Efficacy of Influenza Vaccination and Tamiflu® Treatment – Comparative Studies with Eurasian Swine Influenza Viruses in Pigs

Ralf Duerrwald¹, Michael Schlegel¹, Katja Bauer², Théophile Vissiennon³, Peter Wutzler², Michaela Schmidtke²*

¹ IDT Biologika GmbH, Dessau-Rosslau, Germany, ²Jena University Hospital, Department of Virology and Antiviral Therapy, Jena, Germany, ³Tierpathologie Leipzig, Leipzig, Germany

Abstract

Recent epidemiological developments demonstrated that gene segments of swine influenza A viruses can account for antigenic changes as well as reduced drug susceptibility of pandemic influenza A viruses. This raises questions about the efficacy of preventive measures against swine influenza A viruses. Here, the protective effect of vaccination was compared with that of prophylactic Tamiflu® treatment against two Eurasian swine influenza A viruses. 11-week-old pigs were infected by aerosol nebulisation with high doses of influenza virus A/swine/Potsdam/15/1981 (H1N1/1981, heterologous challenge to H1N1 vaccine strain) and A/swine/Bakum/1832/2000 (H1N2/2000, homologous challenge to H1N2 vaccine strain) in two independent trials. In each trial (i) 10 pigs were vaccinated twice with a trivalent vaccine (RESPIPORC® FLU3; 28 and 7 days before infection), (ii) another 10 pigs received 150 mg/day of Tamiflu® for 5 days starting 12 h before infection, and (iii) 12 virus-infected pigs were left unvaccinated and untreated and served as controls. Both viruses replicated efficiently in porcine respiratory organs causing influenza with fever, dyspnoea, and pneumonia. Tamiflu® treatment as well as vaccination prevented clinical signs and significantly reduced virus shedding. Whereas after homologous challenge with H1N2/2000 no infectious virus in lung and hardly any lung inflammation were detected, the virus titre was not and the lung pathology was only partially reduced in H1N1/1981, heterologous challenged pigs. Tamiflu® application did not affect these study parameters. In conclusion, all tested preventive measures provided protection against disease. Vaccination additionally prevented virus replication and histopathological changes in the lung of homologous challenged pigs.

Introduction

Vaccines and antiviral drugs are essential means for control of influenza [1]. The fast spread and frequent mutation rate of influenza viruses contribute to high incidence and variability of these viruses in seasonal, epidemic, and pandemic influenza [2,3]. The area-wide and permanent circulation of swine influenza A viruses together with the possibility of interspecies transmission and replication of avian and human influenza A viruses enables reassortment of new viruses in pigs [4–9]. As shown by the emergence of pandemic influenza A H1N1(2009) virus (pH1N1/2009) such reassorted viruses can represent a worldwide threat [10–12]. The antigenic properties as well as drug susceptibility of pH1N1/2009 are determined by gene segments of swine influenza A viruses. In particular, pH1N1/2009 became resistant to M2 channel inhibitors [13,14] by accepting the matrix protein-coding gene of European swine influenza A viruses which confers the drug resistance [15,16]. Since H3N2 viruses circulating in humans are also resistant to this drug class [17,18] a situation of nearly 100% prevalence of ion channel inhibitor resistance was caused worldwide and neuraminidase inhibitors (NAI) like Tamiflu® and Relenza® are the only drugs considered for additional prophylactic use at the moment.

The current knowledge about the efficacy of existing NAI against Eurasian swine influenza A viruses is based only on in vitro data [19,20]. To extend this knowledge, in the present study the efficacy of vaccination as well as the application of Tamiflu® against two Eurasian swine influenza A viruses was compared under experimental conditions in their natural host. The protective effect of vaccination was comparatively studied in a vaccine-heterologous as well a vaccine-homologous challenge.

* E-mail: michaela.schmidtke@med.uni-jena.de
Results

Comparison of efficacy of vaccination and Tamiflu® treatment against H1N1/1981 (vaccine-heterologous challenge)

H1N1/1981 had been isolated within the first period after introduction of avian-like viruses into the European pig population [21,22]. Because the vaccine strain H1N1/2003 was isolated after 22 years of evolution of these viruses in pigs and vaccinated pigs do not cross-react in HI with H1N1/1981, challenge with H1N1/1981 allows studying the efficacy of vaccination against heterologous challenge with a not cross-reactive strain of the same influenza A virus subtype in comparison to the prophylactic effect of Tamiflu®.

Just 24 hours after infection with H1N1/1981 unvaccinated untreated pigs developed influenza with dyspnoea diagnosed until day 3 p.i. (Fig. 1A). Coughing was observed rarely in individual animals only (data not shown). Furthermore, a significant rise in body temperature was observed on day 1 p.i. (Fig. 1B). Vaccination and Tamiflu® treatment significantly reduced clinical signs (Fig. 1A and 1B). Reduction of body weight was not observed (data not shown).

Up to 6 days p.i. infected, untreated as well as Tamiflu®-treated pigs shed virus (Fig. 1C). Thereafter, virus titres decreased markedly coinciding with the appearance of first antibodies against the challenge strain (Fig. 2). All (12/12 pigs until day 2 p.i., 7/7 pigs from day 3 to 5 p.i.) untreated and unvaccinated pigs had virus titres in their nasal swabs ranging from 1.3 to 3.7 log10 EID50/ml. On day 6 p.i. 4 of 7 pigs of this group shed virus (0.9–1.3 log10 EID50/ml). On day 7 p.i. virus shedding ceased. Tamiflu®-treated pigs showed following shedding data: day 1 p.i. 9/10 pigs 1.3–2.7 log10 EID50/ml, day 2 p.i. 9/10 pigs 0.7–1.7 log10 EID50/ml, day 3 p.i. 4/5 pigs 1.3–2.7 log10 EID50/ml, day 4 p.i. 5/5 pigs 0.9–2.5 log10 EID50/ml, day 5 p.i. 4/5 pigs 1.3–3.3 log10 EID50/ml, day 6 p.i. 4/5 pigs 0.7–1.7 log10 EID50/ml, day 7 p.i. 0/5 pigs. Vaccinated pigs had following shedding profile: day 1 p.i. 5/10 pigs 1.3–2.5 log10 EID50/ml, day 2 p.i. 2/10 pigs 0.7–3.3 log10 EID50/ml, day 3 p.i. 5/5 pigs 0.7–3.3 log10 EID50/ml, day 4 p.i. 3/5 pigs 1.3–2.9 log10 EID50/ml, day 5 p.i. 2/5 pigs 0.7–0.9 log10 EID50/ml, day 6 p.i. 0/5 pigs, day 7 p.i. 0/5 pigs. At some time points the mean virus titre in nasal swabs of the vaccinated and the Tamiflu®-treated group was significantly lower than in the control group (Fig. 1C). However, one day after Tamiflu® treatment had been stopped, virus titres in nasal swabs increased.

Figure 1. Protective effect of Tamiflu® in 11-week-old, A/swine/Potsdam/15/1981 (H1N1/1981) virus challenged pigs (n = 10) in comparison to RESPIPORC® FLU3-vaccinated (n = 10) and untreated animals (n = 12). Dyspnoea (A), rectal temperatures (B), virus titres in nasal swabs (C), and mean of histopathological scores with standard deviations and representative photographs of formalin fixed, HE stained lungs (D) are shown (am morning; pm afternoon; p statistical probability: *p<0.05, **p<0.01, ***p<0.001, a vaccinated group versus control group, b Tamiflu®-treated group versus control group, c vaccinated group versus Tamiflu®-treated group, Mann-Whitney-U-test). The detection limit of virus titre determination is shown as dotted line (C).

doi:10.1371/journal.pone.0061597.g001
Figure 2. Influence of vaccination and Tamiflu® treatment on antibody kinetics in pigs challenged with A/swine/Potsdam/15/1981 (H1N1/1981) virus. Hemagglutination inhibition assays were performed with serum from pigs vaccinated with RESPIPORC® FLU3 (A), pigs treated with Tamiflu® (B), and untreated pigs (C). Geometric mean and standard deviation of antibody titres determined in serum samples of 10 vaccinated and Tamiflu®-treated or 12 control animals/day are shown until day 2 p.i. and 5 or 7 from day 3–10 p.i., respectively. Mann-Whitney-U-test was used to calculate p statistical probability: *p<0.05, **p<0.01, ***p<0.001. Only significant differences are shown. The detection limit of HI antibody titre determination is shown as dotted line.

doi:10.1371/journal.pone.0061597.g002
Table 1. Summary of the virus titres determined in left and right lung lobes and macroscopic lung lesions at ventral and dorsal view (mean ± standard deviation; n = 5; control group day 10 p.i. n = 7); on day 10 p.i. there was no virus in the lungs anymore (data not shown).

| Challenge         | Experimental group | Lung virus titre (log_{10} EID_{50}/g) | Macroscopic lung lesions (% of affected lung area) on day |
|-------------------|--------------------|---------------------------------------|---------------------------------------------------------|
|                   |                    | on day 2 p.i.                          | 2 p.i.                                                  | 10 p.i.                                                  |
|                   |                    | left lobe                              | ventral                                           | dorsal                                           |
|                   |                    | right lobe                             | ventral                                           | dorsal                                           |
| H1N1/1981         | control            | 3.90±0.51                             | 1.00±1.00                                         | 3.57±1.62                                         |
|                   | Tamiflu-treated    | 4.10±0.35                             | 0.60±0.89                                         | 0.90±1.02                                         |
|                   | FLU3-vaccinated    | 4.46±0.55                             | 2.40±1.82                                         | 0.60±0.82                                         |
| H1N2/2000         | control            | 3.54±0.62                             | 1.90±1.52                                         | 2.71±3.30                                         |
|                   | Tamiflu-treated    | 3.70±0.62                             | 0.55±0.84                                         | 0.40±0.42                                         |
|                   | FLU3-vaccinated    | ≤0.5a*                                | ≤0.5a**                                          | ≤0.5a**                                          |

Statistics, Mann-Whitney-U test, significant differences are shown:
a: vaccinated versus control group;  
b: Tamiflu-treated versus control group; there were no significant differences concerning vaccinated versus Tamiflu-treated group;  
p<0.05;  
p<0.01;  
0.5 detection limit.  
doi:10.1371/journal.pone.0061597.t001

On day 2 p.i., very similar, high virus load was determined in lungs of untreated, vaccinated and Tamiflu-treated H1N1/1981-challenged pigs (Table 1). On day 10 p.i., there was no infectious virus (data not shown).

Macroscopic lung lesions of control pigs mainly affected the margins of the cardiac lobes, followed by lesions on the margins of the apical lobes. The diaphragmatic lobe was only rarely affected near to the cardiac lobe. In general the extent of lung consolidation did not exceed 5–10% of the lung surface. On day 2 p.i., a protective effect was neither observed in Tamiflu-treated nor in FLU3-vaccinated animals. Two vaccinated pigs had larger lung lesions than any other pig. On day 10 p.i., lesions were significantly lower in vaccinated pigs and Tamiflu-treated pigs (Table 1). A histopathological score of about 3 and 2 was detected in untreated, infected as well as Tamiflu-treated pigs on day 2 and 10 p.i. (Fig. 1D). Despite similar virus replication in the lung, a significantly lower histopathological score was observed in vaccinated pigs at both time points (Fig. 1D).

After first vaccination with FLU3 marginal antibody titres to the vaccine strains H1N1/2003 and H1N2/2000 were detected (Fig. 2A). These antibodies had risen to highly significant titres after second vaccine administration and did not differ significantly between H1N1/2003 and H1N2/2000. The challenge induced a strong H1N1/1981-specific antibody response on 6 days p.i. in vaccinated pigs whereas it was observed in pigs of the Tamiflu-treated group; there were no significant differences concerning vaccinated versus Tamiflu-treated group; p<0.05; p<0.01; 0.5 detection limit.  
doi:10.1371/journal.pone.0061597.t001

Comparison of efficacy of influenza vaccination and Tamiflu treatment against H1N2/2000 (vaccine-homologous challenge)

The FLU3 vaccine contains a high passage of strain H1N2/2000. Therefore, challenge with the same H1N2 enables studying the effect of vaccination against homologous virus infection.

Influenza induced by H1N2/2000 in untreated, infected control animals was characterized by two dyspnoea peaks on day 1 and 4 p.i. (Fig. 4A) and temperature >41°C on day 1 p.i. (Fig. 4B). All pigs recovered from clinical signs within 5 days. Clinical signs were neither observed in vaccinated nor in Tamiflu-treated pigs. None of the H1N2/2000-infected animals lost body weight (results not shown).

Vaccination as well as Tamiflu-treatment caused a significant virus titre reduction in nasal swabs (Fig. 4C). All unvaccinated, untreated pigs shed virus from day 1 to 5 p.i. Virus titres ranged from 1.3 to 2.7 log_{10} EID_{50}/ml. Mean virus titres of Tamiflu-treated pigs were significantly reduced from 1 to 4 days p.i. (day 1 p.i. 6 of 10 pigs 6/10 pigs shed virus at titres ranging from 0.7–0.9 log_{10} EID_{50}/ml, day 2 p.i. 5/10 pigs 0.7–1.3 log_{10} EID_{50}/ml, day 3 p.i. 2/5 pigs 0.7–2.1 log_{10} EID_{50}/ml, day 4 p.i. 3/5 pigs 0.9–2.3 log_{10} EID_{50}/ml). After stopping Tamiflu-treatment H1N2/2000 shedding increased in four of five pigs on day 5 (1.3–2.3 log_{10} EID_{50}/ml) but vanished already 6 days p.i. due to the appearing antibodies (Fig. 4C, 5B). In contrast, five of 10 pigs of the vaccinated group did not shed the virus at all. Low virus titres of 0.7 log_{10} EID_{50}/ml were detected in the nasal swabs of three vaccinated pigs 24 h p.i., from a fourth pig from day 2 to 5 p.i.
While vaccination completely prevented H1N2/2000 replication in lungs of vaccinated pigs, the viral titres between the Tamiflu\textsuperscript{H}-treated and untreated animals did not differ (Table 1).

The mean extent of lung consolidation of control animals challenged with H1N2/2000 ranged from 1.9 to 3.0\% (Table 1). Vaccinated pigs had few or no lesions at all on day 2 and 10 p.i. A significantly reduced lung histopathology was also observed in vaccinated pigs in comparison to untreated, unvaccinated pigs on days 2 and 10 p.i. (Fig. 4D). A high mean histopathological score of about 3 was characteristic for lung tissue samples of untreated as well as Tamiflu\textsuperscript{H}-treated pigs on day 2 p.i., an improvement was observed in Tamiflu\textsuperscript{H}-treated pigs (3.2 versus 2.3; not significant, Fig. 4D).

Highly significant NI antibodies were prevalent against all viruses investigated in the vaccinated group (Fig. 3B; “before” versus “vaccination”: \(p<0.001\); statistics for comparison between the groups are not shown). The challenge with H1N2/2000 did not significantly booster these antibodies (Fig. 3B; “vaccination” versus “vaccination and challenge”: \(p>0.05\)). Significant amounts of NI antibodies were induced against N2 in the unvaccinated, untreated control group (Fig. 3B; “before” versus “challenge”: \(p<0.001\)). They did not act against N1.

**Discussion**

High-dose aerosol infection of pigs with H1N1/1981 and H1N2/2000 caused sudden onset of high fever and dyspnoea like influenza in humans [2,3]. Compared with pig infection trials reported so far the observed clinical symptoms were stronger [23–28]. These similarities between influenza in aerosol-infection pig models and influenza in humans and pigs in addition to the similar disease course induced by H1N1/1981 and H1N2/2000 provided a good basis for comparatively evaluating the efficacy of vaccination against heterologous and homologous challenge as well as NAI treatment in the present study. The results reveal different degrees of protection.

Like in humans [29,30], i) vaccination as well as Tamiflu\textsuperscript{H}-treatment significantly reduced clinical symptoms and virus...
shedding whereas (ii) vaccination was less effective when the challenge occurred with heterologous H1N1/1981 than with homologous H1N2/2000. The faster and stronger antibody response against the heterologous challenge strain H1N1/1981 may explain the efficacy of vaccination in the absence of virus-specific HI antibodies against the challenge virus. It suggests a certain degree of reactivity between older and more recent H1N1 strains. The latter could also account for detection of cross-protecting HI antibodies against pH1N1/2009 that concurs strongly with recently published studies [31–34]. Moreover, a higher antibody response against the vaccine strain H1N1/2003 was detected after H1N1/1981 challenge reflecting the “antigenic sin” [35,36]. Additionally, NI can contribute to the protective effect seen after vaccination and heterologous challenge. NI cross-reacting NI antibodies were detected indicating that neuraminidases of H1N1 strains are still antigenetically related to each other. Moreover, major histocompatibility complex restricted epitopes conserved in nucleoprotein and matrix protein could be involved in protection as discussed for human seasonal influenza A viruses and pH1N1/2009 virus [37]. European swine influenza A viruses share similar nucleoprotein, matrix, and polymerase genes.

Marked differences were found comparing the efficacy of the studied preventive measures regarding lung viral load, macroscopic lesions, and inflammation. The lack of virus inhibition in the lung after vaccination and heterologous challenge reflects the pathogenic role of antigenic drift in European swine Influenza A (H1N1) viruses between 1981 and 2003. It also demonstrates a low efficacy of Tamiflu® regarding this study parameter. Macroscopic lung lesions were almost absent after vaccination and homologous challenge. They were also reduced by Tamiflu® treatment as well as in vaccinated, heterologous challenged pigs on day 10 p.i. based on similar observations until day 5 p.i., Gauger et al. postulated that vaccination may potentiate influenza following challenge with divergent homosubtypic viruses that do not share cross-reacting hemagglutinin or serum neutralizing antibodies [32]. But, the significant reduced lung consolidation on day 10 p.i. reported here suggests that this effect is transient and reversed by antibodies specific to the challenge virus. With regard to inflammation, a significant score reduction was found after vaccination but not after drug treatment. Taken together, these results suggest that the pathogenetic processes which lead to induction of disease are blocked at different stages by vaccination and Tamiflu®. The latter prevented disease despite high viral lung load and interstitial lymphoid tissue hyperplasia.

Figure 4. Antiviral activity of Tamiflu® in 11-week-old, A/swine/Bakum/1832/00 (H1N2/2000) virus infected pigs (n=10) in comparison to RESPIPORC® FLU3-vaccinated (n=10) and untreated animals (n=12). Dyspnoea (A), rectal temperatures (B), virus titres in nasal swabs, n=10 animals/group/day until day 2 p.i. and n=5 from day 3 p.i. on, exception: n=12 untreated animals at day 0 to 2 p.i. and n=7 untreated animals/day at 3 to 7 p.i. (C), and mean of histopathological scores with standard deviations and representative photographs of formalin fixed, HE stained lungs (D) are shown (am morning; pm afternoon; p statistical probability: *p<0.05, **p<0.01, ***p<0.001, a vaccinated group versus control group, b Tamiflu®-treated group versus control group, c vaccinated group versus Tamiflu®-treated group, Mann-Whitney-U-test). The detection limit of virus titre determination is shown as dotted line (C).

doi:10.1371/journal.pone.0061597.g004
Two further aspects should be mentioned concerning Tamiflu® treatment. First, the increased virus shedding one day after drug cessation suggest a need for prolonged treatment of pigs until the appearance of protective antibodies in serum. Detection of virus-specific protective antibodies in the blood correlating with virus clearance in the nose underlines this conclusion. Secondly, H1N2/
Flu3 (FLU3, IDT Biologika GmbH, Dessau-Roßlau, Germany) born females; IDT Biologika GmbH, Barsbüttel, Germany) supplemented with 5% fetal bovine serum (Biochrom AG, Berlin, Germany).

Influenza viruses A/swine/Potsdam/15/1981 (avian-like H1N1; H1N1/1981) and A/swine/Bakum/1832/2000 (human-like H1N2; H1N2/2000) (Federal Institute for Risk Assessment, Berlin, Germany, [5]) had been isolated from pig herds in Germany during clinical outbreaks. Additionally, pandemic influenza virus A/Jena/VI5258/2009 (pH1N1/2009) was included in serological studies [Jena University Hospital, Germany]. Virus cultivation in MDCK cells was supported by adding 4 N benzoyl-L-arginine ethyl ester units trypsin (Sigma Aldrich, Munich, Germany).

Animals

64 crossbred swine (Piétrain × Large White; 48 males and 16 females; IDT Biologika GmbH, Dessau-Roßlau, Germany) born on the same farrowing occasion were used in the present study. Pigs had been proved to be free of influenza during their life span as well as free of maternally-derived antibodies against pH1N1/2009, avian H1N1 and human H1N2 influenza A viruses. They were housed in identical isolation rooms based on their challenge status and were provided with feed and water ad libitum.

Compounds

Commercially available Tamiflu® capsules (F. Hoffmann-La Roche AG, Basel, CH, batch B113313, 75 mg oseltamivir per capsule) were used for in vivo antiviral studies according to summary of product characteristics.

Vaccine

The trivalent inactivated swine influenza A virus vaccine RESPIPORC® FLU3 (FLU3, IDT Biologika GmbH, Dessau-Roßlau, Germany; Batch 0050806) was used for vaccination of pigs. It contained the highly passaged vaccine strains A/swine/Bakum/IDT1769/2003 (H3N2/2003), H1N2/2000, and A/swine/Hasenschuette/IDT2617/2003 (H1N1/2003), carbomer 971 P NF (0.998 mg/ml) as adjuvant and thiomersal (0.095 mg/ml) for preservation. In the batch potency testing the guinea pig geometric means of neutralizing units were 11.07 for H1N1/2003, 14.04 for H1N2/2000, and 12.67 for H3N2/2003. FLU3 had a pH of 7.1, was sterile, free of extraneous viruses and complied with the requirements for release.

Experimental Design

**Group classification and experimental conditions.** Two independent trials, one with H1N1/1981 [heterologous challenge with homosubtypic virus not cross-reactive to sera of vaccinated pigs] and another with H1N2/2000 [homologous challenge with the same strain as in the vaccine highly cross-reactive to sera of vaccinated pigs] were performed. The experimental design is summarized in Table 2. In each trial 32 pigs were allotted randomly into 3 groups. One group of 10 pigs was vaccinated i.m. with 2.0 ml of FLU3 21 and 7 days before challenge. Another group of 10 pigs was treated orally with Tamiflu® starting with 2 capsules the evening before challenge. Then, 2 Tamiflu® capsules were administered twice daily for 4 days. The third group included 12 unvaccinated untreated pigs as control.

At an age of 11 weeks, pigs of all 3 groups were simultaneously challenged by one-hour aerosol exposure. Aerosols of H1N1/1981 and H1N2/2000 were dispersed through a flow aerosol generator which produces droplets of 0.5 to 20 μm under atmospheric pressure. H1N1/1981 was nebulised at a dose of 10^7.85 TCID₅₀/ml and H1N2/2000 at a dose of 10^7.34 TCID₅₀/ml.

Experimental infections were done in BSL-2 infection units with High Efficiency Particulate Airfilter H13 filters.

**Study Parameters and Sampling.** After infection, rectal temperatures and signs of respiratory disease, dyspnoea, and cough were recorded twice daily 1–3 days p.i. and daily from 4–10 days p.i. Dyspnoea was assessed as follows: 1, increased respiratory frequency and moderate flank movement; 2, marked breathing difficulty and severe flank movement; 3, laboured breathing affecting the entire body, pronounced flank movement and substantial movements of the snout; 4, extreme breathing difficulty reflecting substantial lack of oxygen. Body weights were recorded daily. Nasal swab samples were collected daily in 2 ml stabilisation medium containing 60 ml Dextran-Sucrose-Glutamate solution (DSG 72: 120 g dextran 40, 1.5 kg sucrose, 3.0 g potassium-L-glutamate-monohydrate, 5 g potassium-dihydrogenphosphate, 12.5 g potassium-monohydrogenphosphate, made up to 10 l with water ad injectionem, IDT Biologika GmbH, Dessau-Roßlau, Germany), 0.2 ml gentamycin (Fagron GmbH, Barsbüttel, Germany), 2 ml amphotericin B (Sigma-Aldrich, Taufkirchen, Germany), made up to 200 ml with cell culture medium (IDT Biologika GmbH, Dessau-Roßlau, Germany, internal use).

In each trial 5 animals of each group were stunned by electrical stunning tongs 2 days p.i. and bled to death. On 10 days p.i. the remaining animals were slaughtered in the same way.

Lung tissue samples were taken from each lobe for virus detection. Samples of the right and left halves of the lungs were pooled, ground with sterile sea sand, and diluted 1:10 in dilution medium (1.0 ml Amphotericin B and 0.1 ml Gentamycin, made up to 100 ml with phosphate buffered saline solution). Additionally, lung tissue was collected and fixed in 10% neutral buffered formalin for histopathological evaluation.
Blood samples for immunological analysis were taken immediately before the first and second vaccinations, 7 days after the second vaccination (before challenge), and 2, 4, 6, 8, and 10 days p.i.

### Hemagglutination Inhibition (HI) Assay

Sera were pre-treated with neuraminidase (Sigma, EC3.2.1.18 Type IV from *Clostridium perfringens*, 14–18 h at 37°C). After adding sodium citrate (1.5%) inactivation was carried out (30 minutes at 56°C), followed by adsorption to chicken erythrocytes (1 h at 4–8°C).

8 hemagglutinating units (HU) of the vaccine strains, H1N1/1961, and pH1N1/2009 were used as antigens and incubated with 1:10 prediluted sera in microtitre plates for 30 min at room temperature. Then a 0.5% chicken erythrocyte suspension was added and incubated for 30 min at room temperature.

### Determination of 50% Egg Infectious Dose (EID50)

Dilution series (log10) from both lung and nasal swab samples were injected into the allantois cavity of 11-day-old chicken embryos (0.1 ml; 5 eggs per dilution). After sealing the perforation with a 0.5% chicken erythrocyte suspension was added and incubated for 30 min at room temperature.

### NA Inhibition (NI)

NI was analyzed using modified protocol of Sandbulte et al. [41]. Briefly, OD was measured at wavelength 550 nm. For data analysis, the absorbance of the fetuin control wells was subtracted from the OD values and the dilution of sera that resulted in a reading equal to 50% of the positive control (virus, no serum) was determined. The inverse of this dilution was the NI titre. Assay validity was supported by positive control samples (virus+fetuin) with mean absorbance of 0.7–1.3, negative control samples (fetuin only) with mean absorbance <0.08, and control serum which did not significantly inhibit NA activity.

### Lung Pathology and Histopathology

The pathology of the lungs was evaluated macroscopically, photographs were taken, and observed lesions were recorded onto a lung diagram. Percentage of affected lung surface area was assessed for each lobe at dorsal and ventral view.

Formalin-fixed lung tissue samples were embedded in paraffin. 5 μm-thick sections were stained with haematoxylin and eosin for light microscopy. Inflammation was scored on a semi quantitative scale from 0–4: 0, no inflammation; 1, discreet interstitial alveolar macrophages; 2, slight interstitial bronchial associated lymphoid tissue hyperplasia; 3, distinct interstitial alveolar macrophages; 4, distinct interstitial and massive broncholuminal alveolar macrophages.

### Statistical Analysis

Mann-Whitney-U-test was performed to evaluate statistical significances.

### Acknowledgments

We thank Kerstin Wieczorek, Roswitha Ulrich, Simone Köppen, and Katrin Schulz (IDT Biologika GmbH, Dessau-Roßlau, Germany) for their technical assistance, Olaf Luder and his co-workers (IDT Biologika GmbH, Dessau-Roßlau, Germany) for their participatory work in the animal studies and Christina Schrader (Federal Institute for Risk Assessment, Berlin, Germany), Jochen Siüs (Friedrich-Loeffler-Institut, Jena), and Andi Krumbholz (Jena University Hospital, Jena, Germany) for providing virus strains.

### Author Contributions

Conceived and designed the experiments: RD M. Schlegel PW M. Schmidtke. Performed the experiments: RD M. Schlegel PW M. Schmidtke. Analyzed the data: RD KB TV M. Schmidtke. Contributed reagents/materials/analysis tools: RD TV PW M. Schmidtke. Performed the experiments: RD M. Schlegel KB TV. Wrote the paper: RD M. Schmidtke.
7. Van Reeth K, Nicoll A (2009) A human case of swine influenza virus infection in Europe—implications for human health and research. Euro Surveill 14.

8. Zell R, Bergmann S, Krumholz A, Wutzler P, Duerrwald R (2008) Ongoing evolution of swine influenza viruses: a novel reassortant. Arch Virol 153: 2005–2009.

9. Zell R, Motzek S, Krumholz A, Wutzler P, Hervig V, et al. (2009) Novel reassortant of swine influenza H1N2 virus in Germany. J Gen Virol 89: 271–276.

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

11. Itoh Y, Shiina K, Kiso M, Watanabe T, Sakoda Y, et al. (2009) In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses. Nature 460: 1021–1023.

12. Smith GJ, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, et al. (2009) Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature 459: 1122–1125.

13. Gubareva LV, Trujillo AA, Okomo-Adhiambo M, Mishin VP, Deyde VM, et al. (2010) Comprehensive assessment of 2009 pandemic influenza A (H1N1) virus drug susceptibility in vitro. Antivir Ther 15: 1151–1159.

14. Nguyen JT, Cemec DF, Barnard DL, Juhander JG, Gross M, et al. (2012) Efficacy of combined therapy with amantadine, oseltamivir, and ribavirin in vivo against susceptible and amantadine-resistant influenza A viruses. PLoS One 7: e31006.

15. Krumholz A, Schmidtke M, Bergmann S, Motzek S, Bauer K, et al. (2009) High prevalence of amantadine resistance among circulating European porcine influenza A viruses. J Gen Virol 90: 900–908.

16. Schmidtke M, Zell R, Bauer K, Krumholz A, Schrader C, et al. (2006) Amantadine resistance among porcine H1N1, H1N2, and H3N2 influenza A viruses isolated in Germany between 1981 and 2001. Intervirology 49: 286–293.

17. Bright RA, Medina MJ, Xu X, Perez-Orozco G, Wallin TR, et al. (2005) Incidence of adamantane resistance among influenza A (H3N2) viruses isolated worldwide from 1994 to 2005: a cause for concern. Lancet 366: 1171–1181.

18. Deyde V, Garten R, Sheu T, Smith C, Myrick A, et al. (2009) Genomic events underlaying the changes in adamantane resistance among influenza A(H3N2) viruses during 2006–2008. Influenza Other Respir Viruses 3: 297–314.

19. Bauer K, Duerrwald R, Schlegel M, Pfarr K, Topf D, et al. (2011) Neuraminidase inhibitor susceptibility of swine influenza A viruses isolated in Germany between 1981 and 2008. Med Microbiol Immunol 201: 61–72.

20. Bauer K, Schrader C, Suess J, Wutzler P, Schmidtke M (2007) Neuraminidase inhibitor susceptibility of porcine H3N2 influenza A viruses isolated in Germany between 1982 and 1999. Antiviral Res 75: 219–226.

21. Pensaert M, Ottis K, Vandeputte J, Kaplan MM, Bachmann PA (1981) Protection against a European H1N2 swine influenza virus in pigs previously infected with H1N1 and/or H3N2 subtypes. Vaccine 21: 1375–1381.

22. Van Reeth K, Labarque G, De Clercq S, Pensaert M (2001) Efficacy of vaccination of pigs with different H1N1 swine influenza viruses using a recent challenge strain and different parameters of protection. Vaccine 19: 4479–4486.

23. Van Reeth K, Van Gucht S, Pensaert M (2003) Investigations of the efficacy of European H1N1- and H3N2-based swine influenza vaccines against the novel H1N2 subtype. Vet Rec 153: 9–13.

24. Weingartl HM, Albrecht RA, Lager KM, Babik S, Marszel P, et al. (2009) Experimental infection of pigs with the human 1918 pandemic influenza virus. J Virol 83: 4287–4296.

25. Hsu J, Santesos N, Mustafa R, Brozek J, Chen YL, et al. (2012) Antivirals for Treatment of Influenza: A Systematic Review and Meta-analysis of Observational Studies. Ann Intern Med.

26. Osterholm MT, Kelley NS, Sommer A, Belongia EA (2011) Efficacy and effectiveness of influenza vaccines: a systematic review and meta-analysis. Lancet Infect Dis 12: 36–44.

27. Duerrwald R, Krumholz A, Baumgarte S, Schlegel M, Vahlenkamp TW, et al. (2010) Swine influenza A vaccines, pandemic (H1N1) 2009 virus, and cross-reactivity. Emerg Infect Dis 16: 1029–1030.

28. Ginger PC, Vincent AL, Loving CL, Lager KM, Janke BH, et al. (2011) Enhanced pneumonia and disease in pigs vaccinated with an inactivated human-like (delta-cluster) H1N2 vaccine and challenged with pandemic 2009 H1N1 influenza virus. Vaccine 29: 2712–2719.

29. Kyriakes CS, Olsen CW, Carman S, Brown IH, Brooks H, et al. (2010) Serologic cross-reactivity with pandemic (H1N1) 2009 virus in pigs. European Emerging Infectious Disease 16: 96–99.

30. Van Reeth K, Labarque G, Pensaert M (2006) Serological profiles after consecutive experimental infections of pigs with European H1N1, H1N2, and H1N2 swine influenza viruses. Viral Immunol 19: 375–382.

31. Francis T (1960) On the doctrine of original antigenic sin. Proceedings of the American Philosophical Society 104: 572–576.

32. Zell R, Motzek S, Krumbholz A, Wutzler P, Schrader C, et al. (2006) Ongoing evolution of swine influenza H1N2 viruses in Germany. J Gen Virol 87: 3641–3647.

33. Nyugen VT, Emsc BF, Barnard DL, Juhander JG, Gross M, et al. (2012) Efficacy of combined therapy with amantadine, oseltamivir, and ribavirin in vivo against susceptible and amantadine-resistant influenza A viruses. PLoS One 7: e31006.

34. Bauer K, Duerrwald R, Schlegel M, Pfarr K, Topf D, et al. (2011) Neuraminidase inhibitor susceptibility of swine influenza A viruses isolated in Germany between 1981 and 2001. Intervirology 49: 286–293.

35. Bright RA, Medina MJ, Xu X, Perez-Orozco G, Wallin TR, et al. (2005) Incidence of adamantane resistance among influenza A (H3N2) viruses isolated worldwide from 1994 to 2005: a cause for concern. Lancet 366: 1171–1181.

36. Deyde V, Garten R, Sheu T, Smith C, Myrick A, et al. (2009) Genomic events underlying the changes in adamantane resistance among influenza A(H1N2) viruses during 2006–2008. Influenza Other Respi Viruses 3: 297–314.

37. Xing Z, Cardona CJ (2009) Preexisting immunity to pandemic (H1N1) 2009. Emerg Infect Dis 16: 1029–1030.

38. Mishin VP, Novikov D, Hayden FG, Gubareva LV (2005) Effect of hemagglutinin glycosylation on influenza virus susceptibility to neuraminidase inhibitors. J Virol 79: 12416–12424.

39. Spearman C (1908) The method of the “right and wrong” cases (“constant stimuli”) without Gaus’s formulae. British Journal Psychology 2: 227–242.