way to wild type, thus explaining the kinetic data. Our structural data also show that, in contrast to a recently reported structure, the active site of 2009 pandemic neuraminidase can adopt an open conformation.

Author summary

Recently, a pandemic A/H1N1 influenza virus was isolated from an immune compromised patient with a novel antiviral resistance pattern to the neuraminidase inhibitor class of drugs. This virus had an amino acid change in the viral neuraminidase enzyme; an isoleucine at position 223 was substituted by an arginine (I223R). Patients infected with such a virus leave physicians with reduced antiviral treatment options, since pandemic viruses are naturally resistant to the other class of antivirals, the adamantanes. Previously, we have shown that this mutant virus retains its potential to cause disease and may still be able to spread in the human population.

Here we used enzyme kinetic measurements and crystal structures of the I223R mutant neuraminidase to determine the resistance mechanism of this amino acid change. We found that the I223R change results in shrinkage of the active site of the enzyme. As a result, binding of the neuraminidase inhibitors is affected. In addition, we found that the active site of our pandemic neuraminidase structure, crystallized in absence of inhibitor, has an extra cavity (150-cavity) adjacent to the active site. Our study could aid in the development of novel inhibitors designed to target the 150-cavity and active site of the enzyme.

1 Introduction

Strategies to combat the burden of disease caused by influenza mainly rely on vaccination [1]. However, in cases when vaccine efficacy is low or vaccine is unavailable, for instance during the first months of a pandemic, antiviral drugs are an important line of defense. For individual patients, especially when the immune system is compromised, antiviral therapy may be life saving [2]. Traditionally, both the adamantane and neuraminidase inhibitor class of drugs have been available for prophylaxis and therapy. However, the majority of recently circulating influenza viruses are resistant to the adamantanes, including the A/H1N1 2009 pandemic influenza virus [3]. This leaves neuraminidase inhibitors (NAIs) as the only option.

Stimulated by the first neuraminidase (NA) crystal structures, the NAIs were designed to interact with the active site of all NA types [4,5]. Because the NA active site is highly conserved, resistant mutants with amino acid changes in the proximity of this active site, were considered likely to be enzymatically compromised and thus predicted to be of marginal epidemiological and clinical significance [6]. Indeed, the first viruses identified as harboring NAI resistant mutations were compromised in their replicative capacity and transmissibility [7,8,9]. Nevertheless, a novel oseltamivir-resistant influenza A/H1N1 variant emerged in the 2007-2008 influenza season and became the dominant virus in some parts of the world [10,11]. Resistance to oseltamivir was caused by a previously identified, and frequently observed, histidine to tyrosine change in the NA at position 275 (H275Y). In contrast to earlier observations on H275Y mutants, this change did not compromise viral replication or transmissibility in the background of the 2007-2008 H1N1 virus [12,13,14]. Additional mutations in the NA were identified that explained why this resistant virus was able to emerge and become widespread [15,16].

With the 2009 pandemic, a novel influenza A/H1N1 virus was introduced into the human population [17]. Given that the appearance of NAI drug resistant mutations varies with the type and structure of the neuraminidase that the virus carries, we were interested to identify and characterize novel patterns of resistance in this virus. We identified a novel isoleucine to arginine change at position 223 (I223R) in the NA of a virus isolated from an immune suppressed child on prolonged oseltamivir and zanamivir therapy [18]. In contrast to the frequently observed H275Y change, which causes selective resistance to oseltamivir, the I223R mutation conferred a resistance phenotype against both oseltamivir and zanamivir. Soon after the identification of this first clinical case, the I223R change was found as a single change or in combination with H275Y, in a number of other cases [19,20]. The

I223R/H275Y double mutant proved to have high levels of resistance to oseltamivir. Both *in vitro* studies and in animal models, viruses with I223R and the combination of I223R with H275Y were found not to be compromised in their replication capacity or transmissibility [21,22].

The structural basis of resistance conferred by the H275Y has been described previously [23]. Here we address the role of the I223R in NAI resistance alone and in combination with H275Y. Both I223R and H275Y changes are near the active site of A/H1N1 pandemic NA. Previously, it was shown that different NA sub types (N1-N9) can be grouped into two genetically distinct groups [24]. The active sites of group-1 NAs (N1, N4, N5, N8) have an extra cavity when crystallized in the absence of inhibitor, because of the 'open' conformation of an active site loop, the 150-loop. This loop, containing residues 148-151, closes a cavity adjacent to the active site upon drug binding (150-cavity). In contrast, in group-2 (N2, N3, N6, N7, N9) NA, this loop adopts the closed conformation in the presence or absence of active site ligands. Recently however, a crystal structure for a ligand-free form of the H1N1 pandemic NA was reported, which showed the active site in a closed conformation [25]. More recently, NMR studies on the pandemic neuraminidase have considered this difference and suggested that the neuraminidase prefers to adopt an open conformation [26]. Our results support this suggestion by indicating that a ligand-free form of the pandemic neuraminidase adopts an open conformation. Together with this structure we present the structures of the I223R mutant neuraminidase in complex with oseltamivir and zanamivir to investigate the resistance mechanism of the I223R mutant neuraminidase. We interpret our results of enzyme kinetic measurements in relation to the structures of the complexes.

2 Results and discussion

The NA studied here originated from a pandemic influenza A/H1N1 virus which was isolated from an immune suppressed child on oseltamivir and intravenously (IV) administered zanamivir antiviral therapy. It contained the mutation I223R [18]. Since this change has been reported both as a single mutation as well as in combination with the substitution H275Y [19], virus recombinants were constructed containing the wild type NA, the I223R or H275Y single mutants and the I223R, H275Y double mutant.

2.1 Enzyme kinetics of the I223R and H275Y mutant neuraminidases

To study the effects of the single mutations I223R and H275Y, and the double mutant on the enzymatic properties of NA, Michaelis-Menten (K_M) constants were determined using MUNANA as a substrate. Relative to wild type NA, K_M values increased marginally for the I223R and H275Y single mutants. There was a maximum 8-fold increase for the double mutant (Table 1). In the 2007-2008 influenza H1N1 season, naturally occurring H275Y variants had emerged, which were not attenuated in virus growth or transmissibility. Our observations suggest that, as was observed with the non-attenuated H275Y variants in the 2007-2008 season, the occurrence of viable NAI resistant pandemic influenza A/H1N1 viruses is not an unlikely event.

The sensitivity to oseltamivir and zanamivir was determined for the single and double mutants. By comparison to the oseltamivir inhibitor constant (K_I) of wild type NA, the K_I of the I223R mutant increased 48 times and the I223R/H275Y double mutant K_I more than 7500 times. The change in the K_I for I223R (48-fold) was largely accounted for by an increased dissociation rate constant for the enzyme-inhibitor

Table 1 Kinetic parameters and oseltamivir/zanamivir drug binding for 2009 pandemic influenza A/H1N1 neuraminidase mutants

		Oseltamivir			Zanamivir		
Virus	**K_M***	**K_I (nM)**	**k_{on} ($\mu M^{-1}s^{-1}$)**	**10^3 x k_{off} (s^{-1})**	**K_I (nM)**	**k_{on} ($\mu M^{-1}s^{-1}$)**	**10^3 x k_{off} (s^{-1})**
Wild type	1.0	0.23 (0.12)	3.10 (0.12)	0.75 (0.20)	0.18 (0.03)	1.31 (0.15)	0.32 (0.10)
I223R	2.1	11.1 (1.10)	1.04 (0.09)	11.1 (0.90)	1.65 (0.40)	0.36 (0.12)	0.78 (0.12)
H275Y	2.6	145 (32)	ND**	ND	0.53 (0.06)	1.02 (0.04)	0.76 (0.21)
I223R/H275Y	8.1	1750 (150)	ND	ND	3.92 (0.15)	0.15 (0.01)	0.72 (0.24)

Measurements were performed in triplicate and values in parentheses are standard deviations from the mean. *K_M values presented relative to wild type. K_M value for wild type=28.0μM. **ND=Not determined.

complex (15-fold) rather than by a reduced association rate constant (k_{on}) (3-fold). In the case of zanamivir, the K_I increased 9-fold for the single and 22-fold for the double mutant relative to wild type. The change in the K_I for I223R (9-fold) was in this case accounted for by a reduced association rate constant (4-fold) and a slightly increased dissociation rate constant (2-fold). Thus the I223R mutation has a markedly greater effect on NA inhibition by oseltamivir than by zanamivir.

Although the K_M-values for MUNANA are increased about equally for both the I223R and H275Y single mutants, the change in oseltamivir inhibitor constant for the H275Y mutant relative to wild type, is approximately 10 times greater than for the I223R mutant (Table 1). This may explain why the H275Y change may be the more likely resistance change in oseltamivir monotherapy and of course, to date, the H275Y mutation is the most frequently detected oseltamivir resistance mutation.

2.2 The active site of the H1N1 pandemic neuraminidase has an open conformation

Crystals of wild type and mutant neuraminidases were grown in the absence or presence of the oseltamivir and zanamivir neuraminidase inhibitors. The crystals yielded high-resolution diffraction data and the structures were solved by molecular replacement and refined by standard procedures (Table 2.) The first striking feature from these data is that the 150-loop in the ligand-free I223R NA adopts an open conformation, as seen in the first reported N1 structure [24], but in contrast to the closed conformation of this loop seen in the recently reported crystal structure of the 2009 pandemic A/H1N1 NA (Figure 1A) [25]. Recent NMR experiments likely clarify the apparent discrepancy in these different studies by showing that the 150-loop is flexible and that an equilibrium between the open and closed conformations exists in solution [26]. Inspection of our current structure shows that a phosphate ion, from the crystallization buffer, interacts with lysine 150 to stabilize the open conformation. We speculate that the different crystallization conditions used in the previous pandemic N1 study led to the stabilization of the closed form [25]. The exact function of the open 150-loop in group-1 neuraminidases is unknown, but opening and closing of the loop may have evolved for natural sialo-glycan substrates to fit into the active site. The open conformation of the 150-loop (residues 148-151) generates an additional cavity at the edge of the active site that we have previously called the 150-cavity (Figure 1A), which we have suggested to be an additional target site for neuraminidase inhibitor design [24,27].

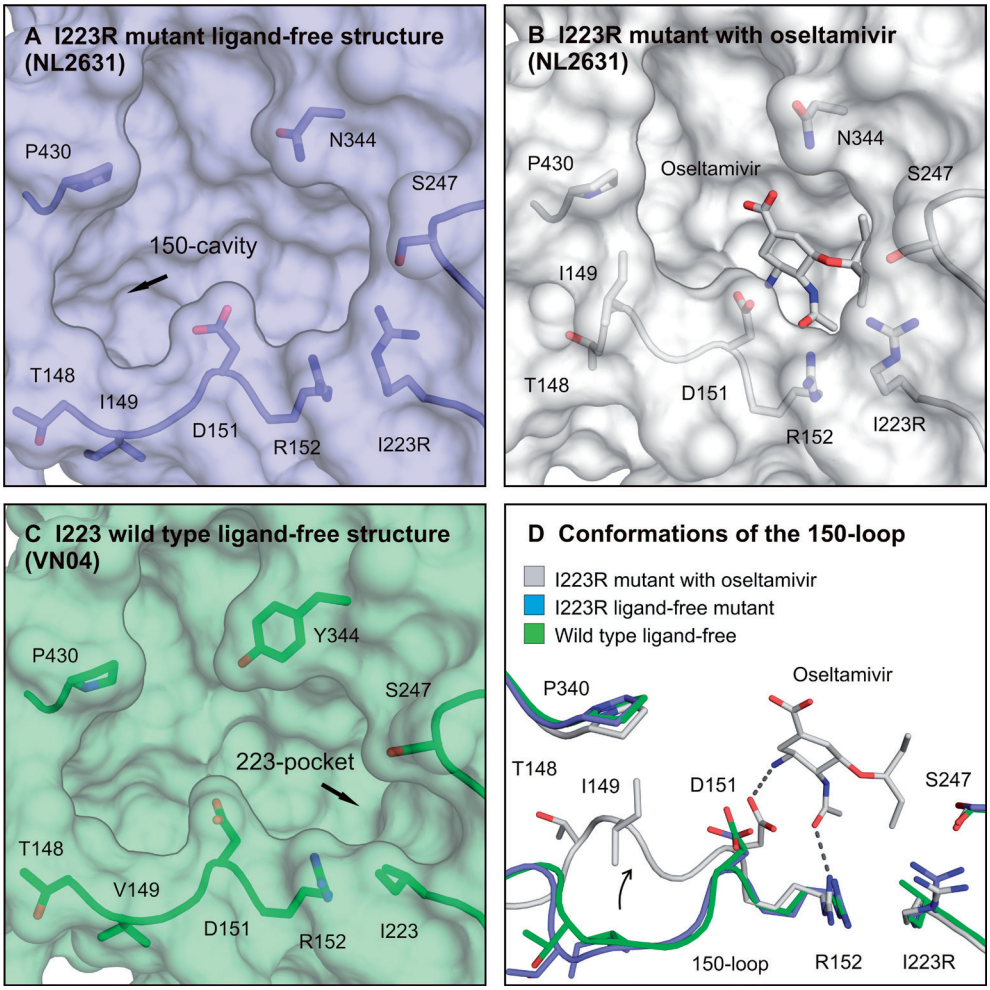

Figure 1 Crystal structures of H1N1 2009 pandemic neuraminidase reveals the open conformational state of its active site

Panel A presents the active site of a 2009 pandemic neuraminidase (PDB ID code 4B7M) in an open conformation. This is the preferred state in the absence of neuraminidase inhibitor. When a protein/inhibitor complex is formed, a flexible loop (150-loop) closes the 150-cavity (panel B). Both the open and closed neuraminidase structures lack a smaller cavity near position 223, due to an isoleucine to arginine change (I223R). This pocket is still present in the wild type N1 neuraminidase (PDB code 2HTY [24]) structure of H5N1 (panel C). Further narrowing of the active site by the I223R change causes antiviral resistance to both oseltamivir and zanamivir. In panel D, an overlay of the 150-loop is presented showing important amino acids in close proximity to the active site. An aspartic acid D151 in the 150-loop and arginine R152 in the 150-loop make hydrogen bond contacts with oseltamivir (or zanamivir) and stabilize the closed conformation of the neuraminidase.

Table 2 (*or as online supporting information*) Data collection and refinement statistics

Virus	NL2631	NL2631	NL2631	Cal07	Cal07
NAI	-	Oseltamivir	Zanamivir	Oseltamivir	Zanamivir
PDB code					
Amino acid differences*	V106I, I223R, N248D	V106I, I223R, N248D	V106I, I223R, N248D	-	-
Data Collection					
Wavelength (Å)					
Space group	$C222_1$	$P42_12$	$P42_12$	$P2_12_12_1$	$P2_12_12_1$
Cell dimensions					
a, *b*, *c* (Å)	118.6, 162.5, 118.9	118.6, 118.6, 67.8	118.4, 118.4, 68.4	83.4, 149.0, 166.8	82.7, 148.8, 166.6
α, β, γ (°)	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90
Resolution (Å)	30.0-2.5 (2.61-2.50)	83.8-2.4 (2.55-2.42)	83.7-2.84 (3.00-2.84)	30.0-1.90 (1.99-1.90)	82.65-2.73 (2.80-2.73)
R_{merge}	14.2 (60.8)	13.0 (41.6)	15.7 (48.1)	12.5 (72.8)	20.5 (53.8)
$I/\sigma I$	9.3 (2.1)	11.4 (4.5)	15.3 (6.0)	13.5 (3.0)	6.1 (2.5)
Completeness (%)	93.3 (92.0)	100 (100)	100 (100)	99.7 (98.0)	99.2 (99.0)
Multiplicity	5.6 (4.6)	10.8 (10.5)	13.3 (13.3)	6.4 (4.8)	6.8 (6.4)
Unique reflections	37388	19102	11923	163254	55091
Refinement					
Resolution (Å)	95.8-2.5	84-2.42	83.6-2.84	111-1.9	82.6-2.7
No. reflections	27395	17197	11333	154943	52204
R_{work}/R_{free}	20.5/23.7	22.8/27.6	18.5/22.4	18.4/20.4	22.4/27.1
R_{free} test set size (%)	4.9	5.1	4.8	5.0	5.1
R_{free} test set count	1412	931	567	8190	2790
No. atoms	6367	3196	3179	13079	12721
B-factor	16.5	13.1	12.7	28.6	35
R.m.s deviations					
Bond lengths (Å)	0.007	0.005	0.006	0.007	0.0054
Bond angles (°)	1.437	0.995	1.037	1.281	0.9922
MolProbity					
MolProbity, clash score	0.16 (100^{th} percentile)	0.33 (100^{th} percentile)	0.65 (100^{th} percentile)	0.08 (100^{th} percentile)	0.33 (100^{th} percentile)
MolProbity score	1.00 (100^{th} percentile)	1.01 (100^{th} percentile)	1.09 (100^{th} percentile)	0.76 (100^{th} percentile)	1.07 (100^{th} percentile)
Ramachandran favored (%)	95.6	95.32	96.35	96.49	95.26
Residues with bad bonds (%)	0.0	0.78	0.0	0.0	0.0
Residues with bad angles (%)	0.0	0.26	0.0	0.0	0.06

*Amino acid differences as compared to Cal07

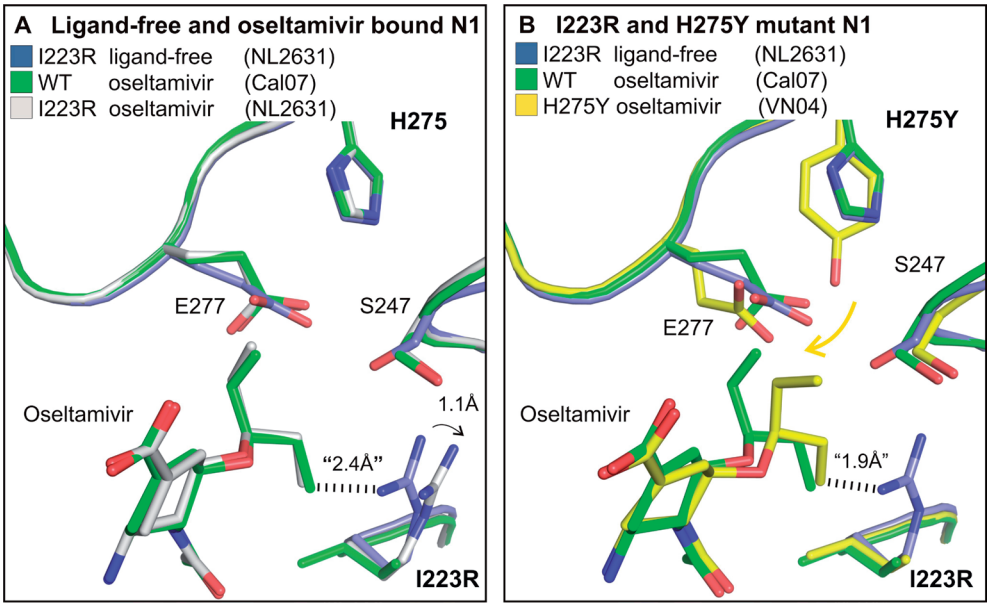

Figure 2 Antiviral resistance mechanism of the I223R mutant. I
n panel A, an overlay of three pandemic neuraminidase structures is presented: I223R mutant ligand-free structure (blue, NL2631, PDB ID code 4B7M) and both wild type (green, Cal07, PDB ID code 4B7R) and I223R mutant (white, Cal07, PDB ID code 4B7J) oseltamivir complexes. The key amino acid residues involved in I223R antiviral resistance pattern are displayed. Binding of oseltamivir to the active site is inhibited by arginine 223 (R223) and serine 247 (S247). The side chain of S247 points towards the active site in the I223 mutant. Oseltamivir binding to the I223R mutant involves reorientation of both R223 and S247 residues. In panel B, an overlay is presented of an H275Y mutant neuraminidase structure in complex with oseltamivir (yellow, VN04, PDB code 2CL0 [23]) with two I223R mutant neuraminidase crystal structures: The ligand-free structure (blue, NL2631, PDB ID code 4B7M) and the structure in complex with oseltamivir (green, NL2631, PDB ID code 4B7J). The resistance mechanisms of the H275Y and I223R mutants act synergistically to gain enhanced levels of oseltamivir resistance.

2.3 Changes in oseltamivir binding by the I223R change

In the wild type N1 neuraminidase structure, a small pocket, the "223-pocket" (Figure 1C), also adjacent to the active site, but distinct from the 150-cavity, contains two water molecules. In the I223R neuraminidase, this pocket is occupied by the side chain of R223. In displacing the two water molecules seen in the wild-type structure, the arginine makes a hydrogen bond with the side chain of serine 247 (S247). As a result, the side chain of S247 adopts a different rotamer and is oriented towards the active site which enables it to make a second hydrogen bond with glutamic acid 277 (E277). These changes in the structure of the I223R neuraminidase result in

shrinkage at the edge of the active site pocket that accommodates the hydrophobic pentoxyl group of oseltamivir. Thus, oseltamivir binding to the active site of I223R neuraminidase requires the reorientation of the S247 and R223 side chains. Moreover, the reorientation of the side-chain of E277, which is required for oseltamivir binding to wild-type neuraminidase, is presumably made even less energetically favorable in the I223R mutant by the need to disrupt the hydrogen bond between S247 and E277. Thus the binding of oseltamivir to the active site of the I223R mutant results not only in changes in the conformation of the side chain of S247 but also in the side-chain of R223 being translated about 1.1Å out of the active site compared to the mutant ligand-free structure (Figure 2A).

S247 therefore plays an important role in the decreased sensitivity of the I223R neuraminidase to inhibitors. Interestingly, a serine to asparagine change at position 247 (S247N) has also been linked to oseltamivir resistance [28]. Like the I223R change, the S247N mutation also causes enhanced resistance to oseltamivir when accompanied by the H275Y change. Both the single and the S247N/H275Y double mutant viruses were found not to be compromised in a ferret model suggesting that the mutant neuraminidases retain most of their normal activity [29]. An asparagine at position 247, an amino acid with a larger polar side chain, can also affect the size of the hydrophobic pocket. Therefore, although there is no structural information in that case we speculate that, like the I223R change, the resistance mechanism of the S247N change also may involve shrinkage of the hydrophobic pocket.

2.4 Structural changes in the I223R/H275Y double mutant

The mechanism whereby the H275Y mutant neuraminidase binds substantially more weakly to oseltamivir than wild type has been described previously [23]. In brief, the introduction of the bulkier tyrosine residue perturbs the position and the reorientation of the acidic side chain of E277 required for oseltamivir binding, such that the hydrophobic pentoxyl substituent of oseltamivir is translated out of the active site towards the pocket occupied in the I223R structure by the side chain of the arginine residue (Figure 2B). The I223R mutation, therefore, appears to restrict the alternative position that the pentoxyl substituent adopts in the H275Y mutant. Furthermore, the binding of oseltamivir by the I223R/H275Y double mutant becomes even less energetically favorable than by either of the two single mutant proteins. Our structural data therefore provide an explanation for the 1750-fold poorer binding of oseltamivir by the I223R/H275Y double mutant by comparison with the wild-type neuraminidase (Table 1).

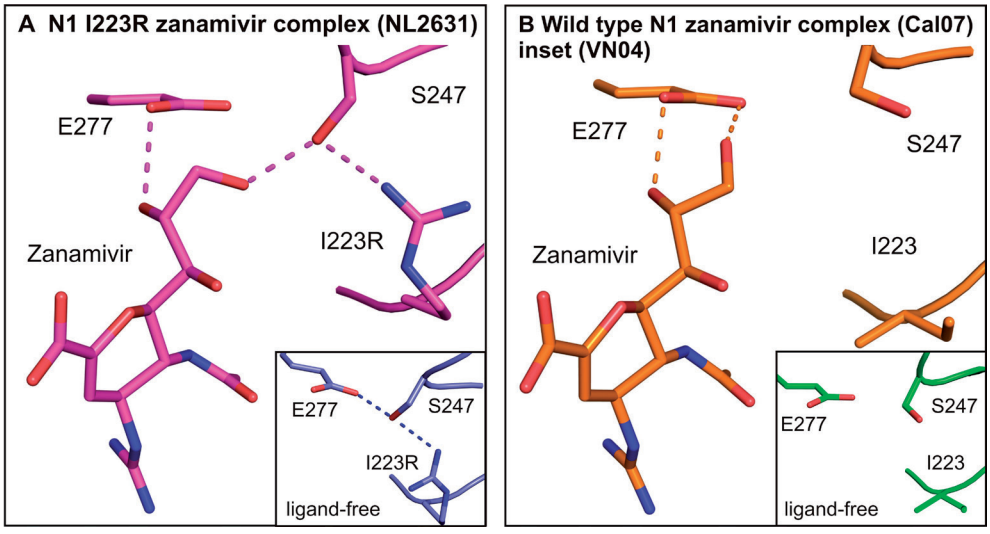

Figure 3 The plasticity of zanamivir enables inhibition of the I223R mutant
In panel A, hydrogen bond contacts are presented (dotted lines), which are formed between zanamivir and active site residues in the I223R mutant (purple, PDB ID code 4B7N). The insert displays the interactions between the same residues in the I223R mutant ligand-free structure (blue, PDB code). In panel B, active site residues and hydrogen bonds are displayed, which are formed in the wild type zanamivir structure complex (brown, PDB ID code 4B7Q). The insert displays the I223, S247 and E277 residues in the wild type ligand-free structure.

2.5 The polarity of the zanamivir glycerol substituent correlates with the smaller effect of the I223R mutation on zanamivir binding

Our structural studies help to explain how the single I223R and I223R/H275Y double mutant neuraminidases bind zanamivir almost as well as wild type. Instead of the hydrophobic pentoxyl substituent present in oseltamivir, zanamivir possesses the same polar glycerol substituent as sialic acid (Figures 2 and 3). Consequently, binding of zanamivir to the active site of neuraminidase does not require the reorientation of the side chain of E277. In the case of the wild type neuraminidase, the glycerol moiety of zanamivir forms two hydrogen bonds with E277. This interaction is unaffected in the H275Y mutant (Figure 3). In the I223R single or double mutant neuraminidases, somewhat different interactions are made by the glycerol moiety: one hydrogen bond is formed with E277 and another hydrogen bond is formed with S247 (Figure 3A). To facilitate zanamivir binding in this way R223 is reoriented only marginally and it still retains the R223 hydrogen bond with S247 (Figure 3).

The plasticity of the pandemic neuraminidase active site, especially around the hydrophobic pocket, allows the emergence of drug resistant influenza variants. In addition to the I223R change, isoleucine 223 changes to valine (I223V) and lysine (I223K) accompanying the H275Y change has also been reported [20]. This further indicates that this residue plays an important role in neuraminidase inhibitor resistance and stresses the importance of a detailed understanding of the mechanism of resistance. Since the single I223R change described here resulted in only a 9-fold increase in the K_I for zanamivir, it is interesting to consider why the I223R mutant virus persisted in the IV zanamivir treated immunocompromised patient. As previously suggested [30], suboptimal drug levels in the patient's respiratory tract, due to the route of administration (inhaled versus IV) as well as the immune status of the patient may have contributed to the emergence and persistence of the I223R mutant virus.

4 Materials and methods

4.1 Antiviral compounds and recombinant viruses

Hoffmann-La Roche Ltd. (Switzerland) kindly provided oseltamivir carboxylate. Zanamivir was kindly provided by GlaxoSmithKline (the Netherlands). Recombinant influenza viruses were generated by reverse genetics essentially as previously described [31]. Each virus contained seven segments of A/WSN/33 and the neuraminidase gene of either A/Netherlands/2631_1202/2010 (NL2631), A/California/07/09 (Cal07) or A/Vietnam/1203/04 (VN04). Recombinant viruses were propagated in embryonated chicken eggs. Allantoic fluids were harvested after 48hrs and cleared from debris by centrifugation at 3000 rpm for 15 minutes. They were then used directly to measure enzyme kinetics or further processed to purify the neuraminidase for crystallography.

4.2 Neuraminidase activity measurements

Michaelis-Menten constants (K_M) were determined at 37°C as previously described [23], using standard initial rate measurements with virus diluted in 32.5mM MES buffer (pH 6.4), 5mM $CaCl_2$ and fluorescent substrate 2'-(4-methylumbelliferyl1)-α-D-N-acetylneuraminic acid (MUNANA) concentrations ranging from 2.0 to 200μM. Inhibition constants (K_I) were determined by following the reduction in MUNANA hydrolysis rate following addition of inhibitor to a virus/MUNANA mixture approximately 100s after initiation of the reaction. K_I was then calculated using equation (1) [32]:

$$V_I = \frac{V_0([S] + K_m)}{[S] + K_m\left(1 + \frac{[I]}{K_I}\right)} \quad (1)$$

where V_I is the new steady state hydrolysis rate in the presence of inhibitor at concentration [I], V_0 is the rate in the absence of inhibitor, [S] is the MUNANA concentration, K_M is the Michaelis-Menten constant and K_I is the dissociation constant for inhibitor binding. The kinetic parameters for inhibitor binding were determined by analyzing the exponential approach to V_I using the following equation [33]:

$$F_t = F_0 + V_I t + (V_0 - V_I)(1 - e^{-k_{OBS}t}) / k_{OBS} \quad (2)$$

where F_o and F_t are the fluorescence signals at time zero and time t, and k_{OBS} is the first-order rate constant. Association (k_{on}) and dissociation (k_{off}) rate constants for

inhibitor binding were then determined by plotting k_{OBS} versus [I] using the following equation [34]:

$$k_{OBS} = k_{off} + \frac{k_{on}K_m[I]}{K_m + [S]} \quad (3)$$

In most cases the kinetically determined K_I values (k_{off}/k_{on}) agree well with those determined using equation (2). Measurements were performed in triplicate using three different inhibitor concentrations.

4.3 Neuraminidase purification

Recombinant virus was harvested from clarified allantoic fluid by centrifugation at 6000 rpm overnight at 4°C and resuspended in 10mM TRIS buffer, (pH 8.0), 150mM NaCl (TRIS-buffer). Virus was then layered over a continuous sucrose gradient (15-40% sucrose in TRIS-buffer) and centrifuged at 25.000rpm for 45 minutes at 4°C. The virus-containing fraction was collected and virus was pelleted by dilution of the remaining sucrose with Tris-buffer and centrifugation at 27.000 rpm for 90 minutes at 4°C. The purified virus was resuspended in Tris-buffer. Next, glycoproteins were released from the virus by bromelain (Sigma-Aldrich) digestion for 1 hour at 37°C and digested viruses were pelleted by centrifugation at 55.000 rpm for 10 minutes. A protease inhibitor tablet was added to the NA containing supernatant to prevent further protein degradation (Roche). NA was purified by layering the supernatant over a sucrose gradient (5-25% sucrose in 10mM TRIS buffer (pH 8.0) and centrifugation at 38000 rpm for 18 hours at 4°C. Subsequently, NA-containing fractions were pooled and further purified using an Q-15 anion-exchange column (Sartorius). Finally, buffer was changed by overnight dialysis against Tris-buffer supplemented with 5mM $CaCl_2$ and neuraminidase protein was concentrated 6mg/ml using a 50kD vivaspin column (Sartorius) for crystallization experiments.

4.4 Protein crystallography

Neuraminidase crystals were obtained by vapor diffusion from sitting drops dispensed with an Oryx 8 robot (Douglas Instruments). The drops consisted of 100nl of protein in the absence or presence of 1mM inhibitor (oseltamivir or zanamivir) mixed with 100nl of reservoir solution. The reservoir solution of the I223R ligand-free crystal consisted of 20% PEG1000, 0.6M Ammonium Phosphate and 0.1M Sodium Acetate (pH 4.6). The reservoir solution of the I223R crystal in complex with zanamivir consisted of 18% PEG3350, 0.2M Sodium Fluoride and 0.1M bis-TRIS

Propane buffer (pH 6.5). The reservoir solutions of the other crystals consisted of 15% PEG3350, 0.1M bis-TRIS propane and 0.1M sodium acetate buffer (pH 4.6). Crystals were transferred into a cryoprotectant that consisted of reservoir solution supplemented with 20% (v/v) ethylene glycol before flash freezing in liquid nitrogen. Data sets were recorded on an ADSC Q315 CCD, Pilatus 6M-F and SLS/Dectris Pilatus miniCBF detectors at the Diamond light source (Oxford, UK). Diffraction images were integrated using iMOSLFM [35] or DENZO and scaled with SCALA [36] or SCALEPACK for the ligand-free I223R structure. Neuraminidase structures were solved by molecular replacement with PHASER [37] using the wild type structure (protein databank (PDB) code 2HU4) as the initial search model. Refinement was performed using Refmac5 [38] or PHENIX Refine [39]. Manual model building was done using Coot [40], structure validation was assessed with MOLPROBIDITY [41] and figures were created using Pymol (http://pymol.sourceforge.net/).

4.5 Acknowledgments

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Chapter 5.1

Multidrug resistant 2009 A/H1N1 influenza clinical isolate with a neuraminidase I223R mutation retains its virulence and transmissibility in ferrets

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Abstract

Only two classes of antiviral drugs, neuraminidase inhibitors and adamantanes, are approved for prophylaxis and therapy against influenza virus infections. A major concern is that influenza virus becomes resistant to these antiviral drugs and spreads in the human population. The 2009 pandemic A/H1N1 influenza virus is naturally resistant to adamantanes. Recently a novel neuraminidase I223R mutation was identified in an A/H1N1 virus showing cross-resistance to the neuraminidase inhibitors oseltamivir, zanamivir and peramivir. However, the ability of this virus to cause disease and spread in the human population is unknown. Therefore, this clinical isolate (NL/2631-R223) was compared with a well-characterized reference virus (NL/602). In vitro experiments showed that NL/2631-I223R replicated as well as NL/602 in MDCK cells. In a ferret pathogenesis model, body weight loss was similar in animals inoculated with NL/2631-R223 or NL/602. In addition, pulmonary lesions were similar at day 4 post inoculation. However, at day 7 post inoculation, NL/2631-R223 caused milder pulmonary lesions and degree of alveolitis than NL/602. This indicated that the mutant virus was less pathogenic. Both NL/2631-R223 and a recombinant virus with a single I223R change (recNL/602-I223R), transmitted among ferrets by aerosols, despite observed attenuation of recNL/602-I223R in vitro. In conclusion, the I223R mutated virus isolate has comparable replicative ability and transmissibility, but lower pathogenicity than the reference virus based on these in vivo studies. This implies that the 2009 pandemic influenza A/H1N1 virus subtype with an isoleucine to arginine change at position 223 in the neuraminidase has the potential to spread in the human population. It is important to be vigilant for this mutation in influenza surveillance and to continue efforts to increase the arsenal of antiviral drugs to combat influenza.

Author summary

Recently, a 2009 pandemic A/H1N1 influenza virus was isolated from an immune compromised patient, with antiviral resistance to the neuraminidase inhibitor class of drugs. This virus had an amino acid change in the viral neuraminidase enzyme; an isoleucine at position 223 was substituted for an arginine (I223R). Patients infected with a pandemic virus that is resistant to all neuraminidase inhibitors, would leave physicians without antiviral treatment options, since these viruses are naturally resistant to the other class of antivirals, the adamantanes. To date, it is unknown if this I223R mutant virus is affected in its ability to cause severe disease and to transmit to other humans. Therefore, we have addressed this question by comparing the I223R mutant virus with a wild type reference virus in a ferret pathogenicity and transmission model. We found that the I223R mutant virus was not severely affected in its pathogenicity, although fewer lung lesions and alveolitis scores were found for the I223R mutant virus. In addition, we demonstrated that this virus transmitted efficiently to naïve ferrets. Consequently, we conclude that this I223R mutant virus has the potential to cause disease and may spread among humans. Therefore, influenza surveillance for this resistance pattern is advised.

1 Introduction

Two classes of antiviral drugs are approved for prophylaxis and therapy of influenza virus infected patients [1]. Antiviral therapy against the new (swine-origin) 2009 pandemic A/H1N1 influenza virus relies on the neuraminidase inhibitor (NAI) class of antiviral drugs only, because this subtype is resistant to the adamantane class (amantadine and rimantadine) of drugs [2]. In 2009 pandemic influenza viruses, this resistance pattern is mainly caused by an asparagine at amino acid position 31 (N31) in the viral M2 membrane protein. Fortunately, NAI treatment, both as prophylaxis and therapy, has been shown to be effective against most 2009 pandemic H1N1 virus infections so far [3,4].

To date, the incidence of NAI resistant 2009 pandemic A/H1N1 viruses is very low. Nevertheless, 565 cases of patients infected with an (H275Y, N1 numbering) oseltamivir (OS) resistant virus have been reported to the World Health Organization [5]. In most of these cases, OS resistance was found in patients receiving prolonged antiviral therapy, in particular patients under immunosuppressive therapy [6]. The H275Y mutant viruses are cross-resistant to peramivir (PER), but remain susceptible

to zanamivir (ZA). Successful clearance of a H275Y mutant virus from a patient treated with ZA was reported previously [7].

Within the first years after approval of the NAIs in 1999, antiviral resistance in influenza viruses at a population level was rare (0.4%). In clinical trials, the incidence of resistant viruses was higher, varying from 0.4 to 1% in adults and up to 18% in young children [8,9]. However, a dramatic increase, up to 100%, of de novo circulating oseltamivir-resistant A/H1N1 viruses characterized the epidemic seasons of 2007-2008 and 2008-2009 [10,11]. This resistance phenotype was also caused by a H275Y mutation. Remarkably, earlier studies on H275Y mutant H1N1 viruses had characterized these viruses as attenuated and not of clinical importance [12-14]. The resistant viruses from 2007-2008 did not seem to be affected in replication capacity, transmissibility and their ability to cause severe disease in humans [15-17]. A compensatory role was assigned to the NA amino acid changes V234M, R222Q and D344N [18,19]. These substitutions may have restored the initial loss of NA activity due to the NAI resistance mutation and facilitated the appearance of the H275Y change in the epidemic influenza A/H1N1 viruses that circulated before the 2009 outbreak of the new pandemic virus. Recently, several research groups have studied the fitness of H275Y mutant pandemic influenza A/H1N1 viruses using both in vitro and in vivo experiments [20-24]. Overall, these data indicate that pandemic viruses with the NA H275Y substitution were comparable to their oseltamivir susceptible counterparts in pathogenicity and transmissibility in animal models.

Recently, the identification of a novel multidrug resistant 2009 pandemic A/H1N1 virus was reported, isolated from an immune compromised child with reduced susceptibility to all NAIs [25]. An isoleucine to arginine substitution at position 223 in NA (I223R, N1 numbering) was detected in the patient after antiviral therapy with OS had failed due to the emergence of the H275Y mutation and therapy was switched to ZA. This I223R containing isolate, in which the H275Y mutation had disappeared, showed reduced susceptibility to OS (45-fold), PER (7-fold) and ZA (10-fold). In vitro analysis showed that reversion of the arginine to isoleucine fully restored NAI susceptibility. In another case, an I223R/H275Y double mutant virus was isolated that showed high resistance to the NAIs [26]. In combination with the natural resistance of pandemic A/H1N1 viruses to adamantanes, an infection of such a multi-drug resistant virus leaves physicians without antiviral treatment options. The emergence of this pandemic 2009 A/H1N1 virus prompted us to investigate the properties of this clinical isolate by evaluating its in vitro replication kinetics and its pathogenicity and transmissibility in the ferret model. We here show that this 2009 pandemic influenza A/H1N1 clinical isolate, harboring a neuraminidase I223R substitution retains its

virulence and transmissibility, but is less pathogenic than a virus prototype without this mutation. In addition, recombinant NL/602/09 with a single I223R amino acid substitution transmitted as well as its recombinant parental virus, suggesting that no additional mutations are needed to compensate for the presence of this I223R mutation in the 2009 pandemic A/H1N1 virus backbone.

2 Results

2.1 Sequence comparison of virus isolates

A pandemic 2009 influenza virus with reduced susceptibility to all NAIs that was isolated from a Dutch immune compromised child was studied here. Full genome sequencing of this clinical isolate A/NL/2631_1202/2010 (NL/2631-R223, GenBank accession numbers JF906180-906187) harboring an I223R mutation in the neuraminidase was performed. Since no drug susceptible virus had been isolated from this patient before start of antiviral therapy, the well-characterized NAI-susceptible virus isolate A/NL/602/2009 (NL/602, GenBank accession numbers CY046940-046945 and CY039527-039528) was used as a reference virus in all experiments. This reference virus is a representative of pandemic H1N1 viruses that circulated in 2009, with only amino acid changes I108V and V407I (N1 numbering) in NA being unusual among the deposited sequences in the Influenza Research Database [27,28]. Pair-wise comparison revealed, in addition to the amino acid change I223R, 5 amino acid differences in NA (V106I, V108I, N248D, N386D and I407V) and 1 in HA (S203T). The NA and HA amino acid positions are given according to the N1 and H1 numbering. Eleven additional amino acid differences were found in gene segments PB2 (3), PB1 (2), PA (2), NP (3) and NS (1) compared to NL/602. None of these mutations have previously been identified as a virulence marker or as a compensatory mutation involved in restoration of NA activity loss, as a result of the presence of resistance mutations. By studying these isolates, a direct comparison could be made between a NAI susceptible and a novel I223R resistant virus, but such comparison does not address the impact of the single I223R mutation directly. Therefore, we introduced the I223R mutation in the recNL/602 backbone, resulting in the drug-resistant recNL602-I223R, to evaluate the impact of the single I223R mutation on virus replication, virus shedding from the upper respiratory tract and transmissibility in the ferret model.

2.2 I223R harboring isolate is not attenuated in vitro

Virus replication was studied in vitro by multi-cycle replication kinetics of the viruses of interest. For this purpose, MDCK or MDCK-SIAT1 cell cultures were inoculated at a multiplicity of infection of 0.001 TCID50 per cell and at fixed time points supernatants were harvested to determine viral titers (Figure 1). Overall, the initial virus replication rates and end point titers were similar for the clinical isolate NL/2631-R223 and recNL/602.

A recombinant derivative of NL/602 with the I223R mutation in NA (recNL/602-I223R) replicated to lower peak titers in both cell lines compared to recNL/602 and NL/2631-R223. In addition, initial virus replication of recNL/602-I223R was delayed by 6 to 12 hours in MDCK-SIAT1 cells.

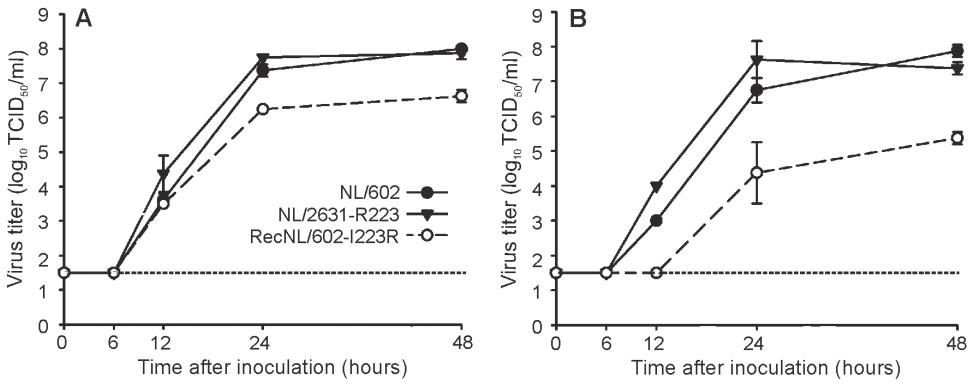

Figure 1 Replication kinetics in MDCK or MDCK-SIAT1 cells
MDCK (panel A) or MDCK-SIAT1 (panel B) cells were inoculated with 0.001 TCID50 virus per cell of recNL/602 (black circles), isolate NL/2631-R223 (black triangles) and recNL/602-I223R (open circles). Supernatants were harvested after 6, 12, 24, and 48 hours post infections and were titrated in MDCK cells. Geometric mean titers and standard deviations were calculated from two independent experiments. The lower limit of detection is indicated by the dotted line.

2.3 No marked differences in virus replication in the respiratory tract of ferrets

The pathogenicity of clinical isolate NL/2631-R223 was compared with NL/602 in the ferret model that was previously established to study the ability of influenza viruses to cause pneumonia [29]. Two groups of 6 ferrets were inoculated intratracheally with 106 TCID50 of virus. The animals were weighed daily as an indicator of disease. Over the 7-day period, no significant differences were observed in weight

loss between the two groups inoculated with either virus. At day 4 post infection (p.i.), when there were still 6 animals present in each group, the mean percentage of weight loss was 8,2±2,4% and 7,6±6,7% for NL/602 and NL/2631-R223-inoculated animals respectively, not statistically significant (Figure 2A and B). In addition, no marked differences were observed for other clinical parameters, such as lethargy, sneezing and interest in food.

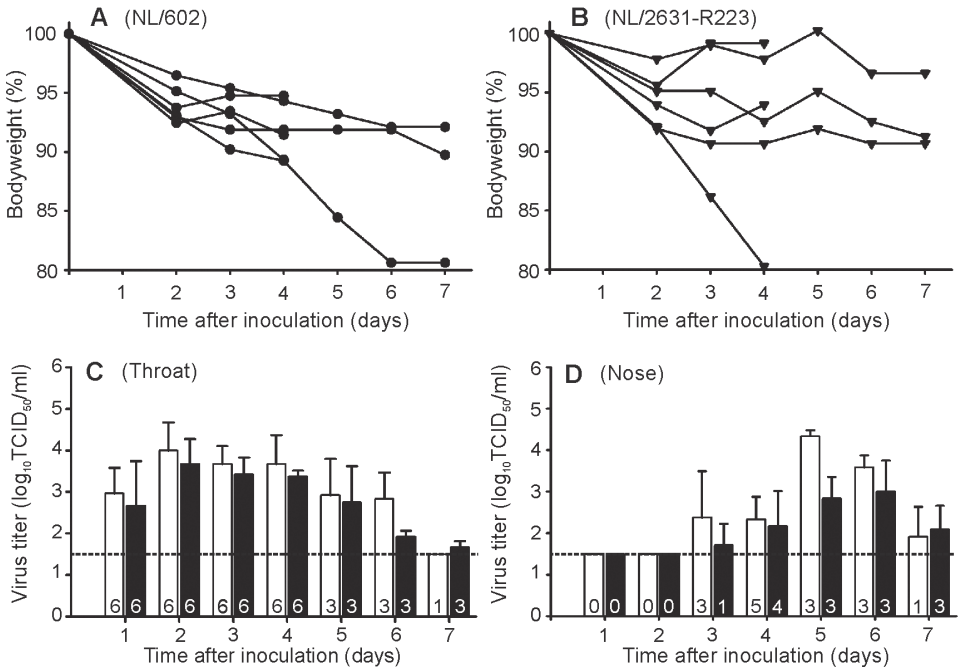

Figure 2 Ferret relative weight loss and virus shedding from the ferret upper respiratory tract
Ferrets were inoculated intratracheally with 1×106 TCID50 of NL/602 or NL/2631-R223. Body weights for NL/602 (Panel A) and NL/2631-R223 (Panel B) inoculated animals are depicted as percentage of body weight relative to the time of inoculation. Data are shown for individual animals until the animals were euthanized at day 4 or 7 p.i.. Virus detection in throat (panel C) and nose swabs (panel D) is indicated for NL/602 (white bars), and NL/2631-R223 (black bars). Geometric mean titers from 6 (day 1 to 4) or 3 animals (day 5 to 7) are displayed and the error bars indicate the standard deviations. The number of influenza virus positive animals per day is depicted in each bar. The lower limit of detection is indicated by the dotted line.

Nose and throat swabs were collected daily from the inoculated animals and virus titers were determined by end-point titration in MDCK cells. Infectious virus shedding from the throat was detected from day 1 p.i. onwards in all ferrets, with

similar patterns of virus shedding from the throat of the animals in the two groups (Figure 2C). At day 4 p.i., 5 and 4 animals were shedding virus from the nose in the NL/602 and NL/2631-R223 inoculated group respectively (Figure 2D). Sequence analysis confirmed the presence of the I223R mutation in the respiratory samples collected at day 7 p.i. from the NL/2631-R223 inoculated ferrets.

At day 4 and 7 p.i., three animals of each group were euthanized and lungs were collected for virological and pathological examination. At day 4 p.i., no marked differences were found between the virus titers for both groups of ferrets (Figure 3A). At day 7 p.i., no virus was detected in the lungs of ferrets inoculated with either virus.

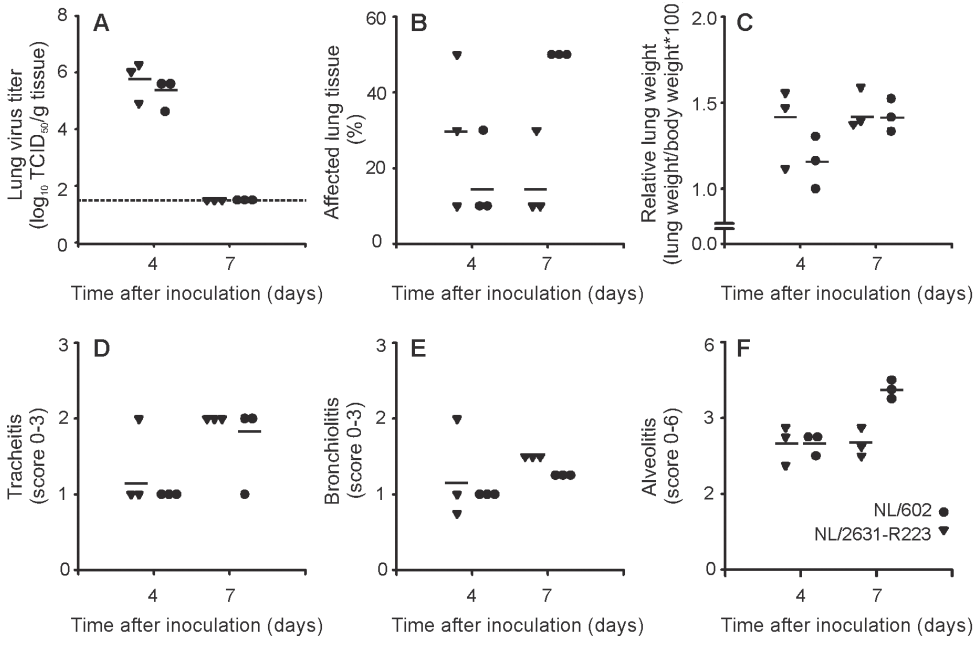

Figure 3 Semi-quantitative lung scores and histological examination of the infected ferret respiratory tract

Lung virus titers (panel A), percentage of affected lung tissue (panel B) and relative lung weights (panel C) were determined for lungs of ferrets inoculated with NL/2631-R223 (triangles) or NL/602 (circles) that were euthanized at day 4 or 7 p.i.. Semi-quantitative assessment of the extent and severity of the tracheitis (panel D), bronchiolitis (panel E) and alveolitis (panel F) are shown. Individual values are displayed. In panel A, the lower limit of detection is indicated by a dotted line.

2.4 Moderate pathogenicity of I223R harboring isolate

Gross pathology of the lungs of all animals revealed pulmonary lesions at day 4 and 7 p.i. (Figure 3B). At day 4 p.i., no marked difference was observed between the groups, but at day 7 p.i., the percentage of affected lung tissue was higher in the group inoculated with NL/602. The mean relative lung weight increased from day 4 to day 7, with no difference between the animals inoculated with NL/602 or NL/2631-R223 (Figure 3C). Histopathological examination of the lungs showed multifocal to coalescing alveolar damage in both groups characterized by the presence of macrophages and neutrophils within the lumina and thickened alveolar walls. At day 4 p.i., the severity of alveolitis did not differ between the two groups (Figure 3D). However, in agreement with the increased percentage of affected lung tissue at day 7 p.i. (Figure 3B), also higher alveolitis scores were determined for the NL/602 inoculated animals at day 7 p.i. (Figure 3D).

The bronchial and bronchiolar epithelium from ferrets in both groups showed slight multifocal necrosis with moderate intra-epithelial infiltrates of neutrophils and multifocal peribronchiolar infiltration of macrophages, lymphocytes, neutrophils and plasma cells. The lumina contained moderate amounts of mucus mixed with cellular debris and few neutrophils. The tracheal epithelium in both groups showed mild neutrophilic infiltrates. The severity of both bronchiolitis and tracheitis increased from day 4 to 7 p.i. in ferrets infected with both viruses, but the differences in scores between groups were minimal (Figure 3E and F).

2.5 I223R harboring isolate is transmissible via aerosols or respiratory droplets

Individually housed ferrets were inoculated with virus isolate NL/2631-R223 or NL/602 and naïve animals were placed in a cage adjacent to each inoculated ferret at day 1 p.i. to allow aerosol or respiratory droplet transmission. All inoculated ferrets started to shed virus at day 1 p.i. with virus titers up to 106 TCID50/ml in throat and nose swabs (Figure 4A and C).

The naïve ferrets became infected, because of aerosol or respiratory droplet transmission, 1, 2 or 3 days p.e. In the naïve animals, virus was detected in 4 (NL/602), or 3 (NL/2631-R223) out of 4 animals (Figure 4B and D). The exposed animal in the NL/2631-R223 transmission experiment, from which no virus could be isolated, did not seroconvert in the course of the experiment. At day 5 p.e., the presence of the I223R mutation was confirmed by sequencing the NA gene of virus isolated from the throat swabs of the positive animals.

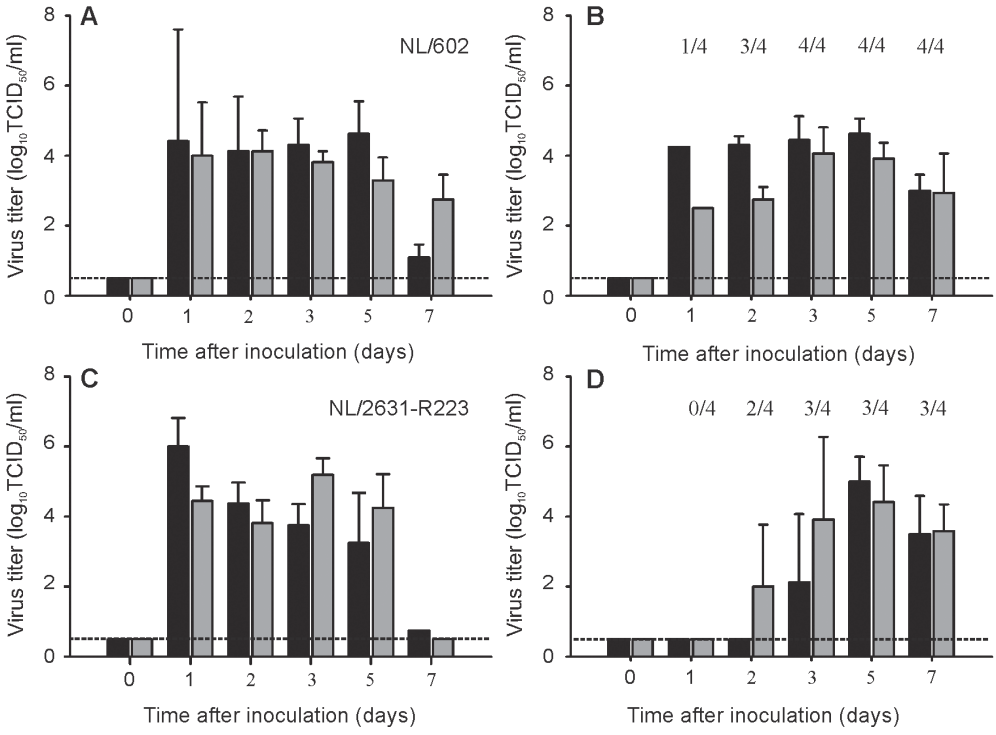

Figure 4 Transmission of NL/602 and NL/2631-R223 by aerosol or respiratory droplets in ferrets
Virus titers in throat (black bars) and nose swabs (grey bars) are displayed for inoculated (Panel A and C) and exposed ferrets (Panel B and D). The geometric mean titers of positive samples are displayed and the error bars indicate the standard deviations. The number of positive exposed animals per day is depicted. The lower limit of detection is indicated by the dotted line.

2.6 I223R mutant transmits as well as parental reference virus

When the multi-cycle replication kinetics were studied of viruses with or without the I223R substitution in MDCK cells, it was noticed that the recombinant virus in which the I223R mutation was introduced, recNL/602-I223R, replicated to lower titers than its parental virus recNL/602 (Figure 1). To address if this difference in in vitro replication capacity could be extrapolated to reduced replication in vivo, the ability of recNL/602-I223R to transmit in the ferret model was studied. It was expected that reduced replication in ferrets would impede the virus to transmit to naïve animals, thereby suggesting that compensatory mutations are needed to balance the fitness loss induced by the I223R mutation. In contrast to the results obtained in MDCK cells, recNL/602-I223R replicated and transmitted as well as recNL/602 when evaluated in the ferret transmission model. Inoculated animals started to shed virus from the

upper respiratory tract from day 1 p.i. onwards and transmission was detected in 4 out of 4 (recNL/602), or 2 out of 2 (recNL/602-I223R) naïve animals from day 2 onwards (Figure 5). The presence of the I223R mutation in the recNL/602 backbone was confirmed in throat samples obtained from these animals at day 5 p.e.

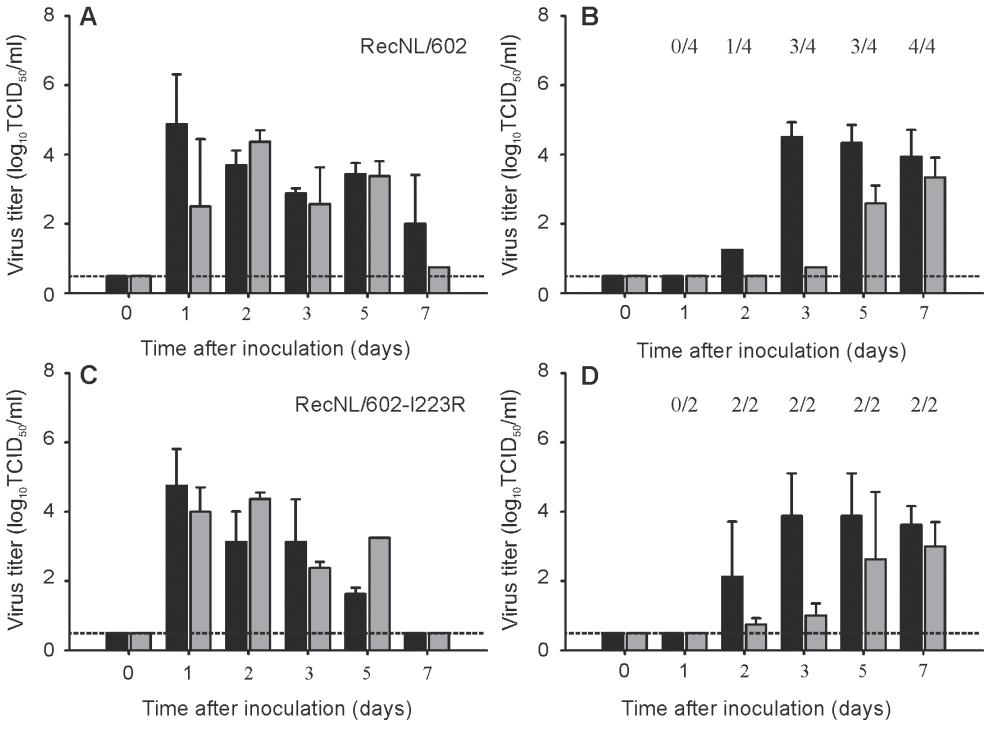

Figure 5 Transmission of recNL/602 and recNL/602-I223R by aerosol or respiratory droplets in ferrets

Virus titers in throat (black bars) and nose swabs (grey bars) are displayed for inoculated (Panel A and C) and exposed ferrets (Panel B and D). The geometric mean titers of positive samples are displayed and the error bars indicate the standard deviations. The number of positive exposed animals per day is depicted. The lower limit of detection is indicated by the dotted line.

3 Discussion

Here, a 2009 pandemic influenza A/H1N1 virus isolate, harboring an I223R multidrug resistance mutation, was characterized by studying its replication capacity in MDCK cells and its pathogenicity and transmissibility in the ferret model. This I223R mutant virus is not attenuated for replication in the ferret respiratory tract and transmitted as well as NAI susceptible reference virus NL/602. Furthermore, it was demonstrated here that compensatory mutations for the I223R mutation are not required, since recombinant NL/602 with a single I223R change transmitted as efficiently as its parental virus in ferrets.

To date, 2009 pandemic viruses with an amino acid substitution at position 223 have only sporadically been isolated from patients. A I223V/H275Y double mutant was detected in two closely residing patients who were treated with OS [30]. Besides the I223R single mutant virus studied here, an I223R/H275Y double mutant was detected in an immune suppressed patient treated with OS and ZA [26]. The combination of these mutations resulted in an increased NAI resistance pattern, as compared to the resistance induced by the single mutations. This emphasizes that neuraminidase position 223 is an important marker for antiviral resistance and may be a key residue in the emergence of influenza viruses with resistance to all NAIs, especially in combination with other resistance-associated mutations. So far, the incidence of 2009 pandemic viruses with a 223 change is very low. Notably, 2009 pandemic viruses were reported with a serine to asparagine change at position 247 [31]. In combination with the H275Y change, these viruses demonstrated resistance patterns similar to the I223R/H275Y mutant.

In a pathogenesis experiment, no statistical significant differences were found when weight loss was compared of ferrets inoculated with clinical isolates NL/2631-R223 or NL/602 (Figure 2A and B). In agreement with high viral loads found in respiratory specimens collected from the patient who was infected with NL/2631-R223, high viral loads were detected in the throat of animals inoculated with the same virus. Overall, identical patterns of virus shedding were observed during the course of the experiment in the throats of animals inoculated with either virus. However, virus shedding from the nose could not be detected in all inoculated animals. Although virus shedding from the nose of NL/2631-R223-inoculated animals seem somewhat delayed in comparison with NL/602-inoculated animals, these differences were not significant due to the large variations within groups and small group size after day 4 p.i. (Figure 2C and D).

Both macroscopic and microscopic evaluation of the lungs of the ferrets at day 4 p.i., revealed no major differences in the percentage of affected lung tissue and relative lung weights between NL/2631-R223 and NL/602 (Figure 3B and C). However, at day 7 p.i. the lungs of ferrets inoculated with NL/2631-R223 had not further deteriorated, whereas the percentage of affected lung tissue had increased to 50% in the NL/602 inoculated animals (Figure 3B). This higher score for affected lung tissue in the NL/602-inoculated animals was also reflected by the higher score for the degree of alveolitis at day 7 p.i. compared to day 4 p.i., whereas the alveolitis scores in the NL/2631-R223-inoculated animals at day 4 and 7 p.i. were similar. To recapitulate, both viruses replicated to the same extent in the respiratory tract of ferrets, but the NL/2631-R223 seemed less pathogenic compared to the NL/602 virus.

Despite the moderate pathogenicity of NL/2631-R223, this virus transmitted to 3 out of 4 exposed animals via aerosols or respiratory droplets (Figure 4B). This result is comparable to the data obtained from NL/602, in which 4 out of 4 exposed animals got infected (Figure 4D) [27]. This ferret transmission model was designed as a qualitative model for transmission and with the limited number of animals, quantitative information on virus transmission could not be obtained. Therefore, from these experiments it was concluded that both NL/2631-R223 and NL/602 transmitted via aerosols or respiratory droplets, although a delay in virus shedding by approximately 1 day was observed in the naïve animals exposed to NL/2631-R223 (Figure 4B and D).

When the impact of the single I223R mutation in the recombinant NL/602 backbone on in vitro replication kinetics was evaluated, a reduction in virus replication in MDCK cells was noticed (Figure 1). In addition, the initial virus replication of NL/602-I223R on MDCK-SIAT1 cells started 6 to 12 hours later as compared to its parental virus (Figure 1B).

These results suggested that compensatory mutations may be required to accommodate the isoleucine to arginine substitution at position 223 in NA and emphasizes the importance of the viral backbone used to study resistance-associated mutations. However, when recNL/602-I223R was tested in the ferret transmission model, the virus transmitted to 2 out of 2 exposed animals (Figure 5B). When these results were compared with transmission data of recNL/602 (Figure 5D) [32], no differences were found in the onset of virus shedding and virus titers that were detected in the collected throat and nose swabs from the exposed animals. This observation demonstrates that the transmissibility of recNL/602-I223R is not significantly diminished or can at least not be studied using a ferret transmission model.

Although these results suggest that introduction of the I223R does not attenuate the virus, it cannot be ruled out that other mutations than 223R in NL/2631-R223 may have compensated for the initial loss of fitness due to the I223R mutation. Sequence comparison revealed 5 amino acid differences between NL/2631-R223 and NL/602. The only amino acid substitution that is located near the active site of the neuraminidase is at position 248, where NL/602 harbors an aspartic acid and NL/2631-R223 an asparagine. Interestingly, neighboring residue 247 has been linked to NAI resistance in combination with the H275Y mutation [31]. Further research is needed to study the I223R resistance mechanism in competitive mixture experiments and potential co-mutations on a molecular level [33].

To note, small differences between NL/602 and recNL/602 could be observed in replication capacity and transmission patterns in ferrets (Figure 4 and 5). Previously, differences were also found in pathogenesis experiments, where the wild type NL/602 was detected more abundantly in the lower airways of ferrets than recNL/602 [34]. These observed differences may be a result of the use of a virus isolate rather than a virus generated by reverse genetics and to a different batch of ferrets used in the different studies. A direct comparison between virus isolates and recombinant viruses can, therefore, not be made.

The different inoculation routes and inoculation doses used for influenza research is subject of debate. The intratracheal route of inoculation is often used to study pathogenicity or to study the efficacy of vaccines to prevent lower respiratory tract infection. In contrast, the intranasal route of inoculation is used when transmissibility is studied. Unfortunately, these inoculation routes and inoculation doses do not accurately mimic the natural way of infection and may mask the fitness differences between the drug-resistant and drug sensitive viruses.

However, the recipient animals in the transmission experiment are infected via the natural route; aerosols or respiratory droplets shed by the donor ferret. The virus secretion pattern, which is the combination of the amount of virus secreted and the duration of virus shedding from the upper respiratory tract, of animals exposed to recNL/602 and rec/NL602-I223R are similar. This suggests that no marked differences in viral fitness are introduced by the single I223R mutation.

The present study demonstrates for the first time that a 2009 pandemic A/H1N1 clinical isolate containing a resistance mutation at position 223 in the NA is not attenuated in its replication capacity and transmissibility in a ferret model. Although the pathogenicity of this virus seems less severe compared to a relevant reference virus in the ferret model, it is unclear whether this moderate pathogenicity has implications for infections with multidrug-resistant viruses in humans. Continuous

surveillance is needed to monitor the emergence of (novel) influenza viruses with reduced susceptibility to the NAIs or mutations that may facilitate the emergence of circulating multi drug resistant influenza viruses.

4 Materials and Methods

4.1 Ethics statement

Animals were housed and experiments were conducted in strict compliance with European guidelines (EU directive on animal testing 86/609/EEC) and Dutch legislation (Experiments on Animals Act, 1997). All animal experiments were approved by the independent animal experimentation ethical review committee 'stichting DEC consult' (Erasmus MC permit number EUR1821) and were performed under animal biosafety level 3+ conditions. Animal welfare was observed on a daily basis, and all animal handling was performed under light anesthesia using ketamine to minimize animal suffering. Influenza virus seronegative 6-month-old female ferrets (Mustella putorius furo), weighing 800–1000 g., were obtained from a commercial breeder.

4.2 Cells and viruses

Madin-Darby Canine Kidney (MDCK) cells were obtained from American Type Culture Collection. MDCK-SIAT1 cells, constitutively expressing the human 2,6-sialyltransferase (SIAT1), were kindly provided by Professor H.D. Klenk, Philipps University Marburg [35]. Both cell lines were cultured in Eagle's minimal essential medium (EMEM) (Lonza, Breda, The Netherlands) supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin, 100 µg/ml streptomycin, 2mM glutamine, 1.5mg/ml sodium bicarbonate (Cambrex), 10 mM HEPES (Lonza) and non-essential amino acids (MP Biomedicals Europe, Illkirch, France). In addition, MDCK-SIAT1 cells were cultured in the presence of 1 mg of antibiotic G418/ml. Influenza virus A/Netherlands/2631_1202/2010 (NL/2631-R223) was isolated from a 5-year-old immune compromised child [25]. Clonal virus of this isolate was obtained by passaging this virus 3 times under limiting diluting conditions in MDCK cells. Full genome sequencing after the last MDCK passage confirmed the absence of mutations. Influenza A/Netherlands/602/2009 (NL/602) was characterized previously [27]. All eight segments of this virus were cloned in a bidirectional reverse genetics plasmid pHW2000 and used to generate recombinant viruses by reverse genetics as described previously [36]. The I223R mutation was introduced in the NA gene of NL/602 using QuickChange multi site-directed mutagenesis kit (Stratagene, Leusden,

The Netherlands) resulting in recombinant viruses recNL/602-I223R. The presence of this mutation was confirmed by sequencing.

4.3 Virus titrations

Virus titers in nasal and throat swabs, homogenized tissue samples, or samples for replication curves were determined by endpoint titration in MDCK cells. MDCK cells were inoculated with 10-fold serial dilutions of each sample, washed 1 hour after inoculation with phosphate-buffered saline (PBS), and grown in 200 µl of infection medium, consisting of EMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1.5 mg/ml sodium bicarbonate, 10 mM HEPES, nonessential amino acids, and 20 µg/ml trypsin (Lonza). Three days after inoculation, the supernatants of inoculated cell cultures were tested for agglutinating activity using turkey erythrocytes as an indicator of virus replication in the cells. Infectious-virus titers were calculated from 4 replicates by the method of Spearman-Kärber [37].

4.4 Replication curves

Multi-cycle replication curves were generated by inoculating MDCK or MDCK-SIAT1 cells at a multiplicity of infection (MOI) of 0.001 50% tissue culture infectious dose (TCID50) per cell. One hour after inoculation, at time point 0, the cells were washed once with PBS, and fresh infection medium was added. The supernatants were sampled at 6, 12, 24, and 48 h post infection and the virus titers in these supernatants were determined by means of endpoint titration in MDCK cells.

4.5 Animal experiments

Pathogenesis

The pathogenesis experiment was done as described previously with some minor changes in the protocol [29]. On day 0, the ferrets were inoculated intratracheally with 106 TCID50 of NL/602 or NL/2631-R223. Throat and nose swabs were collected daily to determine virus excretion from the upper respiratory tract. Animals were weighted daily as indicator of disease and observed for clinical signs. Three animals from each group were euthanized and necropsied at days 4 and 7, and trachea and lung samples were collected to study virus distribution.

Pathology

Necropsy was done by opening the thoracic and abdominal cavities and examining all major organs. Whilst inflated, all lung lobes (left cranial lobe, left caudal lobe, right cranial-, middle- and caudal lobes and accessory lobe) were evaluated. The extent of consolidation was estimated by visual assessment. The lungs were weighed after the trachea was removed at its bifurcation. The relative lung weights were calculated as proportion of the body weight on day of death (lung weight/body weight x 100). Tissues (~0.4 g) from the right lung were collected for determination of lung virus titers at day 4 and 7 p.i. The left lung and trachea were collected for histological examination, and immersed for fixation in 10% neutral-buffered formalin. All samples were sectioned in a standardized way (a total of 4 lung sections per animal; 1 cross section and 1 longitudinal section from both the left cranial and left caudal lobe, and 1 central tracheal cross section) and routinely processed, paraffin embedded and cut to 4 µm hematoxylin and eosin (H&E) stained slides. The samples were histologically examined for the character and severity of influenza virus–associated lesions without knowledge of the identity of the animals. The extent of alveolitis/alveolar damage (0 = 0%, 1 = <25%, 2 = 25-50%, 3 = >50% of a section) and the severity of alveolitis, bronchi(oli)tis (including bronchial submucosal glands) and tracheitis (0 = none, 1 = few, 2 = moderate number, 3 = many inflammatory cells) were scored per slide. The overall histology score for alveolitis is the sum of the scores for the extent and severity of the alveolitis (score 0 to 6).

Transmission

The transmission experiments were done as described previously [27]. The transmission cages were specifically designed to allow transmission experiments to be conducted in negatively pressurized isolator cages (1.6 m × 1 m × 1 m). On day 0, 4 or 2 female ferrets were housed individually in transmission cages (30 cm × 30 cm × 55 cm, W x H x L) and inoculated intranasally with 106 TCID50 of NL/602, NL/2631-R223, recNL/602 or recNL/602-I223R respectively, divided over both nostrils (2×250 µl). On day 1, 4 or 2 naïve female ferrets were individually placed in a transmission cage adjacent to an inoculated ferret, separated by two stainless steel grids. Negative pressure within the isolator cage is used to direct a modest (<0.1 m/sec) flow of high efficiency particulate air (HEPA) filtered air from the inoculated to the naive ferret. This experimental setup was designed to prevent direct contact or fomite transmission, but to allow airflow, thereby permitting transmission via aerosol or respiratory droplets. Nasal and throat swabs were collected on day 0, 1,

2, 3, 5 and p.i. from the inoculated ferrets and on days 0, 1, 2, 3, 5 and 7 p.e. from the naïve ferrets. Inoculated ferrets were euthanized at day 7 p.i., and naive ferrets that were found positive by reverse transcription polymerase chain reaction at day 7 p.e. were also euthanized [38]. Naïve animals that remained negative for virus excretion throughout the experiment were euthanized at day 15 p.e., and a blood sample was collected for serology. Virus titers in the collected swabs were determined by means of endpoint titration in MDCK cells.

4.6 Statistical analysis

For the pathogenesis experiment, statistical analysis was done for each time point, until 4 days after inoculation (when there were still 6 animals present in each group). The Mann-Whitney-U test was used to compare weight losses and virus shedding of the six animals in both groups. P-values less than 0.05 were considered significant.

4.7 Acknowledgments

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4.8 Author Contributions

Conceived and designed the experiments: EvdV EJVK KJS MS ADMEO RAMF CABB SH. Performed the experiments: EvdV EJVK KJS ML AvdL EJAS LML GvA. Analyzed the data: EvdV EJVK TK RAMF CABB SH. Contributed reagents/materials/analysis tools: MS CABB. Wrote the paper: EvdV EJVK TK RAMF CABB SH.

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Chapter 5.2

Prolonged Influenza Virus Shedding and Emergence of Antiviral Resistance in Immunocompromised Patients and Ferrets

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Abstract

Immunocompromised individuals tend to suffer from influenza longer with more serious complications than otherwise healthy patients. Little is known about the impact of prolonged infection and the efficacy of antiviral therapy in these patients. Among all 189 influenza A virus infected immunocompromised patients admitted to ErasmusMC, 71 were hospitalized, since the start of the 2009 H1N1 pandemic. We identified 11 (15%) cases with prolonged 2009 pandemic virus replication (longer than 14 days), despite antiviral therapy. In 5 out of these 11 (45%) cases oseltamivir resistant H275Y viruses emerged. Given the inherent difficulties in studying antiviral efficacy in immunocompromised patients, we have infected immunocompromised ferrets with either wild-type, or oseltamivir-resistant (H275Y) 2009 pandemic virus. All ferrets showed prolonged virus shedding. In wild-type virus infected animals treated with oseltamivir, H275Y resistant variants emerged within a week after infection. Unexpectedly, oseltamivir therapy still proved to be partially protective in animals infected with resistant virus. Immunocompromised ferrets offer an attractive alternative to study efficacy of novel antiviral therapies.

Author summary

Immunocompromised patients, such as transplant recipients on immune suppressive therapy, are a substantial and gradually expanding patient group. Upon influenza virus infection, these patients clear the virus less efficiently and are more likely to develop severe pneumonia than immunocompetent individuals. Existing antiviral strategies are far from satisfactory for this patient group, as they show limited effectiveness with frequent emergence of antiviral resistance. For ethical and practical reasons antiviral efficacy studies are hard to conduct in these patients. Therefore, we developed an immunocompromised ferret, mimicking an immune suppressive regimen used for solid organ transplant recipients. Upon infection with 2009 pandemic influenza A/H1N1 virus these animals, like immunocompromised patients, develop severe respiratory disease with prolonged virus excretion. Interestingly, all immunocompromised ferrets on oseltamivir therapy excreted oseltamivir resistant viruses (H275Y) within one week after start of treatment. Furthermore, high dose oseltamivir therapy still proved to be partially effective against these oseltamivir resistant viruses. These immunocompromised ferrets provide a useful tool in the development of novel antiviral approaches for immunocompromised patients suffering from influenza.

1 Introduction

During the first 12 months of the 2009 influenza A/H1N1 virus (pH1N1) pandemic an estimated 284,000 patients died and hospitalization rates were considerably higher than for seasonal influenza [1]. Although many severe cases were observed in otherwise healthy patients under 50 years of age, most fatal cases during this pandemic were patients belonging to the traditional high risk groups for developing severe disease, like very young children, the elderly and chronically ill patients [2]. In these patients, which in most cases have sub-optimal immune responses, influenza viruses often persists longer and tend to spread more readily into the lower respiratory tract [3-6]. These observations are in contrast to those in otherwise healthy patients younger than 65 years, for which influenza usually remains a self-limiting upper respiratory tract infection [7,8].

It has been recognized that every winter season a significant number of immunocompromised patients are admitted to a hospital with influenza [9,10]. For example, of the total 335 influenza A virus infected patients being diagnosed upon admission to ErasmusMC - a tertiary university hospital - between August 2009

and July 2012, 113 (34%) had an underlying condition that classified them as being immunocompromised [11]. Since immunocompromised patients are more likely to acquire influenza [12,13], showing relatively high influenza-associated mortality [4,14-24], effective antiviral prophylaxis and treatment protocols are of crucial importance for these patients.

Unfortunately, present antiviral strategies are merely based on clinical trials conducted in otherwise healthy patients [25], since randomized clinical trials in immunocompromised patients are, for both ethical and practical reasons, difficult to perform. Furthermore, the degree and cause of a patient's immunocompromised state is variable and consequently, clinical outcome of infection may vary accordingly.

Although antiviral therapy has a documented positive effect on clinical outcome in immunocompromised patients [3,26-28], current antiviral strategies are far from satisfying. This may be explained not only by the lack of evidence based strategies adjusted for immunocompromised patients [4,28], but also by the oral and inhaled administration routes which complicate administration in very young and critically ill patients [29-33]. Furthermore, since physicians may not consider the diagnosis influenza initially, antiviral therapy is often initiated beyond 48 hours [34], and accompanied by the emergence of an oseltamivir resistant virus [35].

We investigated the incidence of prolonged virus shedding and emergence of antiviral resistance by studying the course of infection of the immunocompromised patients infected with pH1N1 virus treated in our university hospital. These phenomena were studied in more detail in immunocompromised ferrets experimentally infected with pH1N1 virus, that closely mimic immunocompromised patients with influenza. These ferrets all showed prolonged virus shedding and emergence of antiviral resistance. Unexpectedly, the group of immunocompromised ferrets treated with an oseltamivir dose equivalent to a 450mg dose (twice daily) in humans, had a higher survival rate than similarly untreated animals when infected with an oseltamivir resistant virus.

2 Results

2.1 Prolonged shedding and resistance development in immunocompromised patients

We quantified prolonged virus replication and resistance development in immunocompromised patients retrospectively, who were infected between August 2009 and July 2012, and hospitalized in our tertiary hospital with influenza A virus. Among the 189 RT-qPCR confirmed influenza A virus infected patients (median age=22.3, range=0-81), 71 (38%) patients were classified as being immunocompromised (Table 1). These patients were either cancer patients on chemotherapy (CC), solid organ transplant (SOT) or patients with an auto-immune disease on immune suppression, HIV-infected patients or patients with another cause of compromised immune status. From 37 (52%) patients, no follow up samples were taken (physician's choice) and 18 (25%) patients had cleared the virus within

Table 1 Immune status and antiviral therapy of 2009 pandemic influenza A virus infected patients hospitalized in our tertiary hospital between August 2009 and July 2012

Total hospitalized influenza A virus infected patients[a,b,c]		**n=189 (%)**
Immunocompromised patients		71 (38)
Cause of compromised immune status (n=71)		
Cancer chemotherapy	41 (58)	
Solid organ transplant recipients	12 (17)	
Auto-immune disease	7 (10)	
HIV/AIDS[d]	3 (4)	
Other[e]	8 (11)	
Antiviral therapy	*Immunocompromised*	*All Hospitalized*
Any	54 (76)	137 (72)
Oseltamivir monotherapy	44 (62)	117 (62)
Zanamivir monotherapy	1 (1)	1 (<1)
Combination therapy	9 (13)	19 (10)
None	17 (24)	50 (26)
Unknown[f]	None	2 (1)

[a]Influenza A virus was detected by an influenza A virus real-time quantitative polymerase-chain-reaction (RT-qPCR) detection assay in respiratory specimens from a total number of 335 admitted patients between August 2009 and July 2012. [b]Clinical data were extracted from hospitalized patient records. [c]Age (mean=29.4, median=22.3, range=0-81). [d]Only classified as immunocompromised when $CD4^+$ cell count was lower than 350 cells per µl. [e]Three patients used high doses of corticosteroids for an endocrine, respiratory or nephrological disease. Three patients had a primary immunodeficiency and 1 patient had a congenital syndrome. One patient was prematurely born. [f]Two patients had been transferred to another hospital.

14 days. Of the immunocompromised patients from whom follow up was available, 11 (15%) had a pH1N1 virus infection and were shedding virus for more than 14 days, despite receiving oseltamivir or oseltamivir/zanamivir therapy (Figure 1). Prolonged replication of influenza A/H3N2 virus was found in 5 (7%) cases (data not shown). In 5 (7%) of the immunocompromised patients with an influenza pH1N1 virus infection the oseltamivir resistance mutation H275Y in the neuraminidase was detected by RT-PCR during oseltamivir mono or oseltamivir/zanamivir combination therapy.

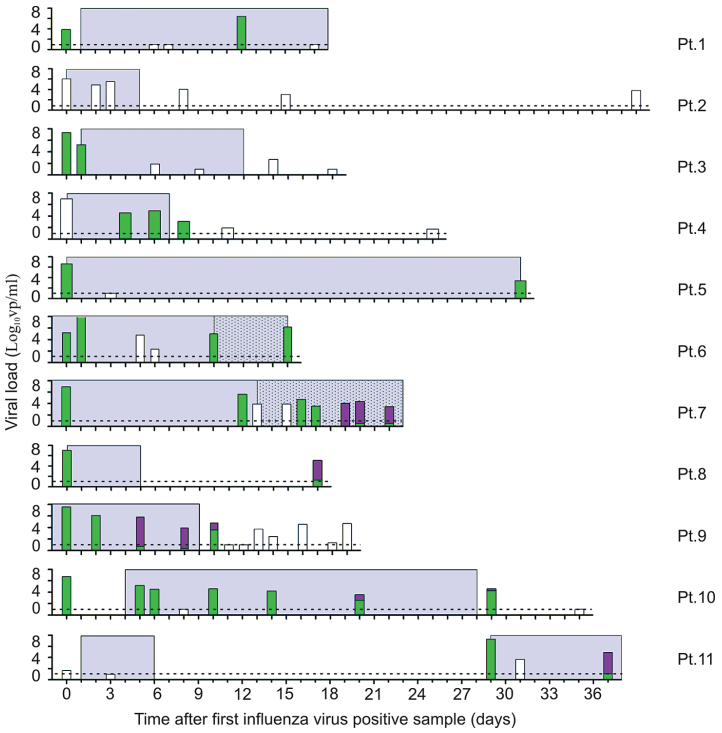

Figure 1 Viral load, antiviral therapy and resistance detection in immunocompromised patients hospitalized with a prolonged pH1N1 virus infection

From 11 immunocompromised patients the viral load in respiratory specimens obtained during the courses of illness are shown in bars. Patients 1, 2 and 10 were solid organ transplant patients. Patients 3, 4, 5, 7, 8, 9 and 11 were cancer patients on chemotherapy. Patient 6 was treated for cerebral vasculitis. The dotted line in each figure indicates the lower limit of detection of the influenza A virus RT-qPCR detection assay. Data are presented as the number of virus particles per ml. Bar colours indicate the absence (green) or presence (magenta) of the H275Y oseltamivir resistance mutation as detected by RT-PCR. If both genotypes were detected in a sample, the proportion is stacked. Bars are coloured white for those respiratory samples in which the H275Y genotype could not be determined or in cases when genotyping was not performed. The duration of oseltamivir monotherapy and oseltamivir/zanamivir combination therapy is indicated, respectively, by blue and dotted blue shading.

2.2 Immunosuppressive treatment of ferrets

We investigated whether the observations on prolonged virus replication and emergence of antiviral resistance in immunocompromised patients could be mimicked in ferrets receiving a cocktail of immunosuppressive drugs similar to that administered to SOT patients (combination of mycophenolate mofetil (MMF), tacrolimus and predisolone). First, pharmacokinetics were studied following oral administration of ferrets (n=4) given 20mg/kg MMF, 1mg/kg tacrolimus and 8mg/kg prednisolone (Table 2 and Supporting information Figure S1). From the concentration over time profiles, peak (C_{max}) levels for MPA, the active form of MMF, were 65±30µg/mL with trough (C_{12}) levels becoming undetectable after 8 hours. Peak and trough tacrolimus levels were 86±30ng/mL and 14±30ng/mL respectively. We determined area under the curve (AUC_{0-12}) values of 54±14µg·h/mL for MPA and 438±265ng·h/mL for tacrolimus. In humans, MPA AUC_{0-12} values between 30-60µg·h/mL and tacrolimus trough levels between 5-15ng·h/mL are proposed[36,37]. Because of a shorter MPA half life ($t_{1/2}$) and high tacrolimus peak levels, we further optimized the ferret regime by administration of the cocktail every 12 hours containing half of the initial tacrolimus dose (0.5mg/kg).

Next, the effects of this cocktail on the ferret immune competence were studied. To this end, 6 groups of ferrets were inoculated intratracheally on day 0 with a wild type or H275Y mutant pH1N1 virus (Figure 2A). Of note, both viruses

Table 2 Steady-state (day 4) pharmacokinetic parameters for mycophenolic acid, tacrolimus, oseltamivir phosphate and oseltamivir carboxylate in ferrets (n=4)

	Dose (mg/kg)[a]	T_{max} (h)	$t_{1/2}$ (h)	C_{max} (ng/mL)	$C_{t=12}$ (ng/mL)	AUC_{0-12} (ng·h/mL)
Immune suppressants						
Mycophenolic acid (MPA)	20.0	0.33 ± 0.18	0.73 ± 0.36	65 ± 20 (x10³)	< 0.1	54 ± 20[a] (x10³)[b]
Tacrolimus	1.0[c]	0.79 ± 0.58	3.60 ± 2.29	86 ± 30	14 ± 14[d]	438 ± 265
Oseltamivir						
Oseltamivir phosphate (OS)	10.0	1.00 ± 0.71	3.22 ± 0.51	2063 ± 579	16 ± 17	6491 ± 1300
Oseltamivir carboxylate (OSC)		4.00 ± 0.00	7.99 ± 1.17	3052 ± 448	833 ± 247	20501 ± 4005[e]

[a]Oral administration twice daily. [b]Proposed human plasma levels (AUC_{0-12}): 30-60µg·h/mL[36]. [c]Dose adjusted to 0.5mg/kg in the infection experiment. [d]Proposed human whole blood trough levels ($C_{t=12}$): 5-15ng/mL[37]. [e]Previously determined human plasma levels (AUC_{0-12}): 3220 ± 982ng·h/mL (75mg b.i.d.), 10100 ± 2710ng·h/mL (225mg b.i.d.) and 19900 ± 4840ng·h/mL (450mg b.i.d.)[53].

had been isolated by clonal culturing from the same respiratory sample taken from an immunocompromised patient on oseltamivir therapy [38]. Three days earlier, immunosuppressive therapy was started for all animals, except for the animals in control groups 1 and 4. On day 13, blood was collected and influenza antibody titers were determined in the ferret sera (Figure 2B). As compared to day 0, both the animals in the control groups (groups 1 and 4) and those on immunosuppressive therapy (groups 2, 3, 5 and 6), developed serum hemagglutination inhibiting (HI) antibody titers against the inoculated virus. However, antibody titers in the animals

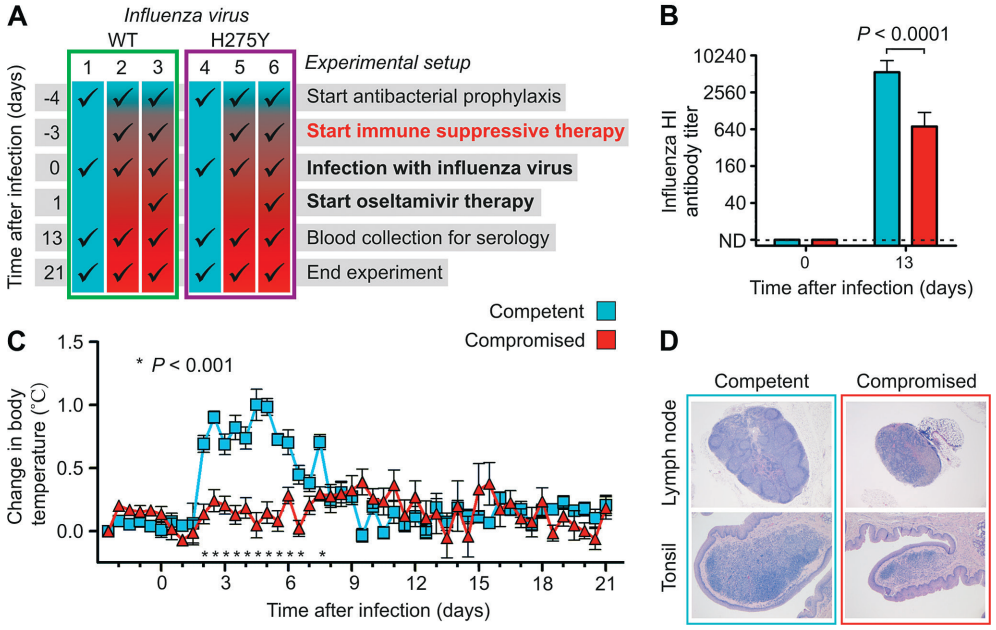

Figure 2 Ferrets on immune suppressive therapy show defective immune function

(a) Schematic of the experimental setup in which 6 groups of ferrets (n=6) were infected on day 0 with wild type (WT; groups 1-3; green) or oseltamivir resistant (H275Y) mutant virus (groups 4-6; magenta). Three days before, immune suppressive therapy was started and added to the antibacterial cocktail of groups 2, 3, 5 and 6. Oseltamivir therapy was added to the drug regime of groups 3 and 6, 24 hours after infection. (b) Reduction of pH1N1 virus specific serum hemagglutination inhibiting (HI) antibody titers in the remaining (n=14) immunocompromised ferrets compared to control (n=12) ferrets. Data are mean ± s.e.m. The *P* value was calculated by Mann-Whitney U test. (c) Body temperature profiles show the absence of body temperature rise during the acute stage of infection in immunocompromised ferrets. Data are mean ± s.e.m. The *P* value was calculated by unpaired Student's *t* test. (d) Lymphoid tissues show deficient lymphoid follicle formation in lymph nodes and lymphocyte depletion in the tonsils of immunocompromised ferrets. Representative photomicrographs of tracheobronchial lymph nodes (original magnification 25x) and tonsils (original magnification 50x). Tissues were stained with hematoxylin and eosin (H&E).

on immunosuppressive therapy were significantly lower ($P < 0.0001$) (Figure 2B and S2A-D). As an indication of an activated immune response, body temperatures of all animals in the control groups raised from day 2, as compared to baseline, and remained higher until day 6 (Figure 2C and S2E-F). No significant change in body temperature was detected in the infected animals on immunosuppressive therapy, as is often seen in immunocompromised patients[39]. Finally, pathological examination of lymphoid tissues of animals sacrificed on day 21 revealed deficient lymphoid follicle formation in tracheobronchial lymph nodes and lymphocyte depletion in the tonsils of animals on immunosuppressive therapy (Figure 2D).

2.3 Prolonged virus shedding in immunocompromised ferrets

The animals of groups 1, 2 and 3 had been inoculated with oseltamivir sensitive (wild type; H275) and the animals in groups 4, 5 and 6 with oseltamivir resistant virus (mutant; H275Y) (Figure 2A and 3). On day 2 post infection (p.i.), all 6 animals were found positive by virus culture from their throat (Figure 3 and S3). On day 3 p.i., virus was detected in the nose of five (group 1) wild type inoculated animals and one (group 4) animal inoculated with oseltamivir resistant virus (Figure S3A and C). In the animals infected with wild type virus, which were treated with oseltamivir (group 3), replication of virus in the nose was delayed as compared to the oseltamivir treated animals inoculated with mutant virus (group 6). By day 9, all animals in the control groups had cleared the virus. The surviving immunocompromised ferrets in the other groups were shedding virus for at least another 7 days, except for those in group 5. These animals had cleared the virus from the nose and throat by day 13 (Figure 3C and D).

2.4 Oseltamivir therapy and emergence of oseltamivir resistance

In the animals of groups 3 and 6, oseltamivir therapy (10mg/kg twice daily) was started 24 hours after infection and continued for 21 days (Table 2 and S2C). Until day 7, when still 6 animals were alive in each group, the viral loads in the oseltamivir treated animals infected with wild type virus was significantly lower on days 3, 4 and 5 in the nose and on days 3 to 7 in the throat ($P < 0.001$). Such difference was not observed in the animals infected with oseltamivir resistant virus (Figure 3C and D).

Emergence of oseltamivir resistance was studied in the animals infected with wild type virus (groups 1, 2 and 3) using an RT-PCR assay specifically detecting the H275Y oseltamivir resistance mutation (Figure 4)[40]. From day 8 onward, the H275Y mutation emerged in the virus population of all oseltamivir treated animals in

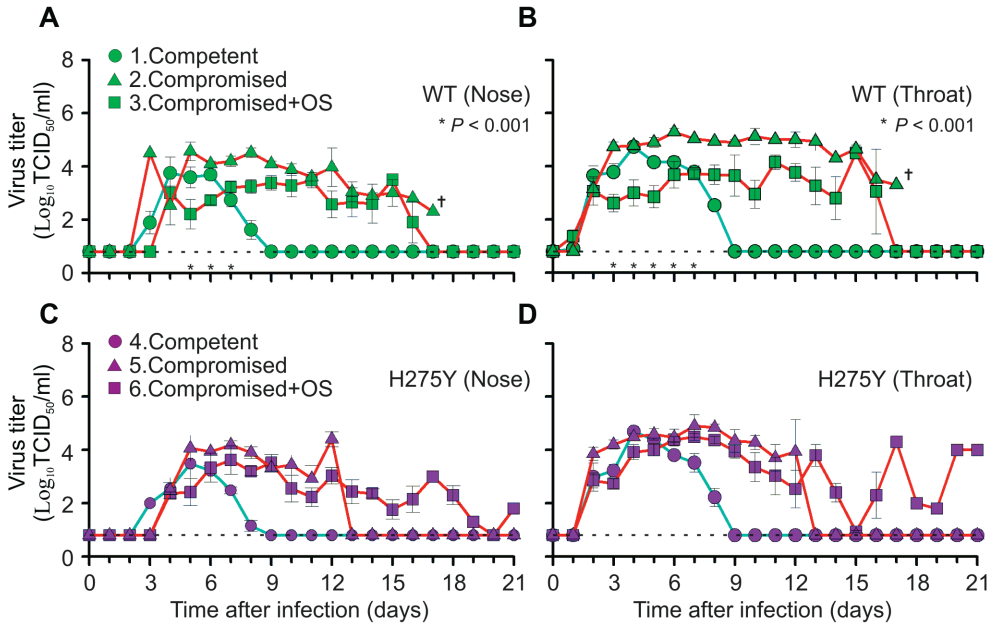

Figure 3 Prolonged virus replication in the upper respiratory tract of immunocompromised ferrets

Influenza virus titers were determined in nose and throat swabs daily taken from immunocompetent (blue lines) and immunocompromised ferrets (red lines). The animals were infected either with wild type (WT; green; a, b) or mutant virus (H275Y; magenta; c, d). The dotted line in each figure indicates the LLOD. Data are mean ± s.e.m. *P* values were calculated, until day 7, when all animals (n=6) were still present in each group, by two-tailed Mann-Whitney U test comparing virus titers between untreated and oseltamivir (OS) treated immunocompromised ferret groups. By day 17, no ferrets were remaining in group 2.

both the nose and throat. The H275Y mutant became the major genotype 2 or 3 days later. No oseltamivir resistant viruses were detected in the immunocompromised wild-type virus infected animals that did not receive oseltamivir.

2.5 Increased survival of immunocompromised animals treated with oseltamivir.

Unexpectedly, oseltamivir treatment appeared to have a positive effect on the proportion of surviving immunocompromised animals and animal body weight loss (Figure 5). Without oseltamivir treatment, half of the wild type infected group of animals had succumbed by day 11 and none of the remaining animals survived the complete 21 day experiment (0/6) (Figure 5A). However, when oseltamivir therapy was started 24 hours after infection, half of the animals were still alive at day 16 and

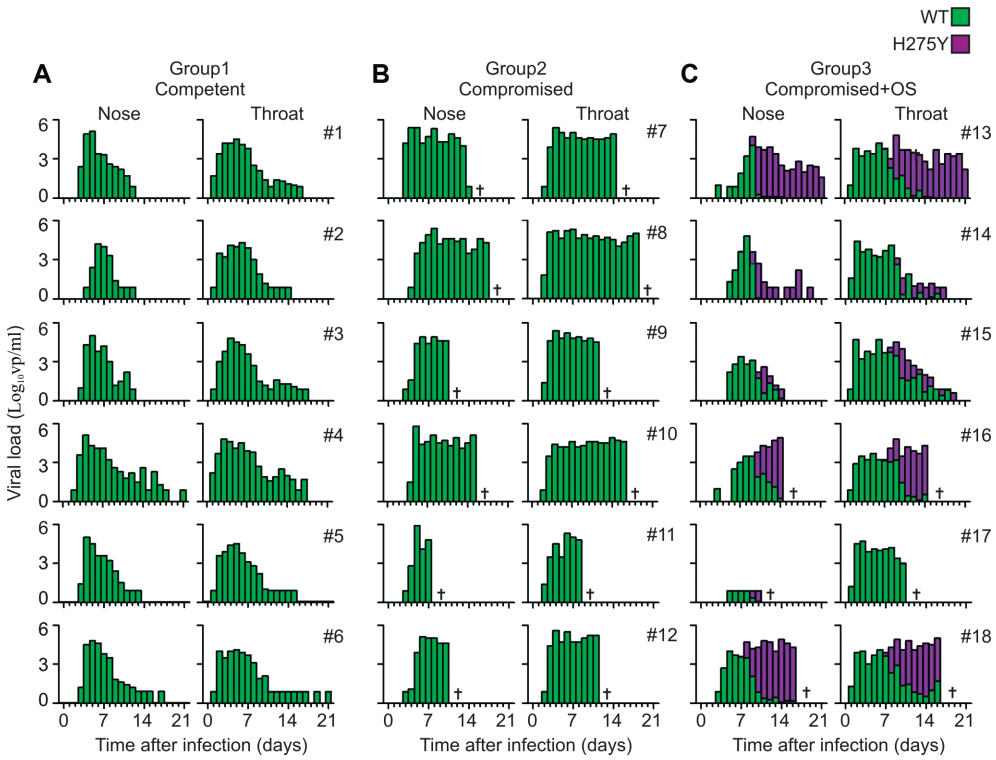

Figure 4 Emergence of oseltamivir resistance mutation H275Y in influenza virus quasispecies from ferrets infected with wild type virus

Viral RNA was detected using RT-qPCR in nose and throat swabs taken from immunocompetent (group 1; **a**) or immunocompromised ferrets (groups 2; **b** and 3; **c**). Ferrets in group 3 were treated with oseltamivir (OS). Bar colours indicate the absence (green) or presence (magenta) of the oseltamivir resistance mutation H275Y as detected by RT-PCR[40]. If both genotypes were detected in a sample, the proportion is stacked.

these remaining animals all survived until day 21 (3/6). Of the immunocompromised ferrets, which were infected with the resistant virus (Figure 5B), half of the untreated animals had died before day 13, but two of the untreated animals and four of the treated animals survived the complete 21 day experiment.

In addition, oseltamivir treatment appeared to have a protective effect on body weight loss of the immunocompromised animals (Figure 5C and D). This trend was observed for both wild type and oseltamivir resistant virus infected groups, although statistical significant differences was found only for the wild type infected animals on day 12 when three animals were still alive (groups 2 versus 3; $P = 0.03$), and not for the oseltamivir resistant virus infected animals (groups 5 versus 6; $P = 0.07$).

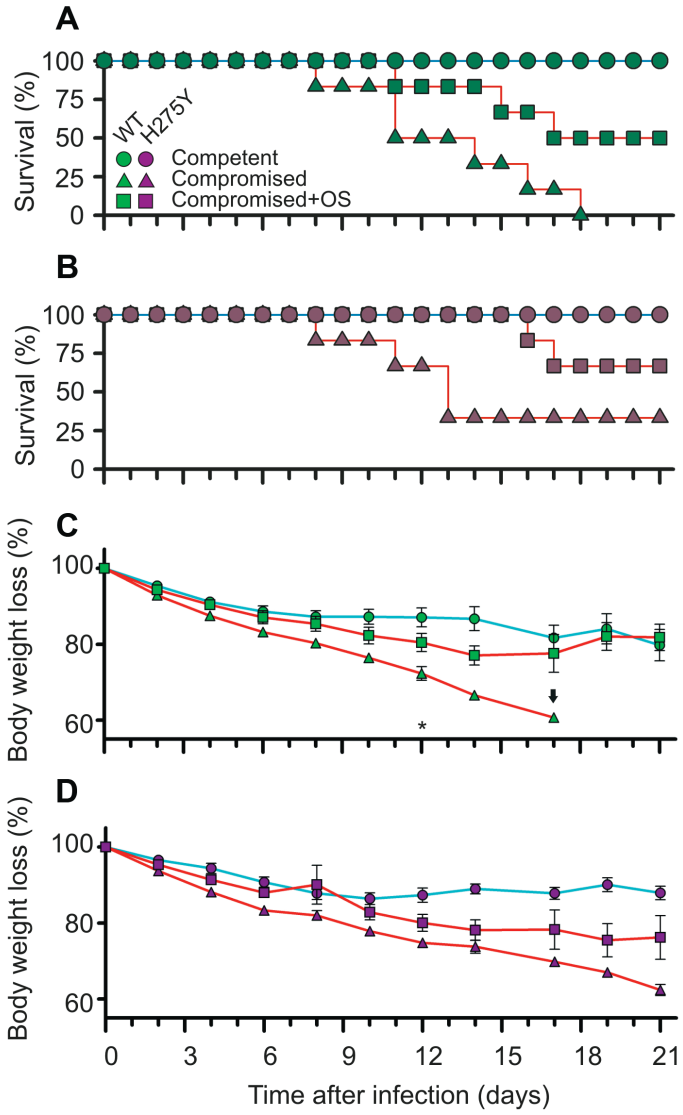

Figure 5 Increased survival and reduced loss of body weights of immunocompromised ferrets treated with oseltamivir

Kaplan-Meier survival curves for groups of immunocompetent ferrets (circles), immunocompromised ferrets (triangles) and immunocompromised ferrets treated with oseltamivir (squares) (a;b). Groups of ferrets were infected with wild type (green;a;c) or oseltamivir resistant (H275Y) mutant virus (magenta;b;d). Body weights are displayed from day 0 to day 21, with a two or three days interval (c;d). Data points represent mean ± s.e.m. of percentage body weight loss. Body weights at day 0 were set at 100%. The arrow indicates data point of only a single animal. The asterisk indicates significant difference between untreated and oseltamivir treated immunocompromised animals ($P = 0.03$). The P value was calculated by a two-tailed Mann-Whitney U test, when at least 3 animals were present in each group.

3 Discussion

Here we show that prolonged influenza virus shedding and the emergence of oseltamivir resistance are two phenomena commonly observed in immunocompromised patients during antiviral therapy. As resistance development is low (19 out of 874 (2.2%)) treated influenza virus infected patients[41], the incidence in immunocompromised patients appears to be considerably higher. Of all 71 immunocompromised patients infected with an influenza A virus in our hospital, at least 16 (23%) (11 pH1N1 and 5 H3N2) showed virus persistence for longer than 2 weeks with 5 of them harbouring oseltamivir resistant virus (5/16) (31%). Because 52% of the included patients were sent home before a virus negative follow-up sample had marked the end of infection, final conclusions on the true incidence of prolonged virus shedding and development of oseltamivir resistant virus cannot be made. However, this observation is in line with previously observed high resistance levels (33%) among paediatric cancer patients [35], and stresses the importance of a thorough evaluation of the currently used antiviral therapies in immunocompromised patients.

Since for both logistic and ethical reasons randomized studies are difficult to perform in often critically ill immunocompromised patients, we showed that prolonged influenza virus replication is also a common feature in immunocompromised ferrets. We observed an absence of a rise in body temperature, reduction of lymphocyte proliferation and follicle formation in ferret lymphoid tissues, which are also hallmarks in immunocompromised patients [42] and found a significant reduction of influenza virus specific antibodies in serum.

In the 2007/2008 H1N1 virus season an H275Y oseltamivir resistant mutant emerged, which had completely overtaken the circulating virus population by the end of 2008 [43]. This introduction of the H275Y mutation disqualified oseltamivir as the first line antiviral drug. It might happen again with pH1N1 virus if it would also harbour the H275Y mutation without loss of viral fitness. Recent reports on clusters of transmitted pH1N1 H275Y mutant viruses are the first indication that this may not be an unlikely scenario [44,45]. Early studies on H275Y pH1N1 viral fitness were performed on viruses isolated shortly after the start of the 2009 pandemic. These viruses were found to be at least slightly compromised in their pathogenicity and replication capacity [46-49]. We also used an early H275Y virus isolate in our experiment [38]. If virus replication is not tempered by adequate immune responses, duration of virus shedding will eventually be restricted by an exhaustion of susceptible host target cells. This then may explain why replication

of this apparently less pathogenic virus lasted longest in our immunocompromised ferrets (Figure 3D). Priority should now be given to study the overall fitness of these recent pH1N1 viruses that appear not to be affected by the H275Y change. The use of our immunocompromised ferrets seems to be a very suitable strategy for this purpose, because subtle fitness costs may be amplified in these animals[50].

In our study, immunocompromised ferrets were treated with an oseltamivir dose of 10mg/kg twice daily. This dose is equivalent to a much higher human dose than the currently recommended dose of 75mg twice daily [51]. We observed that, for animals infected with either wild type or H275Y mutant virus, high dose oseltamivir treatment was still beneficial. Currently the World Health Organisation recommends switching to zanamivir when dealing with such a resistant virus [52]. However, in the light of our data, discontinuation of oseltamivir therapy may not always be the best strategy. An increased oseltamivir dose for the treatment of immunocompromised patients may be considered as a future antiviral strategy. However, obviously this will need further clinical investigation first. Of note, an increased dose of oseltamivir is well tolerated in humans [53]. We observed lower mortality in the treated animals infected with H275Y mutant virus without an observed difference in virus titers in the upper respiratory tract. In these ferrets, oseltamivir carboxylate plasma levels peaked (C_{max}) from 3052ng/ml in 4 hours to 833ng/ml 12 hours after administration (Table 2 and Figure S1C). These levels were still about 100 and 30 times higher than the 50% inhibitor concentration of an H275Y mutant virus, which is roughly 30ng/ml (~100nM) [54]. It is therefore plausible that oseltamivir therapy reduced H275Y mutant virus titer in the lungs, but not in the upper respiratory tract. This had been observed before and could explain the lower mortality rates in the treated animals [55]. It will therefore be of interest to study penetration of oseltamivir throughout the ferret respiratory tract in more detail, which may be expected to have a direct impact on the effectiveness of the presently recommended human dose in immunocompromised ferrets. In conclusion, both our clinical observations and ferret experiments show that viral clearance cannot be achieved in the immunocompromised host solely by the use of currently used antiviral therapy. Our immunocompromised ferrets may therefore be an excellent alternative to evaluate and explore novel therapeutic and immunization strategies for immunocompromised patients.

4 Materials and methods

4.1 Patient inclusion criteria and diagnostics

We identified patients hospitalized in the Erasmus Medical Centre (ErasmusMC), a large (> 40,000 admissions in 2011) tertiary university hospital in the Netherlands, with an influenza A virus positive respiratory specimen taken between August 2009 through July 2012. Patients had a prolonged virus infection if the virus could still be detected after 14 days. Virological data, patient immune status and administration of antiviral therapy were obtained by reviewing medical records. Immunosuppression was defined as any of the following: receipt of treatment for any cancer, the use of any immunosuppressive medication to prevent transplant rejection or for management of pulmonary or autoimmune conditions, premature birth and below gestational age or a diagnosis of AIDS. Influenza A virus and the H275Y oseltamivir resistance mutation were detected by reverse transcriptase RT-PCR assays. These assays have been described previously[40,56]. Informed consent was waived because patient inclusion was performed retrospectively and data were anonymously stored as agreed by the hospital medical ethical board (MEC-2012-463)

4.2 Viruses used in ferret experiment

Two biologically cloned pH1N1 influenza viruses were used in this study. Both viruses were isolated from an oseltamivir treated patient during the first wave of the pandemic in October 2009. Wild type influenza virus A/Netherlands/1715b/2009 (genbank ID code: CY065810) and H275Y mutant virus were isolated from the original quasispecies by co-cultivation of a respiratory sample in a Madin-Darby Canine Kidney (MDCK) cell culture in a single passage[38]. Biological clones were then obtained by 3 additional MDCK passages performed under limiting virus concentrations. As determined by full-genome Sanger sequencing, the mutant virus contained, additional to mutation H275Y in the neuraminidase, an L233M mutation in PB2 and a V541L mutation in HA.

4.3 Ferrets

Animal were housed and experiments were conducted in strict compliance with European guidelines (EU directive on animal testing 86/609/EEC) and Dutch legislation (Experiments on Animals Act, 1997). The protocol was approved by the independent animal experimentation ethical review committee from the Netherlands Vaccine Institute (permit number 200900201). All experiments were

performed under animal bio-safety level 3 conditions. Animal welfare was observed on a daily basis, and all animal handling was performed under light anaesthesia using a mixture of ketamine and medetomidine to minimize animal suffering. After handling atipamezole was administered to antagonize the effect of medetomidine. All ferrets were eleven-month-old purpose-bred males (body weights between 1562 and 2362g) and were seronegative for Aleutian disease virus and circulating influenza virus (sub) types A/H1N1, A/H3N2 and B virus. The animals were maintained in standard housing and were transferred to negatively pressured glove boxed on the day immunosuppressive therapy was started. They were provided food *ad libidum* with commercial food pellets and water. Approximately three to four weeks prior to the experiment a temperature logger (DST micro-T ultra small temperature logger; Star-Oddi, Reykjavik, Iceland) was placed in the peritoneal cavity of the animals. This device recorded the body temperature of the animals every 10 minutes. From day -8 to day -4, an average baseline temperature was recorded for each group of 6 animals.

4.4 Immunosuppressive, antibiotic and antiviral drugs

The following immunosuppressive drugs were used to suppress the immune system of ferrets: Mycophenolate mofetil (MMF) (CellCept, Roche, The Netherlands) powder for infusion, tacrolimus concentrate (5mg/ml) for infusion (Prograft, Astellas Pharma BV, Leiderdorp, The Netherlands) and prednisolone sodium phosphate (5mg/ml) oral solution (Hospital Pharmacy, UMCN St Radboud, Nijmegen, The Netherlands). All ferrets received an antibiotic prophylaxis of amoxicillin supplemented with 62.5 mg clavulanic acid (250/62.5mg per 5ml) oral suspension (Pharmachemie BV, Haarlem, The Netherlands). Prodrug oseltamivir phosphate, used in the ferret experiments, was kindly provided by Hoffman-La Roche LtD. (Tamiflu, Basel, Switzerland).

4.5 Other chemicals and reagents

Oseltamivir standards for mass spectrometry, oseltamivir phosphate (OS), oseltamivir-d3 (OS-d3), oseltamivir carboxylate (OSC) and oseltamivir carboxylate-d3 (OSC-d3) were purchased from Toronto Research Chemicals (Toronto, Canada). Mycophenolic acid (MPA) standard and internal standards (MPAC) were purchased from Sigma Aldrich (Zwijndrecht, the Netherlands) and from Hoffman-La Roche LtD. respectively. The tacrolimus standards and internal standards (Ascomycin) were purchased from, respectively, Chromsystems and Sigma Aldrich. ULC/MS grade methanol and water containing 0.1 % formic acid were obtained from Biosolve

(Valkenswaard, the Netherlands). Trichloro acetic acid (TCA) and formic acid (> 96%, HCOOH) were obtained from Sigma Aldrich and both were from ACS reagent quality.

4.6 Administration of the drugs

A schematic of the ferret experiment is presented in Figure 2A. Shortly before gavage, drugs were prepared as follows: MMF was dissolved in a 5% glucose solution (Baxter, Unterschleisheim, Germany) to 33mg/ml. Amoxicillin/clavulanic acid was diluted 5 times in water to obtain a suspension containing 50/12.5mg/ml amoxicillin/clavulanic acid. Oseltamivir phosphate was dissolved in 5% glucose to 20mg/ml. These intermediate preparations and the ready-to-use tacrolimus and prednisolone solutions were then used to dose the animals orally and twice daily, as follows: four days before infection, all 6 groups received 10/2.5mg/kg amoxicillin/clavulanic acid diluted in 5% glucose to a final administration volume of 4ml/kg. One day later, antibiotic prophylaxis was supplemented to the regimes of groups 2, 3, 5 and 6 with 20mg/kg MMF, 0.5mg/kg tacrolimus and 8mg/kg prednisolone retaining the administration volume of 4 ml/kg. On day 1, 24 hours after infection, therapy of group 3 and 6 was further supplemented with 10mg/kg oseltamivir phosphate for the remaining 20 days of the experiment. The dose of prednisolone was halved every 7 days from 8mg/kg in the first to 1 mg/kg in the last week.

4.7 Virology and serology

On day 0, three days after start of immunosuppressive therapy, ferrets were intratracheally infected with $1x10^4$ $TCID_{50}$ of wild type (groups 1, 2 and 3) or mutant virus (groups 4, 5 and 6). Each day, pharyngeal and nasal swabs were collected just before administration of the drugs. Swabs were resuspended in 3ml virus transport medium [57], and aliquots were made and used either directly for online detection of viral RNA by RT-PCR or stored at -80°C for retrospective virus titration. An electron microscopy counted influenza A virus stock was run in parallel to convert RT-PCR cycle threshold (CT) values into a viral particle count. Blood samples for serum and plasma were collected on day 13 after infection. Influenza antibody titers were determined as described previously[58].

4.8 Oseltamivir blood plasma levels

Oseltamivir and MMF plasma levels and whole blood tacrolimus levels were determined in a pharmacokinetic pilot study. For 4 days, four groups of ferrets (n=4) received MMF, tacrolimus, or oseltamivir in combination with amoxicillin/

clavulanic acid and prednisolone or as the complete cocktail. On day 4, blood was collected from these animals after 0, 10, 20, 30 minutes and 1, 2, 4, 5, 8 and 12 hours after administration of the drugs in order to determine MMF and tacrolimus levels, as described previously [59]. Ferret oseltamivir plasma levels were determined as described previously with some modifications [60]. Calibrators used for the determination of the calibration curve of OS and OSC were prepared from one single stock solution in plasma each containing 50µg/ml. Calibrators were then prepared by serial dilutions using drug-free plasma. Calibrators for OS and OSC yielded following concentrations: 5000, 1500, 750, 500, 250, 150, 50, 12.5, 2.5 and 0ng/ml (blank). Aliquots of 50µl were spiked with 5µl internal standard solution containing 50µg/ml OS-d3 and OSC-d3. To calibrators and ferret plasma (K_2EDTA) samples, 5µl of a 50 % TCA solution (w/v) was added for plasma protein precipitation. Precipitated plasma proteins were removed by centrifugation for 10 minutes at 2000xg at ambient temperature. De-proteinized plasma samples (20µl) were 2.5 times diluted with ultrapure water and 40 µl of the diluted samples were injected by an auto-sampler (kept at 4 °C) into the liquid chromatography mass spectrometry (LC-MS) system. The calibration curves for OS and OSC showed a linear relationship between the SRM peak area of ratios between OS/OS-d3 and OSC/OSC-d3, respectively (OS; r^2=0.9966 and OSC; r^2=0.9970). The LLOQ and LOD were determined according to FDA guidelines and were, respectively, 2.5 and 1.0ng/ml for both OS and OSC. The LC-MS system used was an 4000 API triple quadruple mass spectrometer containing a Turbo V electron spray ion source (ESI) (AB Sciex, Concord, Canada) operating in the positive ionization mode using selected reaction monitoring (SRM) in combination with a Dionex Ultimate 3000 UHPLC system (Amsterdam, the Netherlands) using an Ascentis Express RP-C18 column (100x2.1, 2.7µm, Supelco, Munich) applying a gradient separation at 30°C.

4.9 Pathology

Samples for histological examination of the tonsils and tracheobronchial lymph nodes were taken to evaluate the immune status and were stored in 10% neutral-buffered formalin. Subsequently, these were routinely processed and embedded in paraffin wax, sectioned at 4µm and stained with haematoxylin and eosin (HE) for examination by light microscopy.

4.10 Statistical analysis

Data are reported as mean ± standard error of the mean (s.e.m). The *P* values for comparison of influenza HI antibody titers in figure 2B, virus titers in figure 3 and body weight loss in figure 5C were calculated using Mann-Whitney U test only if at least three animals were remaining in each experimental group. $P \leq 0.05$ was considered significant.

4.11 Acknowledgements

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5 Supplemental figures

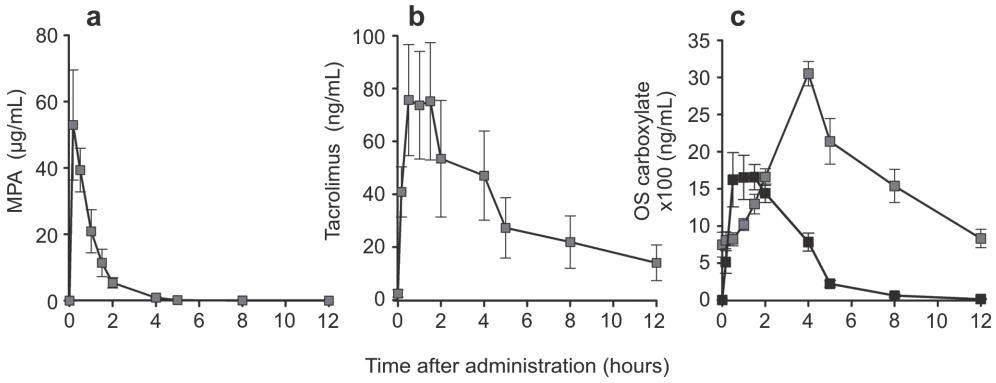

Figure S1 Mean steady state (day 4) pharmacokinetics of MMF, tacrolimus and oseltamivir in ferrets

Twice daily, four ferrets were given a cocktail of antibacterial prophylaxis, immune suppressive therapy (a; b) and oseltamivir phosphate (c) for 4 days. On day 4, blood was collected after 0, 10, and 30 minutes and 1, 2, 4, 5, 8 and 12 hours after the final cocktail was administered. Plasma levels of the active form of mycophenolate mofetil (MMF), the metabolite mycophenolic acid (MPA) (a), whole blood tacrolimus levels (b), oseltamivir phosphate (black squares; c) and its metabolite oseltamivir carboxylate (grey squares; c) plasma levels were determined by mass spectrometry. Area under the curve (AUC_{0-12}), peak (C_{max}) and trough (C_{12}) levels and half-life ($t_{1/2}$) values are presented in table 2. Data are mean ± s.e.m..

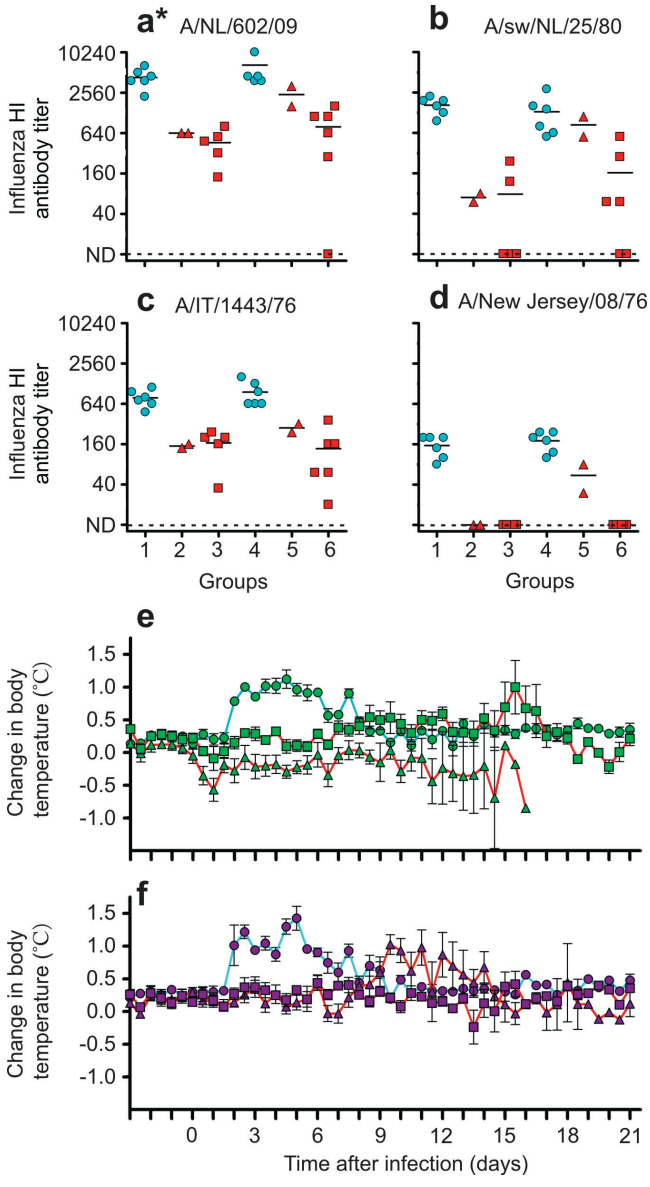

Figure S2 Ferrets on immune-suppressive therapy show reduced antibody titers
Reduction of serum hemagglutination inhibiting (HI) antibody titers against pH1N1 A/NL/602/2009 virus (a) and more distant viruses A/sw/NL/25/80 (b), A/IT/1443/76 (c), and A/New Jersey/08/76 (d). Individual data points represent antibody titers for each animal and horizontal bars represent the mean titer per group. Body temperature profiles show the absence of fever during the acute stage of infection in ferrets infected with wild type (e) or mutant (f) virus. Animals in this experiment were either immunocompetent (circles), immunocompromised (triangles) or immunocompromised and oseltamivir treated (squares). Data are mean ± s.e.m. Data used for Figure 2B are marked with an asterisk.

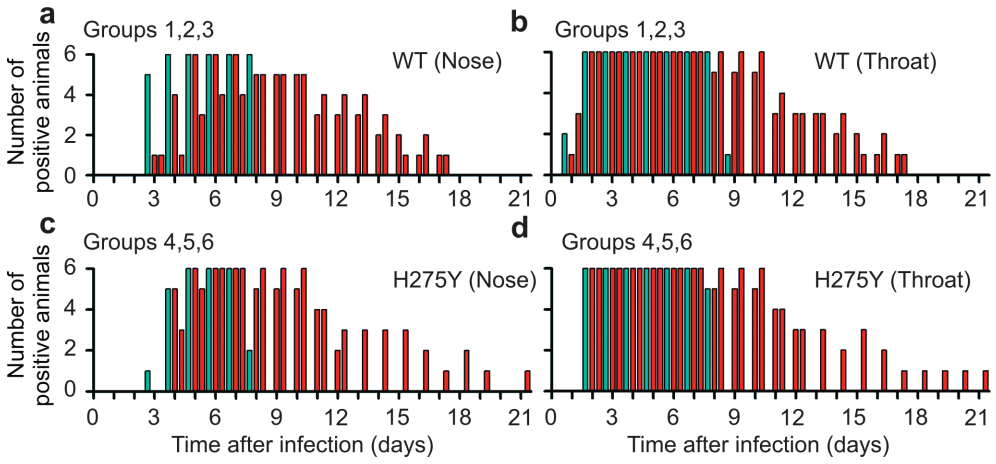

Figure S3 Total number of animals positive for replication competent influenza virus in the upper respiratory tract

Influenza virus titers ($TCID_{50}$/ml) were determined in nose and throat swabs daily taken from immunocompetent (blue bars; group 1 and 4) and immunocompromised ferrets (red bars; group 2, 3, 5 and 6). The animals were infected either with wild type (WT; a, b) or mutant virus (H275Y; c, d). Ferrets in groups 3 and 6 were treated with oseltamivir (10mg/kg twice daily) starting 24 hours after inoculation.

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Chapter 6

Summary of this thesis

Each year, approximately 5-10% of the world population is infected with the influenza viruses resulting in significant morbidity and an estimated 250.000 to 500.000 deaths every year. Among individuals at increased risk of developing severe influenza disease are those with a compromised immune system. For them being able to effectively suppress viral replication antiviral therapy can be crucial. However, in immunocompromised patients the currently available antiviral drugs show limited effectiveness. The emergence and spread of antiviral resistant viruses limit current therapeutic intervention even more. The aim of this thesis is to improve our understanding of influenza antiviral resistance. We developed new molecular tools to aid in influenza patient management, characterised a novel I222R antiviral resistance mutation and developed an immunocompromised ferret model. Finally, a key role for the influenza hemagglutinin in neuraminidase inhibitor resistance is proposed in the general discussion of this thesis. The contribution of the hemagglutinin in neuraminidase inhibitor resistance may explain the emergence of the H274Y oseltamivir-resistant influenza A/H1N1 virus in the winter season of 2007/2008.

Improving influenza diagnostics

Rapid, sensitive and reproducible detection assays are essential for the initial diagnosis of the disease, especially since they facilitate a timely initiation of antiviral therapy. Subsequently, they can used to monitor viral response and sensitivity to antiviral therapy during follow-up of patients on antiviral therapy. **In chapter 2**, the design, validation and evaluation of a set of real-time RT-PCR assays for quantification and subtyping of human influenza viruses are described. We have introduced a quantification standard, based on a commercially available electron microscopy counted influenza virus stock, and added internal quality controls to monitor longitudinal assay performance. Four assays were included to detect oseltamivir resistance mutations H274Y in pre- and post-pandemic influenza A/H1N1 and mutations E119V and R292K in the influenza A/H3N2 virus neuraminidase. As the set of clinical cases illustrate in **chapter 3**, this complete set of RT-PCR assays were a powerful tool for enhanced influenza virus surveillance and individual patient management. In addition, these assays formed the basis of the IRIS project. In the first three years after the 2009 influenza pandemic world wide oseltamivir resistance was found to be low (2.2%) and mostly found as minority variants in treated patients aged 1-5 years.

Antiviral resistance in the clinic setting

A year before the IRIS study had enrolled its first patient an oseltamivir resistant seasonal influenza A/H1N1 virus had emerged and spread, which carried a H274Y oseltamivir resistance mutation. In **chapter 3.1**, the fatal course of illness in an immunocompromised patient is described who became infected with this resistant virus. This case illustrates the pathogenic nature of this resistant virus despite carrying the H274Y oseltamivir resistance mutation.

In response to the outbreak of the 2009 pandemic influenza A/H1N1 virus intravenously (IV) administered zanamivir was made available as an add-on investigational drug for emergency compassionate use. **In chapter 3.2,** we have retrospectively analyzed the virological response to IV zanamivir treatment in ICU patients with severe influenza in the Netherlands. A consistent virological response was seen in only 6 out of 13 patients, most of whom were immunocompromised. We found a trend towards an increased likelihood of response for those who started IV zanamivir at an earlier stage of disease, but overall, the use IV zanamivir as late add-on therapy for 2009 pandemic influenza A/H1N1 virus infected patients in the ICU showed limited antiviral effectiveness. In one of these IV zanamivir treated patients a novel I222R neuraminidase inhibitor resistance mutation was identified. The course of illness of this case was described in **chapter 3.3**.

The I222R influenza virus neuraminidase mutant

After the identification of this I222R change questions were raised concerning the level of resistance it induced, the molecular resistance mechanism and its effect on viral fitness. To answer the first two questions, in **chapter 4** the I222R resistance mechanism was studied by both enzyme kinetics and X-ray protein crystallography. We showed that the I222R caused a 50-fold and 10-fold increase in IC_{50} to oseltamivir and zanamivir. As revealed by X-ray crystal structures, the I222R mutation causes shrinkage of the active site pocket leading to decreased oseltamivir binding. This finding was in accordance with the neuraminidase enzymatic changes in on- and off-rates for the neuraminidase inhibitors. Zanamivir binding was less affected, because it maintained its ability to form hydrogen bonds in the smaller active site pocket of the mutant neuraminidase. These crystal structures also revealed why the I222R mutation could act synergistically with the H274Y change in elevating the levels of oseltamivir resistance dramatically (1750-fold increase in IC50).

Finally, to answer the third question, virus pathogenicity and transmissibility of the I222R mutant was addressed and described in **chapter 5.1**. As compared to a

reference wild type virus (A/Netherlands/602/2009) the I222R mutant was found to be less pathogenic, but maintained its transmissibility in the ferret model.

Emergence of antiviral resistant viruses in the immunocompromised host

Highlighted throughout this thesis, the subject of antiviral resistant influenza viruses is of particular concern when treating immunocompromised patients. These patients tend to suffer from influenza longer with more serious complications than otherwise healthy patients. Although these patients should benefit most from existing antiviral treatment current strategies are far from satisfactory for this patient group. These therapies show limited effectiveness with frequent emergence of antiviral resistance. Tailored antiviral therapies for these patients do not exist, however, since for ethical and practical reasons antiviral efficacy studies are hard to conduct. To aid in the development of new antiviral strategies for the immunocompromised patients an immunocompromised ferret model was established as described in **chapter 5.2**. Here we also show that the emergence of antiviral resistant viruses and prolonged virus infections occurs frequently in hospitalized immunocompromised patients. These phenomena were then reproduced in the immunocompromised ferret model. One of the striking findings was that high dose oseltamivir therapy still proved partially protective in ferrets when these animals were infected with an oseltamivir-resistant virus. We anticipate that this model will aid in the development of more effective antiviral therapies for immunocompromised patients with influenza.

Chapter 7

Discussion of this thesis

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During the influenza season of 2007/2008 surprisingly an influenza A/H1N1 variant emerged, which showed resistance to the neuraminidase inhibitor (NAI) oseltamivir. This oseltamivir-resistant virus, harbouring the neuraminidase H274Y amino acid change, was initially detected in routine influenza surveillance in Norway [1], but rapidly spread throughout Europe [2]. By the end of that season it had become the dominant seasonal influenza A/H1N1 virus world-wide [3]. However, due to the introduction and spread of the novel oseltamivir-sensitive pandemic H1N1 in 2009 the oseltamivir-resistant variant was replaced [4]. Therefore, at least from the therapy perspective the 2009 pandemic came as a blessing in disguise.

Since then multiple researchers have questioned what mechanisms caused the development and spread of this oseltamivir-resistant virus [5-7]. Unfortunately as we still do not fully understand the underlying mechanisms it leaves the possibility that in the future another epidemic with a drug-resistant virus may occur. Here we propose a mechanism explaining the emergence and spread of this resistant virus.

A functional balance between the hemagglutinin and neuraminidase glycoproteins

The neuraminidase and the hemagglutinin are two glycoproteins present on the influenza virion [8]. During the initial stage of infection binding to sialic acids by the hemagglutinin is required for attachment to the cell. At the end of the virus replication cycle removal of these sugars by the neuraminidase is a requirement for efficient release of progeny viruses. Because the neuraminidase can also exert its sialidase function at the stage of virus attachment it has the potential to affect the efficacy of viral entry [9]. Therefore, a tight functional balance between these two glycoproteins has been proposed [10-12].

Perhaps one of the strongest lines of evidence for this functional balance was observed in early experiments in the 1990s when influenza viruses were cultured in the presence of a neuraminidase inhibitor (NAI) with the aim of selecting antiviral resistant viruses [13]. Surprisingly, the mutations appearing first were not located in the neuraminidase, but in the hemagglutinin. Importantly, characterization of these hemagglutinin mutants revealed that these viruses showed reduced ability to bind to sialic acids, thus facilitating viral release in the absence of sialidase activity, but in parallel, reducing the efficacy of viral attachment. Interestingly, these mutants were found to be dependent on the inhibition of NA for efficient replication, illustrating the balance between NA and HA activity.

In this example the functional balance between hemagglutinin and neuraminidase, initially disturbed by the presence of the neuraminidase inhibitor, was restored by mutations that reduced hemagglutinin binding. Sialic acid binding and removal were in balance on the virion only in the presence of NAI.

Hypothesis

If a traditional weigh balance is a metaphor representing the interactions between the two proteins correctly than the inverse order of events may also be possible. Here we propose that the appearance of the H274Y mutation in the 2007/2008 H1N1 neuraminidase, which reduced enzyme activity, was a response to enforced changes in the hemagglutinin by immune pressure pre-existing in the population. These changes in turn decreased hemagglutinin receptor binding as a bystander effect. By changing the neuraminidase at position 274 the virus adapted to changes in the hemagglutinin, thus reinstituting the functional balance between the two glycoproteins on the virion.

The attenuated phenotype of NAI resistant influenza viruses

For experts in the field of influenza antiviral research the sustained spread of these oseltamivir-resistant viruses was unexpected. Before 2007 it was generally believed that NAI-resistant viruses were unlikely to emerge and survive sustainably [3,14]. This concept arose on the basis of the first studies [15], and from characterization of early antiviral resistant viruses in the ferret model [16].

The non-attenuated phenotype of the oseltamivir-resistant 2007/2008 viruses

The dogma that resistant viruses were attenuated clearly did not hold up for the 2007/2008 H1N1 viruses. Based on several *in vitro* replication and animal studies it was shown that these H274Y mutant harbouring viruses were not attenuated in their ability to replicate *in vitro* and as pathogenic as their wild type counterparts *in vivo* [17]. Contra-intuitively, given that the mutant virus had completely replaced these oseltamivir-sensitive variants by the end of 2008 it remains possible that the H274Y mutant virus may even have gained viral function [2,3,18].

It is interesting to note, that for another recent H1N1 isolate, obtained during an outbreak of 2009 pandemic H274Y H1N1 viruses in Australia [19], enhanced transmission was observed in ferrets [20].

Mutations which alter neuraminidase activity

Recognizing another feature of the H274Y mutation is key for the understanding this hypothesis. Since amino acid position 274 is located close to the active site of the enzyme it also reduces sialidase activity. For the H274Y mutation this is shown by an increased Michaelis-Menten (K_M) constant of enzyme [6,7]. As described above the neuraminidase and the hemagglutinin both share the sialic acid target, a reduction of sialidase activity on the virion will also affect virus binding. Vice versa, a change in receptor binding properties of the hemagglutinin asks for adaptation of virus sialidase activity in order to allow efficient release of progeny viruses.

Pre-existing immunity forced antigenic drift and evoked the H274Y in the neuraminidase

Recently, it was shown that the hemagglutinin binding properties indeed can be altered by antigenic drift mutations emerging under antigenic pressure [21,22]. In order to escape pre-existing immunity (herd immunity) the virus mutates at antigenic sites, which are clustered at antigenic sites. Importantly, these are located in close proximity to the hemagglutinin receptor binding site. For instance for recent H3N2 viruses these antigenic drift mutations have resulted in a complete loss of binding to chicken red blood cells [23]. Prior to the emergence of the H274Y mutation two hemagglutinin mutations R192K and K144E emerged in antigenic sites Sb and Ca (Figure 1).

It remains to be seen if the selection of these mutations led to the emergence of the H274Y mutation. A more obvious selective pressure for its emergence seems be the use of oseltamivir, since the H274Y mutation rapidly emerges in infected patients on oseltamivir therapy [25]. As shown in chapters 3.2, 3.3 and 5.2 of this thesis, oseltamivir-resistance mutations frequently emerge in immunocompromised patients on antiviral therapy. These patients are often treated for extended periods of time, far longer than the recommended five day regime. In addition, as shown in chapter 5.2, these patients tend to develop a persistent infection as well, which increases the chance of viruses with a primary resistance mutation to subsequently acquire compensatory mutations. However, if oseltamivir would have been the evolutionary force, as proposed for adamantane-resistance emergence [26], one would expect the virus to have initially emerged in countries where oseltamivir use is high, such as US or Japan, rather than Norway, where it was barely prescribed prior to the emergence of the oseltamivir-resistant virus [27].

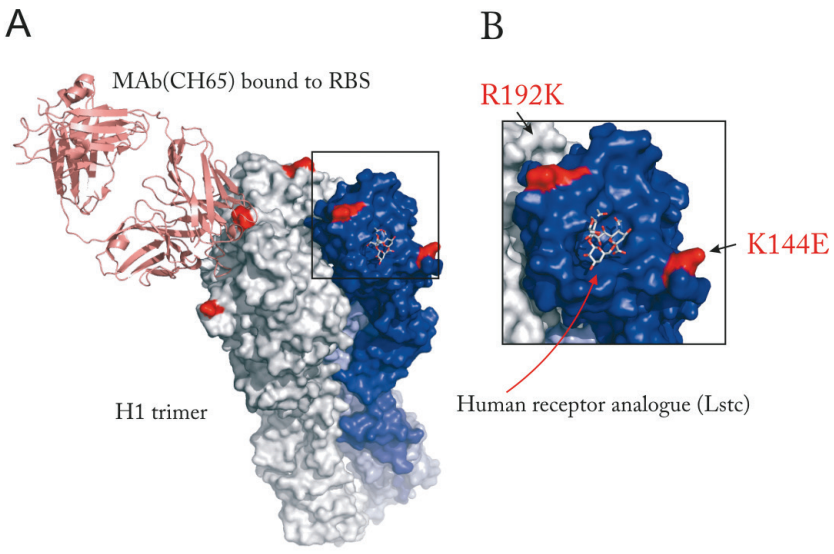

Figure 1 Monoclonal antibody (MAb) CH65 in complex with H1 hemagglutinin trimer at the receptor binding site (RBS)
Crystal structure of monoclonal antibody CH65 in complex with an H1 hemagglutinin trimer (A) (PDB ID code 3SM5 [24]). Amino acid residues which changed prior to the emergence of the H274Y oseltamivir-resistance mutation are in red. Antigenic sites (Sb and Ca) around the receptor binding site are coloured yellow.

Like the H274Y mutation, for several other changes in the neuraminidase it was shown that they can affect sialidase activity of the neuraminidase [5-7]. Since these mutations had appeared before the 2007/2008 and were shown to increase sialidase activity, these have mutations been proposed to act as compensatory mutations for the decreased activity of the H274Y mutated neuraminidase [5-7]. In the light of the hypothesis proposed here these mutations may have acted similar to the H274Y mutation, but in a mutually opposing manner.

Nowadays, state of the art influenza research makes use of recombinant viruses. This powerful tool enables studying of different mutations in an identical background. In order to test the hypothesis one could study the impact on virus function of single hemagglutinin and neuraminidase mutation, which appeared in the virus prior the 2007/2008 season. However, effects of single changes may be masked by other mechanisms involved in maintaining the functional balance of the hemagglutinin and neuraminidase. Furthermore, the use of virus recombinants could lead to misinterpretation if particular phenotypes like oseltamivir-resistance cannot be linked to a single mutation. Experiments performed with recombinant antiviral-

resistant viruses have probably contributed to the dogma that NAI-resistant viruses have reduced fitness.

Our hypothesis implies that the oseltamivir-resistance was only a by-product, unrelated to the use of oseltamivir. Theoretically, for other known NAI-resistance mutations in the neuraminidase, such as the E119V and R292K mutations in H3N2 or H7N9 viruses, the same principle may apply. These changes have been shown to alter neuraminidase activity and could act as compensatory mutations for reduced receptor binding upon changed hemagglutinin antigenicity.

Potential involvement of the hemagglutinin in the emergence of the 2009 I222R mutated virus

In chapter 5.1, the potential compensatory roles for mutations in the neuraminidase were assigned for the clinical isolate (NL2631-R223). This isolate carried the I222R amino acid change. We found that the I222R mutation, when introduced as a single mutation in recombinant virus A/NL/602/2009 (NL/602), replicated to lower virus titers than the recombinant NL/602 wild type or the patient isolate (Chapter 5.1; Figure 1). We questioned whether this compromised phenotype could be restored by introducing all mutations of the NL/2631-R223 neuraminidase into NL-602. We have not performed *in vitro* replication studies to address this, but instead, we have compared the neuraminidase crystal structure of Cal07, similar to that of NL602, with the neuraminidase of isolate NL2631-R223. Of the five mutations, N248D and N386D seemed the most likely candidates to play compensatory roles. Next to amino acid position 248, position 247 has been linked to I222R mediated resistance in chapter 4. Mutation N386D destroys a potential glycosylation site. However, no structural changes were observed at position N248. In addition, in the X-ray structure we found no evidence that the asparagine at position 386 was glycosylated.

However, it may well be that the attenuated phenotype of recombinant virus NL602 with the neuraminidase of the clinical isolate was the result of the presence of the hemagglutinin of NL602 instead of the hemagglutinin of NL2631-R223. As mentioned before, reverse genetics is an extremely powerful tool to study influenza virus in detail. However, caution should be made when translating obtained results to the clinic, especially if multiple proteins are involved. From this perspective, it may also be valuable to study the receptor binding properties of the virus isolate NL2631-R223. This data may better explain why this isolate could have emerged during intravenously administered zanamivir therapy.

A call for novel antiviral therapies

The clinical cases presented in this thesis are clear examples of the shortcomings of the current available antiviral therapies. These have been approved for non-risk patients only for whom influenza usually is a self-limiting disease. However, antiviral strategies for patients who could clearly benefit even more, such as immunocompromised patients, are less well defined. Currently, several new strategies, inhibiting various stages of the influenza virus life cycle, are under development. These include virus inhibition at the stage of attachment, membrane fusion and gene transcription [28,29]. Examples of these are bacterial sialidase DAS181 [30-32] and the RNA polymerase inhibitor favipiravir (T-705). Moreover, exciting new generations of monoclonal antibodies have recently been identified. These antibodies were shown to neutralize a wide range of influenza viruses [33].

These new drugs may be used in combination with other drugs preferably with a non-overlapping resistance profiles. This may decrease the chance of antiviral resistance emergence and may act synergistically [34]. Clinical studies addressing effectiveness of combination therapy are still very limited with conflicting outcome [35,36].

Global incidence of adamantane and neuraminidase inhibitor resistance from 2008

To date, most circulating influenza viruses are inherently adamantane resistant or in case of influenza B virus simply not sensitive to this drug class [37,38]. In a recent paper by Whitley *et al.* we have reported on the incidence of current NAI resistance in a global multicentre study carried out between 2008 and 2012 [4,38]. For influenza A viruses, oseltamivir resistance was found to be rare (2.2%) and only detected in relation with antiviral therapy (16/656 patients). Emergence of oseltamivir resistance was highest in children (10.1%) between 1-5 years of age (14/138). The study did not detect NAI resistant influenza B viruses. No zanamivir resistance was detected either. However, others have reported reduced zanamivir susceptibility for influenza viruses in recent years [39-44] [45]. Bearing in mind the 2007/2008 emergence of oseltamivir-resistance the recent outbreaks of 2009 pandemic H1N1 viruses with an H274Y oseltamivir-resistance mutation are alarming [19,46].

Finally, influenza antiviral surveillance networks, such as the Neuraminidase Inhibitor Susceptibility Network (NISN) and Influenza Resistance Information Study (IRIS) remain important for monitoring the incidence of primary resistance in circulating influenza viruses. However, a better understanding of the impact

of antiviral resistance mutations on the therapeutic effectiveness will be useful for treatment of critically-ill patients. In addition, a better understanding of the potential permissive amino acid changes in the circulating viruses, such as in the hemagglutinin, and experiments to prove the strict interaction between hemagglutinin-neuraminidase balance, may aid in a better understanding of the emergence of non-compromising antiviral resistance and may even be of predictive value.

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Chapter 8

Summary in Dutch / Nederlandse samenvatting

Jaarlijks raakt 5 tot 10 procent van de wereldbevolking geïnfecteerd met het influenzavirus (griepvirus). Normaal gesproken veroorzaakt het virus een lokale infectie waarbij de vermeerdering van het virus (virusreplicatie) beperkt blijft tot de bovenste luchtwegen. De klachten en symptomen die bij influenza horen, zoals hoofdpijn, hoesten, spierpijn en koorts houden normaal gesproken niet veel langer aan dan een week, maar volledig herstel kan ook veel langer duren.

Influenzavirusinfecties kunnen soms ook tot complicaties leiden. Vaak treden deze op wanneer het virus afzakt naar de lagere luchtwegen en een longontsteking veroorzaakt. De Wereldgezondheidsorganisatie (WHO) schat dat jaarlijks tussen de 250.000 en 500.000 patiënten wereldwijd overlijden aan de gevolgen van de griep. Veelal zijn deze doden te betreuren onder patiënten met een verhoogd risico op een ernstig ziektebeloop. Mensen die in deze categorie vallen zijn onder andere ouderen boven de 65 jaar en mensen die anderzijds een verzwakt immuunsysteem hebben. Daarom wordt deze mensen geadviseerd zich te laten vaccineren.

Naast deze jaarlijkse griepprik, waarmee infectie kan worden voorkomen, zijn er twee klassen antivirale middelen beschikbaar voor de behandeling van influenza: de M_2-kanaal- en neuraminidaseremmers (NRs). Deze middelen zijn een waardevolle aanvulling op de behandeling van patiënten met influenza. De effectiviteit van deze middelen laat echter te wensen over. En één van de oorzaken hiervoor is het ontstaan van influenzavirussen die resistent zijn tegen één of meerdere van deze middelen.

Het influenzavirus

Virussen van de *Orthomyxoviridae* familie van virussen, waartoe influenzavirussen A, B en C behoren, hebben een gesegmenteerd enkelstrengs RNA genoom omsloten door een membraan. Influenza A virussen worden verder onderverdeeld in subtypen op basis van twee membraaneiwitten: het hemagglutinine (H/HA) en neuraminidase (N/NA). Een derde membraaneiwit is het M_2-kanaal. Op de voorkant van dit proefschrift zijn de dwarsdoorsneden van het influenzavirus zichtbaar gemaakt met een elektronenmicroscoop. Ze zijn gemiddeld 100nm in dwarsdoorsnede. HA en NA zijn zichtbaar als de kleine stekeltjes aan de buitenkant van het virus.

Tot nu toe zijn er 17 verschillende influenza HA en 10 NA subtypen bekend. Influenza A H17N10 virus is onlangs geïsoleerd uit een vleermuis. De andere subtypes komen voor in wilde vogels. Deze worden dan ook gezien als het natuurlijke reservoir van waaruit influenza A virussen kunnen overspringen naar zoogdieren en mensen. Een dergelijke introductie in de humane populatie kan leiden tot een pandemie.

Het virus moet dan wel over de eigenschap beschikken dat ook van mens op mens overdraagbaar is. In 2009 nog zorgde de introductie van een influenza A virus van het subtype H1N1 voor de eerste influenza pandemie van de 21ste eeuw.

Replicatiecyclus

De replicatiecyclus begint wanneer een influenzavirus zijn receptor bindt. Deze bestaan uit vertakte suikerketens gebonden aan membraaneiwitten op onder andere de epitheelcellen in de luchtwegen. Meestal eindigen deze suikerketens met een siaalzuur. Deze suikergroep staat centraal in de replicatiecyclus. Binding aan siaalzuren komt tot stand in het receptor bindingsdomein van HA. Eenmaal gebonden dringt het virus de cel binnen via endocytose. Aan het eind van de replicatiecyclus, vlak voordat nieuwe virussen uit de geïnfecteerde cel vrijkomen, verwijdert NA deze siaalzuren. Dit voorkomt voortijdige siaalzuurbinding en aggregatie van nieuw gevormde virussen op het moment dat zij de geïnfecteerde cel verlaten. Door de tegengestelde functies van HA en NA op het virus is er een strikte functionele balans nodig tussen beide membraaneiwitten voor een efficiënte ronde van virusreplicatie.

Influenza pandemieën en epidemieën

Introductie van een nieuw influenza A virus kan leiden tot influenzapandemie. Bekend zijn de pandemieën van 1918 en meest recentelijk, die van 2009. Beide pandemieën werden veroorzaakt door een H1N1 virus. Influenzapandemieën worden meestal geassocieerd met een hoger sterftecijfer, doordat immuniteit voor deze nieuwe virussen in meer of mindere mate ontbreekt. Griepgolven die volgen zwakken af bij een toenemende immuniteit en dan vormen de griepepidemieën. Op dit moment vormen de H3N2 en H1N1 virussen jaarlijkse griepepidemieën tijdens de winterperiode samen met het influenza B virus.

Antivirale resistentie ontwikkeling

Sinds 2003 heeft de resistentieontwikkeling tegen de M_2-kanaalremmers een dramatische wending genomen. Het percentage resistente influenza A virussen is inmiddels gestegen tot 100%. Meer recentelijk zorgde een uitbraak in het winterseizoen 2007/2008 van een griepvirus met een H274Y oseltamivir-resistentie mutatie ervoor dat veel H1N1 virussen in dat seizoen resistent waren tegen oseltamivir (Tamiflu). Een beter begrip van het ontstaan van antiviraal resistente virussen, de moleculaire resistentiemechanismen en de gevolgen voor de behandeling van patiënten is daarom gewenst.

Ontwikkeling van moleculaire influenza diagnostiek

Aan de basis van dit promotieonderzoek ligt de ontwikkeling van het juiste gereedschap om antivirale resistente virussen in patiëntenmaterialen te kunnen detecteren en het verdere beloop van de infectie te kunnen volgen.

In hoofdstuk 2 van dit proefschrift is het ontwerp, de validatie en de evaluatie beschreven van een uitgebreide set aan moleculaire tests die het mogelijk maakt de aanwezigheid van influenzavirus RNA in respiratoir patiëntenmateriaal aan te kunnen tonen. Door de introductie van externe en interne standaarden zijn resultaten kwantitatief en reproduceerbaar, waardoor verschillende resultaten met elkaar vergeleken kunnen worden. Deze informatie maakt het bijvoorbeeld mogelijk het effect van antivirale therapie te volgen tijdens behandeling met een antiviraal middel. Onderdeel van de set zijn een viertal testen die bekende antivirale resistentie mutaties detecteren in de H1N1 en H3N2 subtypes, waaronder de H274Y mutatie in het H1N1 virus.

Deze set aan moleculaire testen vormden het fundament voor de "Influenza Resistance Information Study" (IRIS), een door Hoffmann-La Roche gesponsorde studie naar het wereldwijde voorkomen van antivirale resistentie in ziekenhuizen. In de eerste drie jaar na het uitbreken van de influenza pandemie in 2009 was dit percentage over het algemeen laag (2.2%). Resistentie was vooral aanwezig (12%), als kleine subpopulatie in respiratoir materiaal afgenomen van jonge oseltamivir behandelde kinderen behandeld met oseltamivir (1 tot 5 jaar).

In het bijzonder is de test voor detectie van de H274Y oseltamivirresistentie-mutatie in het 2009 H1H1 virus bruikbaar gebleken. Deze is geïmplementeerd in de diagnostiek van verschillende Nederlandse en buitenlandse ziekenhuizen. Hiermee zijn bijvoorbeeld de eerste patiënten met een oseltamivir-resistent 2009 H1N1 virus gedetecteerd in Nederland en in het Verenigd Koninkrijk.

Antivirale resistente griepvirussen in de kliniek

De uitbraak van een oseltamivir-resistent H1N1 virus in het winterseizoen van 2007/2008 was onaangekondigd. Voor dat seizoen werd algemeen gedacht dat virussen resistent tegen de neuraminidaseremmers een verlaagde virulentie hadden en minder goed konden verspreiden. In hoofdstuk 3.1 is een beschrijving van een fatale infectie van een immuungecompromitteerde patiënt met een dergelijk virus gegeven. De beschrijving van deze casus was illustratief voor het virulente karakter van dit virus ondanks dat het de H274Y mutatie droeg.

In een reactie op het uitbreken van de 2009 pandemie kwam zanamivir behandeling door middel van intraveneuze toediening tijdelijk beschikbaar voor patiënten op de intensive care met ernstige influenza. In hoofdstuk 3.2 is de respons op deze therapie geëvalueerd voor 13 ernstig zieke, voornamelijk immuungecompromitteerde patiënten. Voor deze ernstig zieke patiëntengroep, waarvan de meesten al enig tijd op de intensive care lagen, bleek deze therapie weinig effectief. In een van de patiënten werd een 2009 H1N1 virus ontdekt die resistent bleek te zijn tegen de neuraminidaseremmers oseltamivir en zanamivir. Een nieuwe aminozuurverandering in het neuraminidase van een isoleucine naar arginine op positie 222 (I222R) zorgde voor dit antivirale resistente fenotype. In hoofdstuk 3.3 is de identificatie van deze mutatie en het ziektebeloop van de patiënt beschreven.

De I222R antivirale resistentiemechanisme

Van deze I222R mutatie was het resistentiemechanisme nog onbekend. Ook was niet duidelijk wat het effect van deze mutatie was op de virulentie en eventuele kans op verspreiding van dit virus. Om meer inzicht te krijgen in het antivirale resistentiemechanisme is het effect van de I222R mutatie op neuraminidase enzymatische activiteit onderzocht. Met behulp van eiwitkristallografie zijn de veranderingen in de eiwitstructuur bestudeerd. De resultaten hiervan zijn beschreven in hoofdstuk 4. De mutatie zorgde voor een 50- en 10-voudige verlaging van de gevoeligheid voor respectievelijk oseltamivir en zanamivir. Zichtbaar gemaakt door middel van eiwitkristallografie de I222R mutatie zorgde voor een verkleining van het actieve centrum van het enzym, waardoor voor oseltamivir minder goed het actieve centrum kon binden. Verschillen in de structuur tussen de twee neuraminidaseremmers maakt het dat zanamivir minder gevoelig is voor de I222R mutatie. De kristalstructuren maakten ook zichtbaar waarom de I222R met de H274Y mutatie samen tot een dramatische verhoging van de resistentie tegen oseltamivir kunnen zorgen.

In hoofdstuk 5.1 is gekeken naar het effect van de I222R mutatie op de virulentie en overdracht van dit virus in een frettenmodel. In vergelijking met een wild type virus (A/Netherlands/602/2009) bleek de I222R mutant minder pathogeen. Echter, het virus werd net als het wild type virus goed overgedragen in het fretten transmissiemodel.

Antivirale resistentie in de immuungecompromitteerde gastheer

Resistentieontwikkeling komt niet alleen vaker voor bij jonge kinderen. In patiënten met een gecompromitteerd immuunsysteem komt dit ook regelmatig voor. In deze patiënten kan het virus gedurende langere tijd blijven repliceren omdat het immuunsysteem niet in staat is het virus te klaren. Dit vergroot de kans op het ontstaan van resistente virussen. In hoofdstuk 5.2 is een inventarisatie gemaakt van het aantal gevallen van resistentie in opgenomen immuungecompromitteerde patiënten die langer dan 14 dagen influenzavirus positief waren. In 45% van deze groep patiënten opgenomen in het Erasmus MC (2009-2012) bleek een resistent virus aangetoond te kunnen worden (5 uit 11 patiënten).

Antivirale therapieën zouden juist effectief moeten zijn in deze patiënten, maar de huidige antivirale strategieën laat veel te wensen over. Antivirale therapieën afgestemd op deze patiënten ontbreekt, niet op de minste plaats omdat klinische onderzoeken naar de effectiviteit van deze middelen in deze patiënten moeilijk uitvoerbaar zijn. Daarom is in hoofdstuk 5.2 een nieuw diermodel beschreven waarmee virusinfecties, behandelingsmethoden en de ontwikkeling van antivirale resistentie in een immuungecompromitteerde gastheer kunnen worden bestudeerd. Door gebruik te maken van dit model kon een verlengde virusreplicatie en ontwikkeling van antivirale resistentie, zoals gevonden in immuungecompromitteerde patiënten worden nagebootst in deze fretten. Een interessante observatie was dat fretten geïnfecteerd met een oseltamivir-resistent virus, toch profijt hadden bij oseltamivir behandeling.

Samengevat heeft het onderzoek beschreven in dit proefschrift bijgedragen aan de kennis van influenza en antivirale resistentie. De diagnostische tests beschreven in dit proefschrift zijn een waardevolle toevoeging op influenzadiagnostiek. Illustratief hiervoor zijn de beschreven patiëntencasussen in dit proefschrift. Deze laten ook zien dat huidige mono-therapieën voor de behandeling van chronische influenzavirusinfecties te wensen over laat. Een beter begrip van het ontstaan van resistente influenzavirussen voor de ontwikkeling van meer effectieve antivirale therapieën is essentieel.

Chapter 9

Word of thanks / Dankwoord

Voor wat bij velen vloeiend in elkaar over lijkt te gaan, voor mij was de overgang van kersverse doctorandus naar onderzoeker-in-opleiding geen een-tweetje. Achteraf gezien heb ik mijn promotietijd bij de Rotterdamse virologen voor geen goud willen missen. Maar toen Martin mij belde voor een eerste kennismaking-gesprek dacht ik daar nog heel anders over. Natuurlijk wil ik verderop iedereen bedanken die aan de totstandkoming van dit proefschrift heeft bijgedragen. Maar eerst neem ik u graag nog even mee naar het moment waarop mijn telefoon overging.

Het was op een donkere najaarsavond toen, iets ten noorden van Terschelling, de windkracht net was toegenomen tot een stevige 4 Beaufort. Ik voer die dag mee van Amsterdam naar de Eemshaven aan boord van het schip *The Atlantic Rose*. Een maand of twee daarvoor had ik mij ingeschreven voor een krankzinnig wereld zeilproject op deze schoener en de eerste trainingen waren begonnen. Hieraan meedoen, dat was de ultieme vluchtpoging om maar niet te hoeven denken aan een toekomstige baan. Toch had ik nog net, met lichte tegenzin weliswaar, een sollicitatiebrief in een fles gestopt, de kurk erop gedrukt en overboord gegooid.

Vluchtpogingen had ik eigenlijk genoeg ondernomen. Direct na mijn studie moleculaire biologie was ik vertrokken en in een aaneengesloten ketting van reizen uiteindelijk langs de kant van de weg in Mali beland. Ik was in een paar maanden tijd van Frankrijk, door Syrië naar Cairo gelift en was daarna per vliegtuig de Sahara overgestoken. Na een cursus Frans bij mijn tante in Dakar volgde avontuur nummer drie. Echter, halverwege deze trip, met eindbestemming Timboektoe, brak de ketting van aaneengeregen reisavonturen. De achteras van de touringcar bus, afgeladen met schapen, mensen en handelswaar brak in tweeën. Als een geluk bij een ongeluk strandde ik daarom bij de familie Tapily, een traditionele Malinese jagersfamilie uit Bandiagara.

Het waren de wijze woorden van grootvader Tapily die me deden beseffen dat ik met al dat gereis was afgedwaald en iets van een doel miste. Hij was het die me wees op de treffende gelijkenis tussen mezelf en een andere vreemde Hollandse vogel die ook bij de Tapily's was beland. Een vogel met zowel dezelfde herkomst als hetzelfde doodlopende vluchtpatroon.

Het bewijs hiervan vond ik om het uitgedroogde vogelpootje dat om de nek van grootvader Tapily hing. Hij had de vogel rond het jaar 2000 vijfhonderd meter van zijn hut geschoten. Het bleek, tot verbijstering van de gehele familie, een ring te dragen (zie rug van dit proefschrift). Met behulp van de code op de ring (Vogeltrekstation Arnhem-Holland 5.332.004) kwam ik er thuis achter dat het een kleine mantelmeeuw betrof. Het was een paar weken voor zijn fatale vlucht in het

nest geringd op de voormalige afvalstortplaats de Prinsesseplaat nabij Bergen op Zoom. Nog steeds een van de langst geregistreerde vluchten van een Hollandse kleine mantelmeeuw ooit. Op zijn trektocht naar de Afrikaanse kust moet het zijn afgedwaald en net als ik, dwars over de Sahara zijn gevlogen. Uitgeput was het bij de Tapily's ook op zoek geweest naar wat rust. Hoe de vogel er had uitgezien? Grootvader Tapily antwoordde resoluut: "L'ouissaux était grand, blanc et très très fatigué!" Achterafgezien sloeg die beschrijving dus niet alleen op die grote witte kleine mantelmeeuw, maar dus ook op die andere grote blanke avonturier.

Terug naar die bewuste dag in het najaar van 2007. Het weer was dus onstuimig en we waren juist langs de Richel, tussen Vlieland en Terschelling door, de Noordzee op gevaren. We zaten precies ten noorden van de Brandaris. Ik was al een beetje misselijk toen ik Martin aan de telefoon kreeg en me vroeg of ik nog trek had de lange reis van Groningen naar Rotterdam te maken. Twee maanden later woonde ik dus op Zuid en ging de tijd ineens zo snel. Ik heb me geen moment meer heb afgevraagd hoe die wereldreis zou zijn geweest. In ieder geval ben ik niet, zoals die arme meeuw, als talisman geëindigd.

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Lieve Anne, paranimf, dit proefschrift was er echt niet gekomen zonder jou. Bedankt voor alles wat je voor me gedaan hebt en voor de fijne tijd die we samen waren. Ik zal de drieweekse bikkeltocht over de GR20 nooit vergeten.

Alle collega's van de diagnostiek en de kliniek: Ontzettend bedankt voor het gedogen van een jonge verstrooide wetenschapper in een diagnostisch lab. Bedankt voor de fijne tijd en voor alles wat ik van jullie heb geleerd. Stagiaires Ralf, Jeer, Iris, Jurriën, Nezha en Geny, ontzettend bedankt voor jullie inzet. Bianca en Koos, ik mis jullie.

Beste FluFighter en andere virologen, heb je ook wel eens gemerkt dat mensen buiten de afdeling raar opkijken als je hen vertelt over het werk? Bijvoorbeeld wanneer je zegt hotelbedden te delen met een collega tijdens een congres? Of dat je na een dagje werken heerlijk hebt gedanst in de liftschacht rondom een zelfgebouwde bar van pallets? De legendarische sinterkerstfeesten worden überhaupt nooit begrepen. Vooral op dat soort momenten lijkt me werken buiten de virologie ontzettend saai! Ook al is mijn verhuizing van het ziekenhuis naar de 17de tot tweemaal toe gestrand ik vond het fantastisch om met jullie samen te werken, congressen te bezoeken en feestjes te bouwen. In het bijzonder wil ik graag bedanken de collega's met wie ik meer dan alleen labresultaten heb gedeeld: Corine, Rogier, Joost, Sander, Eefje, Miranda, Imke, Lonneke, Patrick, Judith, Martin, Bjorn, Leslie, Frank en Stella en Debby. Theo, mijn totaal aan georganiseerde borrels loopt niet synchroon met mijn lijst aan gepubliceerde artikelen. Ik hoop dat mijn promotiefeest enigszins zal kunnen compenseren.

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Dear Steve G., John, Steve M., Patrick, Sebastian, Alex, Phil, Lesley H., Lesley C., Peter, Roxy, and many others from the departments of Virology, Biophyscial chemistry and Molecular Structure of the National Institute of Medical Research in London, UK. Thank you all for hosting the Flying Dutchman. Learning from you about enzymology and X-ray crystallography it all was a great adventure as were Steve's pub crawls in the heart of old city London. I really hope we could continue our projects together.

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Mijn lieve Toon, wat ging alles toch ineens snel! Dacht ik 4 jaar lang, in alle rust te kunnen broeden op mijn proefschrift, knalde jij daar uit het ei. En ik al die tijd maar denken tijdens dat gezwoeg, straks dat boekje, dat maakt me blij. Wat een dom idee nu, want de meeste blijdschap, die breng jij. Bedankt voor het geven van het nieuwe doel om voor te leven!

Chapter 10

About the author

1 Curriculum vitae

The author of this thesis was born on 16 August 1980 in Assen, the Netherlands. After finishing high school in his home town in 1998 (Chr. Scholengemeenschap Vincent van Gogh) he started his study in Physics at the University of Twente (the Netherlands) and continued his studies in Molecular biology at the University of Groningen (RUG, the Netherlands). At the RUG he performed internships at the departments of molecular microbiology and developmental genetics. During a nine month externship at the department of biochemistry at the University of Uppsala (Sweden) he developed a method to construct tailor-made gene transcription factors. He finished his Master of Science degree by the beginning of 2006. Before starting his scientific career he worked as a nature guide on the Dutch wads and hitchhiked to Cairo. In January 2008 he started his PhD in the Erasmus MC department of Viroscience (Rotterdam, the Netherlands) under supervision of Prof.Dr. A.D.M.E Osterhaus and Prof.Dr. C.A.B. Boucher. Guided by Dr. M. Schutten in the unit clinical virology he developed influenza diagnostic assays used in the Influenza Resistance Information Study; a clinical study to monitor the incidence of global influenza antiviral resistance. There he also identified a virus with a new antiviral resistant mechanism elucidated by protein enzymology and X-ray crystallography. This work was carried out at the national institute of medical research (NIMR, London, UK) where he was supervised by Dr. J.J. Skehel and Dr. S.J. Gamblin. In collaboration with Viroclinics Biosciences B.V. he developed a ferret model to study influenza virus persistence and intervention in an immunocompromised host. The results obtained at the Erasmus MC, NIMR and during collaboration with Viroclinics Biosciences B.V. are presented in this thesis.

2 PhD Portfolio

Name:	Erhard van der Vries
Research group:	Department of Viroscience
Research school:	Post-Graduate Molecular Medicine
PhD period:	2008-2013
Promotors:	Prof.Dr. A.D.M.E. Osterhaus
	Prof.Dr. C.A.B. Boucher
Co-promotor:	Dr. M. Schutten

Education

2010-2013	Externship at the department of Molecular Structure, MRC-National institute of Medical Research, Mill Hill, London, United Kingdom
2008-2013	PhD position at the department of Viroscience, Erasmus Medical Center Rotterdam, the Netherlands

In-Depth Courses

2012	One Health Course (Antigone)
2012	Course in Photoshop/Illustrator (Molmed)
2010	Course in Virology (Molmed)
2008-present	Internal and external presentations at the department of Viroscience (Viroscience lab)

Scientific presentations

2013	Options for the control of Influenza VIII, Cape Town	(poster)
2013	XV Macrea meeting on respiratory infections, Rotterdam	(oral)
2012	Orthomyxovirus Research Conference, Montreal	(oral)
2012	Influenza Antiviral Drug Resistance Workshop (ISIRV), Hanoi	(oral)
2011	4th European Influenza Conference (ESWI), Malta	(poster)
2011	Influenza Antiviral Drug Resistance Workshop, Rio de Janeiro	(oral)
2010	Options for the control of Influenza VII, Hongkong	(poster)
2009	Orthomyxovirus Research Conference, Freiburg	(poster)
2009	13th Molecular Medicine Day, Rotterdam	(poster)
2008	3th European Influenza Conference (ESWI), Villamoura	(poster)
2008	12th Molecular Medicine Day, Rotterdam	(poster)

Teaching

2013 2nd year medicine students, Rotterdam, The Netherlands
2012 One Health course, Rotterdam, The Netherlands
2010 User training of an influenza resistance assay (NA-star) at the department of the Virology, Universitado, Sao Paolo, Brazil

National and international collaborations

2013-present Department of Molecular Nanofabrication, University of Twente, Enschede, the Netherlands
2011-present Department of Molecular Structure, National Institute of Medical Research, London, United Kingdom
2009-present Influenza Resistance Information Study (IRIS): Following global emergence of influenza antiviral resistance in the clinic

Grants and Awards

2013 MacCrae Young Scientist Award
2011 ESWI Young Scientist travel grant (4th European Influenza Conference)

Miscellaneous

2013 Board member of MUGAS an initiative on review and statistical analysis of oseltamivir data
2013 Co-chair at the XV Macrea meeting on respiratory infections, Rotterdam
2009 Finalist Dutch Academic Year Award (*The FluFighters*)
2012 Inventor on patent application PCT/EP2012/063497
2008-2013 Supervision of technicians and HLO students during the course of my PhD period

3 List of publications

2013

1. **van der Vries E**, Stittelaar KJ, van Amerongen G, Veldhuis Kroeze EJ, de Waal L, et al. (2013) Prolonged influenza virus shedding and emergence of antiviral resistance in immunocompromised patients and ferrets. PLoS Pathog 9: e1003343.

2. **van der Vries E**, Schutten M, Fraaij PL, Boucher CAB, Osterhaus ADME (2013) Influenza virus resistance to antiviral therapy. Adv Pharmacol 67: 217-246.

3. Fraaij PL, **van der Vries E**, Osterhaus ADME (2013) Reply to chan-tack et Al. J Infect Dis 207: 198-199.

4. **van der Vries E**, Anber J, van der Linden A, Wu Y, Maaskant J, et al. (2013) Molecular assays for quantitative and qualitative detection of influenza virus and oseltamivir resistance mutations. J Mol Diagn 15: 347-354.

2012

5 **Van der Vries E,** Collins PJ, Vachieri SG, Xiong X, Liu J, et al. (2012) H1N1 2009 pandemic influenza virus: resistance of the I223R neuraminidase mutant explained by kinetic and structural analysis. PLoS Pathog 8: e1002914.

2011

6. Fraaij PL, **Van der Vries E**, Beersma MF, Riezebos-Brilman A, Niesters HGM, et al. (2011) Evaluation of the antiviral response to zanamivir administered intravenously for treatment of critically ill patients with pandemic influenza A (H1N1) infection. J Infect Dis 204: 777-782.

7. Meijer A, Jonges M, Abbink F, Ang W, van Beek J, et al. (2011) Oseltamivir-resistant pandemic A(H1N1) 2009 influenza viruses detected through enhanced surveillance in the Netherlands, 2009-2010. Antiviral Res 92: 81-89.

8. **Van der Vries E**, Schutten M, Boucher CAB (2011) The potential for multidrug-resistant influenza. Curr Opin Infect Dis 24: 599-604. *Review.*

9. **Van der Vries E**, Veldhuis Kroeze EJ, Stittelaar KJ, Linster M, Van der Linden A, et al. (2011) Multidrug resistant 2009 A/H1N1 influenza clinical isolate with a neuraminidase I223R mutation retains its virulence and transmissibility in ferrets. PLoS Pathog 7: e1002276.

2010

10. Jonges M, Liu WM, **Van der Vries E**, Jacobi R, Pronk I, et al. (2010) Influenza virus inactivation for studies of antigenicity and phenotypic neuraminidase inhibitor resistance profiling. J Clin Microbiol 48: 928-940.

11. **Van der Vries E**, Jonges M, Herfst S, Maaskant J, Van der Linden A, et al. (2010) 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 47: 34-37.

12. **Van der Vries E**, Schutten M (2010) Satisfying the need for rapid diagnosis of new variant influenza A H1N1. Expert Rev Mol Diagn 10: 251-253. *Review*.

13. **Van der Vries E**, Stelma FF, Boucher CAB (2010) Emergence of a multidrug-resistant pandemic influenza A (H1N1) virus. N Engl J Med 363: 1381-1382.

2008

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