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

Erhard van der Vries[1], Koert J. Stittelaar[2], Geert van Amerongen[2], Edwin J. B. Veldhuis Kroeze[2], Leon de Waal[2], Pieter L. A. Fraaij[1,3], Roland J. Meesters[4], Theo M. Luider[4], Bart van der Nagel[5], Birgit Koch[5], Arnold G. Vulto[5], Martin Schutten[1], Albert D. M. E. Osterhaus[1,2*]

[1]Department of Virology, ErasmusMC, Rotterdam, The Netherlands, [2]Viroclinics Biosciences B.V., Rotterdam, The Netherlands, [3]Department of Paediatrics, ErasmusMCSophia, Rotterdam, The Netherlands, [4]Department of Neurology, ErasmusMC, Rotterdam, The Netherlands, [5]Department of Hospital Pharmacy, ErasmusMC, Rotterdam, The Netherlands

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.

MATERIALS AND METHODS

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.

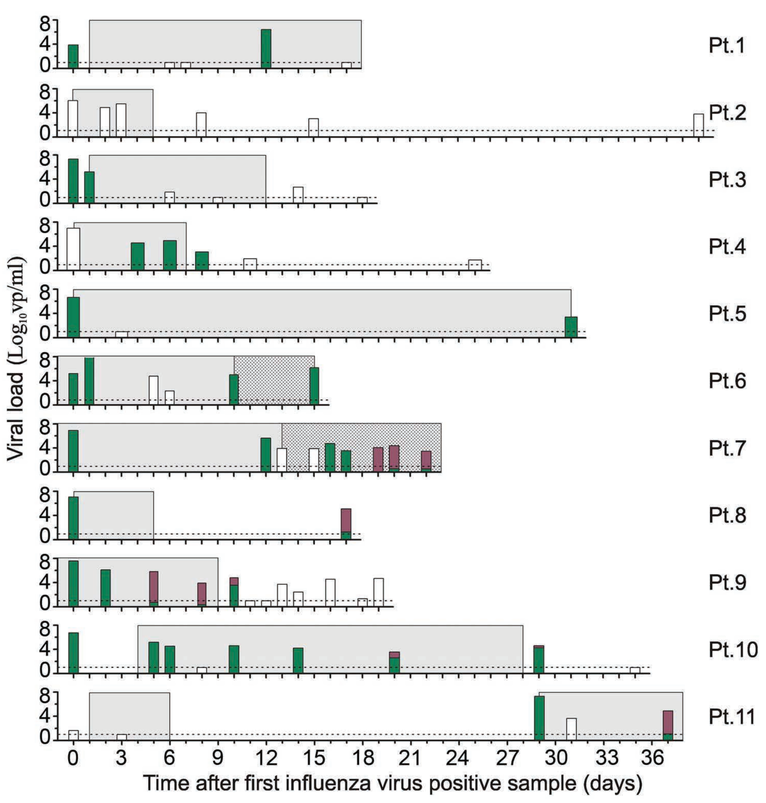
Immunosuppressive, antibiotic and antiviral drugs

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 (5 mg/ml) for infusion (Prograft, Astellas Pharma BV, Leiderdorp, The Netherlands) and prednisolone sodium phosphate (5 mg/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 (250/62.5 mg per 5 ml) 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).

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 4 mm and stained with haematoxylin and eosin (HE) for examination by light microscopy.

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



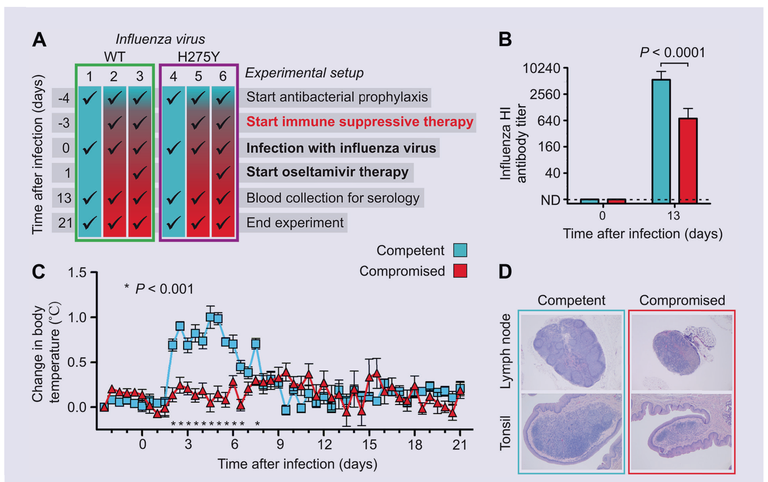
From 11 immunocompromised patients the viral load in respiratory specimens obtained during the courses of illness are shown in bars. Patients 1, 2 and 10 were solid organ transplant patients. Patients 3, 4, 5, 7, 8, 9 and 11 were cancer patients on chemotherapy. Patient 6 was treated for cerebral vasculitis. 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. doi:10.1371/journal.ppat.1003343.g001

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)

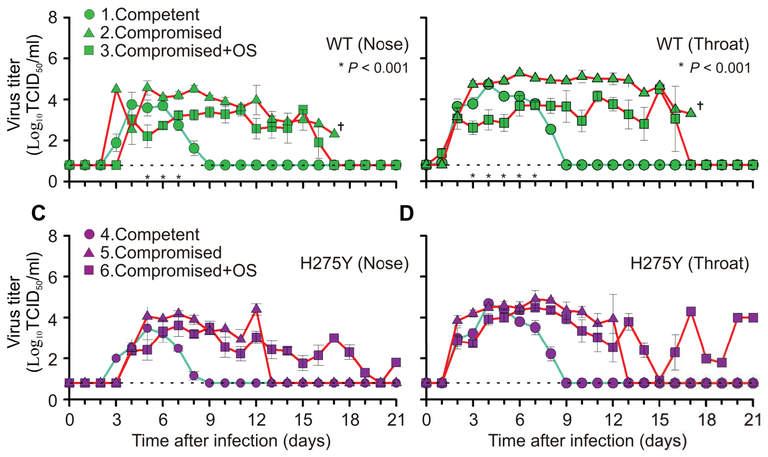
a Influenza A virus was detected by an influenza A virus real-time quantitative polymerase-chain-reaction (RT-qPCR) detection assay in respiratory specimens from a total number of 335 admitted patients between August 2009 and July 2012.
b Clinical data were extracted from hospitalized patient records.
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 ml.
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.
doi:10.1371/journal.ppat.1003343.t001

Ferrets on immune suppressive therapy show defective immune function.



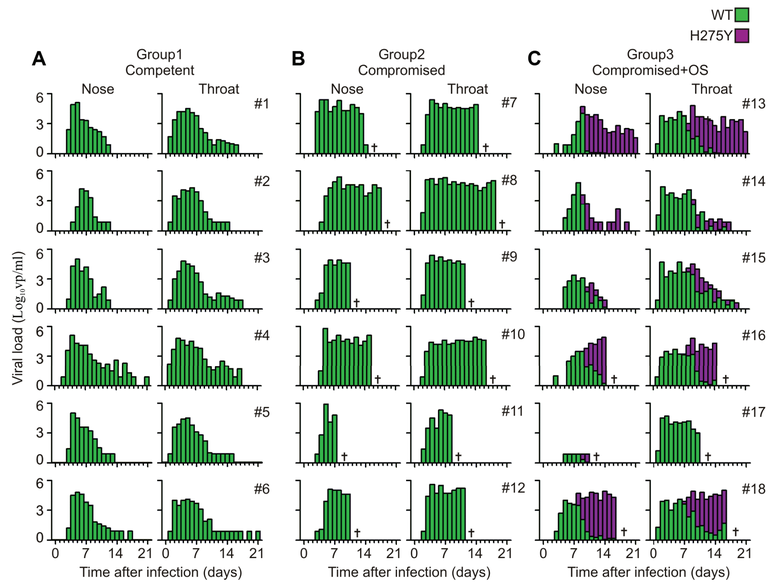
(a) Schematic of the experimental setup in which 6 groups of ferrets (n = 6) were infected on day 0 with wild type (WT; groups 1–3; green) or oseltamivir resistant (H275Y) mutant virus (groups 4–6; magenta). Three days before, immune suppressive therapy was started and added to the antibacterial cocktail 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 6 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 6 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 256) and tonsils (original magnification 506). Tissues were stained with hematoxylin and eosin (H&E). doi:10.1371/journal.ppat.1003343.g002

Prolonged virus replication in the upper respiratory tract of immunocompromised ferrets.



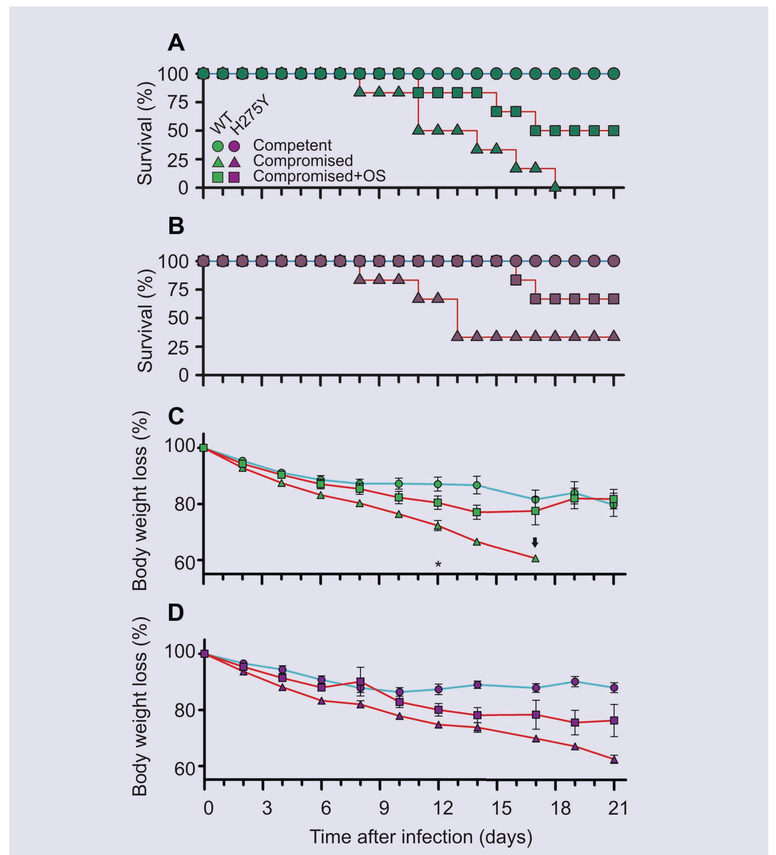
Influenza virus titers were determined in nose and throat swabs daily taken from immunocompetent (blue lines) and immunocompromised ferrets (red lines). The animals were infected either with wild type (WT; green; a, b) or mutant virus (H275Y; magenta; c, d). The dotted line in each figure indicates the LLOD. Data are mean 6 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. doi:10.1371/journal.ppat.1003343.g003

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. doi:10.1371/journal.ppat.1003343.g004

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 6 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. doi:10.1371/journal.ppat.1003343.g005

For the immunocompromised ferret model a patent is pending (PCT/EP2012/063497)