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Supporting Online Material for

Pathogenesis and Transmission of Swine-Origin 2009 A(H1N1) Influenza Virus in Ferrets

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SUPPORTING ONLINE MATERIAL

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Materials and Methods

Viruses

Influenza virus A/Netherlands/26/07 (seasonal A/H1N1 virus) was isolated from a nasopharyngeal swab, collected from a patient during the 2006-2007 influenza season. The virus was passaged in MDCK cells three times and had a titre of $10^{7.5}$ $TCID_{50}$/ml. Influenza virus A/Netherlands/602/2009 (2009 A/H1N1 virus) was isolated from the first case of laboratory-confirmed 2009 A/H1N1 virus infection in The Netherlands. The patient was a 3-year-old child that travelled with its family from Mexico to The Netherlands, and developed fever (>39.5 °C) and respiratory symptoms upon return. The patient was treated with oseltamivir and recovered uneventfully. The virus was isolated from a specimen collected before the start of drug treatment by inoculation of 11-day old embryonated chicken eggs and passaged once in MDCK cells, to yield a virus titre of $10^{7.8}$ $TCID_{50}$/ml. The virus used differs by eight amino acid positions from A/California/4/2009 (P100S, T214A and I338V in HA, I108V and V407I in NA, T373I in NP, P224S and M581L in PA; GISAID accession numbers EPI178246-250, EPI178467, EPI178290 and EPI178291). None of the differences map to known pathogenicity markers of influenza A virus (*1*). A/Netherlands/602/2009 is more representative (i.e. more consensus-like) than A/California/4/09; most amino acid changes in A/Netherlands/602/2009 as compared to A/California/4/2009 were also found in the majority of sequenced strains in the database. The only unique sequence changes in A/Netherlands/602/2009 are I108V and V407I in NA, which can be considered conservative changes, not previously associated with virulence. Until now,

the 2009 A/H1N1 viruses show low genetic diversity and we consider the isolate used to be representative of the currently circulating 2009 A/H1N1 virus.

Cells

Madin-Darby Canine kidney (MDCK) cells were cultured in Eagle's minimal essential medium (EMEM, Cambrex, Heerhugowaard, The Netherlands) supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1.5 mg/ml sodiumbicarbonate, 10 mM Hepes and non-essential amino acids.

Ferret models

To determine pathogenicity, groups of six adult female ferrets (*Mustela putorius furo*) were inoculated intranasally with 10^6 $TCID_{50}$ of seasonal A/H1N1 or 2009 A/H1N1 virus by applying a 250 µl volume of virus dilution to each nostril. The animals were observed for clinical signs and weighed daily as an indicator of disease. Nose and throat swabs were collected daily and transferred to 1 ml of transport media (Hanks balanced salt solution containing 0.5 % Lactalbumin, 10 % glycerol, 200 U/ml penicillin, 200 µg/ml streptomycin, 100 U/ml polymyxin B sulfate, 250 µg/ml gentamycin, 50 U/ml nystatin (ICN, the Netherlands) and virus titers were determined by end-point titration in MDCK cells. Three animals from each group were euthanized at 3 and 7 days post inoculation (d.p.i.) and virus titers in the lungs, trachea, nasal turbinates, spleen, liver, kidneys and brain were determined.

For aerosol or respiratory droplet transmission experiments, four female adult ferrets were inoculated intranasally with 10^6 $TCID_{50}$ of seasonal A/H1N1 or 2009 A/H1N1 virus by applying a 250 µl volume of virus dilution to each nostril. Each ferret was

then placed in a transmission cage (Fig. S3). At 1 d.p.i., one naive ferret was placed opposite each inoculated ferret. Each transmission pair was housed in a separate transmission cage designed to prevent direct contact between the inoculated and naive ferrets but allowing airflow (<0.1 m/sec) from the inoculated to the naive ferret. Nose and throat swabs were collected on 0, 1, 2, 3, 5, 7 and 9 d.p.i. for inoculated ferrets and on 0, 1, 2, 3, 5, 7 and 9 days post exposure (d.p.e.) for the naive ferrets. Virus titers in these swabs were determined by end-point titration in MDCK cells. Inoculated ferrets were euthanized at 9 d.p.i.; naive ferrets were euthanized at 15 d.p.e. to allow seroconversion. The transmission model was validated with seasonal human A/H1N1 (Fig. S4) and A/H3N2 viruses (data not shown), which resulted in transmission consistently, and with highly pathogenic avian influenza A/Indonesia/5/05 (H5N1) virus that did not result in transmission, as expected (Fig. S5).

An independent Animal Ethics Committee approved all animal studies. All experiments were performed under BSL3 conditions.

Histopathology and immunohistochemistry

Animal necropsies were performed according to a standard protocol. Samples for histological examination were stored in 10% neutral-buffered formalin (lungs after inflation with formalin), embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin (H&E) for examination by light microscopy or with an immunohistochemical method using a monoclonal antibody against the nucleoprotein of influenza A virus (*2*)

Virus titrations

Virus titrations were performed by end-point titration in MDCK cells. MDCK cells were inoculated with tenfold serial dilutions of homogenized tissues, nose and throat swabs. One hour after inoculation, cells were washed once with PBS and grown in 200 µl of infection media, consisting of EMEM (Cambrex, Heerhugowaard, The Netherlands) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2mM glutamine, 1.5mg/ml sodiumbicarbonate (Cambrex), 10mM Hepes (Cambrex), non-essential amino acids (MP Biomedicals) and 20 µg/ml trypsin (Cambrex). At 3 d.p.i., the supernatants of infected cell cultures were tested for agglutinating activity using turkey erythrocytes as an indicator of infection of the cells. Infectious titers were calculated from five (homogenized tissues) or four (swabs) replicates by the method of Spearman-Karber (*3*).

Statistical analysis

Viral titers and body weight were compared between the groups using the Mann-Whitney test. The Area-Under-The-Curve (AUC) of the absolute viral titer values was calculated for each ferret using the trapezoidal rule. The Mann-Whitney test was used to compare the AUC values between the groups.

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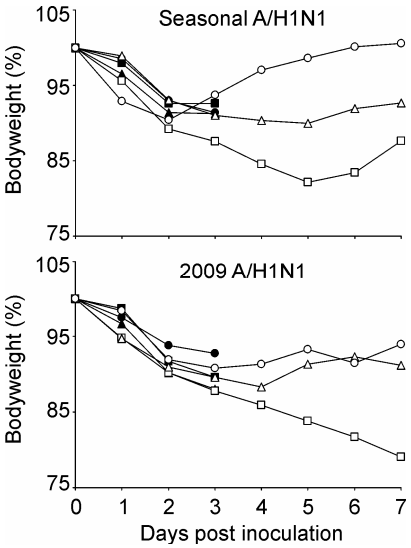

Figure S1. Weight loss of individual ferrets inoculated with seasonal and 2009 A/H1N1 virus. Bodyweight of individual ferrets is indicated as a percentage of their weight at the start of the experiment.

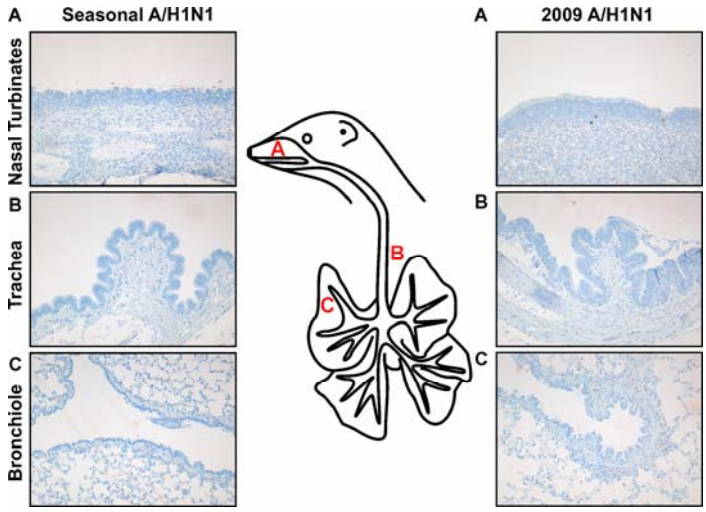

Figure S2. Immunohistochemical analysis of respiratory tract tissues of ferrets inoculated with seasonal or 2009 A/H1N1 virus, collected at 7 d.p.i. Tissue sections of the nasal turbinates (A), trachea (B), and bronchi (C) were stained with a monoclonal antibody against influenza A virus nucleoprotein. The same figure for tissues collected at 3 d.p.i. is shown in the manuscript. At 7 d.p.i. most virus was cleared from the respiratory tract.

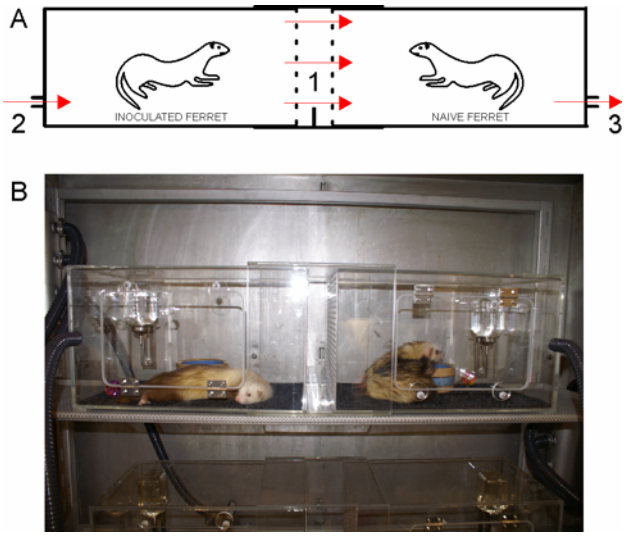

Figure S3. Schematic presentation (A) and a photograph (B) of transmission cages.

The transmission cages were specifically designed to allow transmission experiments to be conducted in negatively pressurized isolator cages (1.6m x 1m x 1m) present in the ABSL3 facility of Erasmus MC Rotterdam. The ferrets are housed in clear Perspex transmission cages, in which each inoculated animal was housed individually with a naive ferret. Each ferret cage was 30 cm x 30 cm x 55 cm (W x H x L) and the two cages of the inoculated and naive transmission pair were separated by two stainless steel grids (1), with a grid size of 0.5 cm^2, 10 cm apart. 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 (2) from the inoculated to the naive ferret. The outlet airflow (3) is HEPA filtered to prevent continuous circulation of infectious influenza A virus particles and to prevent cross-contamination with other transmission cages placed in the same isolator cage. Animals are housed on solid rubber floor tiles, which do not generate dust and avoid unwanted fomite transmission among animals. Arrows indicate airflow.

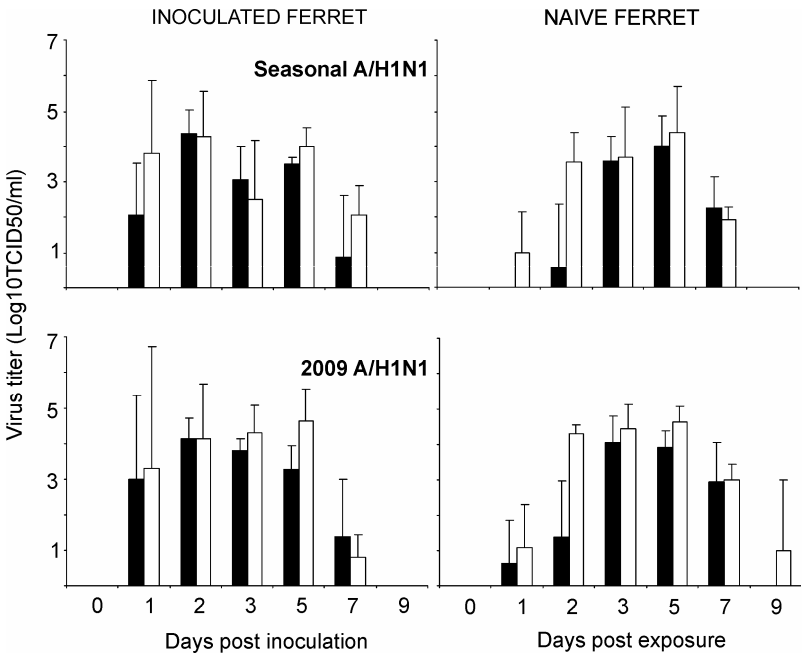

Figure S4. Shedding of seasonal A/H1N1 and 2009 A/H1N1 virus in ferrets in transmission experiments. Nose (white bars) and throat swabs (black bars) were collected on 0, 1, 2, 3, 5, 7 and 9 d.p.i. for inoculated ferrets and on 0, 1, 2, 3, 5, 7 and 9 d.p.e. for naive ferrets. Virus titers were determined by end-point titration in MDCK cells. Geometric mean titers are given; error bars indicate standard deviation.

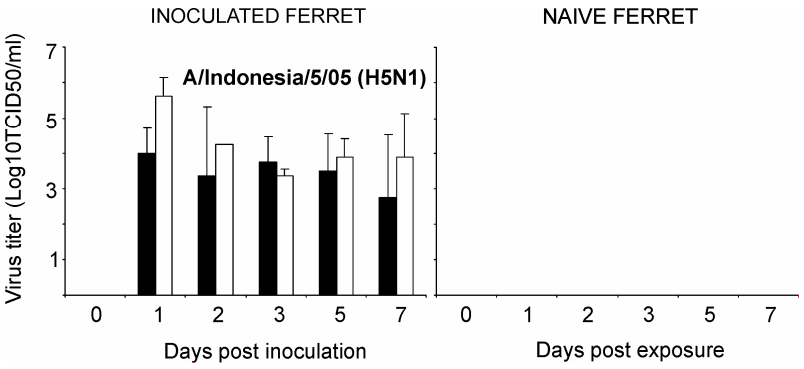

Figure S5. Shedding of influenza virus A/Indonesia/5/2005 (H5N1) in ferrets in transmission experiments. Nose (white bars) and throat swabs (black bars) were collected on 0, 1, 2, 3, 5, and 7 d.p.i. for inoculated ferrets and on 0, 1, 2, 3, 5, and 7 d.p.e. for naive ferrets. Virus titers were determined by end-point titration in MDCK cells. Geometric mean titers are given; error bars indicate standard deviation. Lack of transmission of A/H5N1 virus to naive ferrets was confirmed by serology.

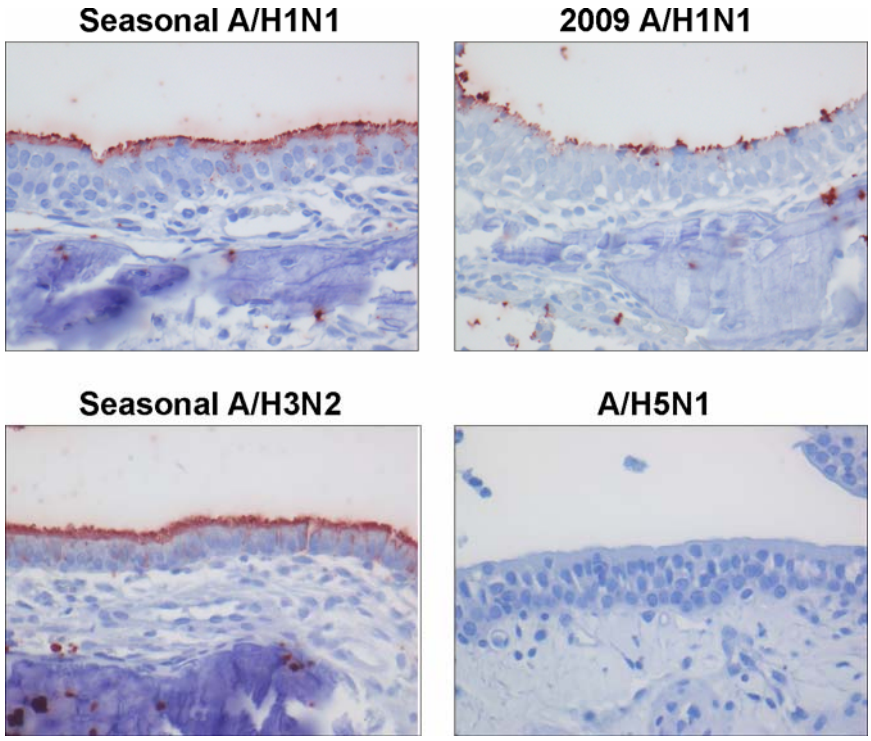

Figure S6. Attachment of the 2009 A/H1N1 virus to the nasal turbinates of ferrets, compared with seasonal human influenza A viruses and A/H5N1 virus. Formalin-fixed, paraffin-embedded tissue sections were incubated with formalin-inactivated fluorescein isothiocyanate (FITC)–labelled viruses as described previously (*4*). Virus binding to receptors is shown as a red-brown staining. Viruses used: A/Netherlands/602/2009 (H1N1), A/Netherlands/26/2007 (H1N1), A/Netherlands/213/2003 (H3N2), and A/Indonesia/5/2005 (H5N1). The 2009 A/H1N1 virus displayed similar receptor binding to seasonal human influenza viruses that predominantly use α2,6-linked sialic acid receptors, distinct from binding of an A/H5N1 virus that preferentially binds α2,3-linked sialic acid receptors.