

Short Communication

Pathogenesis and transmission of the novel swine-origin influenza virus A/H1N1 after experimental infection of pigs

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Influenza virus A/H1N1, which is currently causing a pandemic, contains gene segments with ancestors in the North American and Eurasian swine lineages. To get insights into virus replication dynamics, clinical symptoms and virus transmission in pigs, we infected animals intranasally with influenza virus A/Regensburg/D6/09/H1N1. Virus excretion in the inoculated pigs was detected in nasal swabs from 1 day post-infection (p.i.) onwards and the pigs developed generally mild symptoms, including fever, sneezing, nasal discharge and diarrhoea. Contact pigs became infected, shed virus and developed clinical symptoms similar to those in the inoculated animals. Plasma samples of all animals remained negative for virus RNA. Nucleoprotein- and haemagglutinin H1-specific antibodies could be detected by ELISA 7 days p.i. CD4⁺ T cells became activated immediately after infection and both CD4⁺ and CD8⁺ T-cell populations expanded from 3 to 7 days p.i., coinciding with clinical signs. Contact chickens remained uninfected, as judged by the absence of virus excretion, clinical signs and seroconversion.

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Influenza A/H1N1 viruses were first isolated from swine in 1930 (Shope, 1931). Between 1930 and the late 1990s, these classical swine influenza viruses circulated in pigs in the USA and remained relatively stable. This relative antigenic stasis of classical influenza A/H1N1 viruses in swine until 1998, during the time when significant antigenic drift of influenza H1 viruses in humans was observed, created a substantial antigenic gap between classical swine and human seasonal H1 viruses. Thus, swine became a reservoir of influenza H1 viruses with the potential to cause significant respiratory disease or even pandemics in humans (Garten *et al.*, 2009).

Sporadic cross-species transfer of swine and avian influenza viruses to humans has been documented repeatedly during the last decades. Despite the development of severe clinical signs and fatal pneumonia in some patients, the infections lacked the critical capacity to spread efficiently from human to human in order to pose a threat for medical care (van Reeth, 2007; Irvine & Brown, 2009). Since its identification in April 2009, a novel swine-origin influenza virus A/H1N1 containing a unique combination of gene

segments from both North American and Eurasian swine lineages has continued to circulate in humans (Cohen, 2009; Garten *et al.*, 2009). Similarity analyses between the novel influenza virus A/H1N1 and its nearest relatives indicated that it may have been circulating undetected for an extended period of time (Smith *et al.*, 2009). As of 3 July 2009, there have been 89 921 laboratory-confirmed cases in over 100 countries, resulting in more than 380 deaths. A key determinant of the current infections is the transmission rate of the novel influenza virus A/H1N1 in humans. Due to the unprecedented worldwide spread of the virus in humans, the WHO raised the influenza alert to the highest pandemic phase level about 10 weeks after the first detection of the virus outside Mexico.

The objectives of the current studies were to investigate (i) whether experimental intranasal infection of pigs with the novel influenza virus results in clinical signs and leads to virus excretion, (ii) whether infection causes alterations in T- or B-lymphocyte subsets and (iii) whether the infection would be transmitted to naïve contact pigs and chickens. For this purpose, five 10-week-old pigs were infected intranasally with 10⁶ TCID₅₀ influenza virus A/Regensburg/D6/09/H1N1 in the BSL3+ facilities at the Friedrich-Loeffler-Institut. The pigs were obtained from a

The GenBank/EMBL/DDBJ accession numbers for the complete genome sequence of novel influenza virus A/H1N1 described in this study are FN401574–FN401581.

commercial farm and tested negative for pre-existing antibodies against influenza A viruses. The animal experiments were approved by the regional ethical committee. The virus had been isolated from a patient in Germany and propagated on Madin–Darby canine kidney (MDCK) cells. The complete genome sequence of the virus used for the experiments revealed high similarity to the novel influenza H1N1 viruses characterized worldwide and has been submitted to GenBank under accession numbers FN401574–FN401581. From day 1 post-infection (p.i.), three naïve pigs and five naïve chickens were housed together with the infected animals in direct contact without any cages in the same room. From all pigs, oropharyngeal swabs were taken daily and EDTA–blood samples were obtained on days 1, 2, 3, 5, 7, 10, 14 and 21 p.i. From the chickens, cloacal and oropharyngeal swabs were sampled daily and all animals were assessed for disease signs by following a clinical score that included nasal discharge, sneezing, salivation, diarrhoea, fever, emaciation, lid oedema and/or compromised general condition.

Real-time RT-PCR analysis of the swab samples using primers (<http://offlu.net>) designed to specifically amplify the haemagglutinin gene of the novel influenza virus A/H1N1 could detect virus excretion by day 1 p.i. in two of the five inoculated pigs (Table 1). Possibly, these two pigs were infected experimentally and subsequently passed the virus to the other pigs. Within 4 days p.i., the swab samples of all infected and contact pigs were positive. Positive RT-PCR results were detected until day 11 p.i., with intermittent days without detectable virus excretion in individual animals (Table 1). Virus could be reisolated from the swabs in MDCK cells with titres $\geq 10^{1.6}$ TCID₅₀ from day 3 p.i. onwards from the infected animals and from day 5 p.i. onwards from the contact pigs. The last positive virus isolations were obtained on day 11 p.i. All plasma samples remained negative for virus RNA. The experimentally infected pigs developed clinical symptoms from day 3 p.i. None of the animals displayed more than four of the clinical symptoms used as clinical-score criteria at the same time. Clinical signs were mild and generally resembled those described for other swine influenza virus infections (van Reeth *et al.*, 2003; Zell *et al.*, 2008). Nasal discharge, sneezing and fever were observed as the main clinical signs between days 4 and 5 p.i. All three naïve contact pigs also developed similar clinical signs with a delay of 2–3 days. Diarrhoea developed between days 3 and 7 p.i. in several of the infected and contact pigs. Examination of the faeces did not reveal any pathogenic bacteria. Most probably, the general compromised condition induced by the infection in these animals supported the development of diarrhoea, which has also been reported in humans infected with recent triple-reassortant swine influenza A (H1) viruses in the USA (Shinde *et al.*, 2009).

The development of an influenza virus-specific immune response was analysed by two commercially available ELISAs (Table 1). Anti-nucleoprotein (NP) antibodies were investigated with the ID Screen Influenza A Antibody

Table 1. Virus excretion in pigs after experimental infection with influenza virus A/Regensburg/D6/09/H1N1

Real-time RT-PCR results are given as +++ (C_t values <30), ++ (C_t values 30–<35), + (C_t values 35–<40) or – (C_t values ≥40). The ELISA columns represent the results of the antibody ELISA tests performed on days 0, 7, 10 and 14 p.i. The NP-ELISA results are given as – (inhibition 0–<60%), + (inhibition 60–<85%) or ++ (inhibition ≥85%); H1-specific ELISA results are given as + or – in parentheses.

Pig no.	Type	Time post-infection (days)																				
		0	ELISA	1	2	3	4	5	6	7	ELISA	8	9	10	ELISA	11	12	13	14	ELISA		
1	Infected	–	(–)	+	++	++	++	++	++	–	(–)	+	+	–	+	+	–	–	–	+	+	(+)
2	Infected	–	(–)	–	–	+	+	+	–	–	(–)	–	+	–	–	–	–	–	–	–	–	(–)
3	Infected	–	(–)	–	–	+	+	+	+	–	(–)	–	–	–	–	–	–	–	–	–	–	(+)
4	Infected	–	(–)	+	++	++	++	++	++	+	(–)	–	+	–	–	–	–	–	–	–	–	(+)
6	Infected	–	(–)	–	–	+	+	+	–	–	(–)	–	+	–	–	–	–	–	–	–	–	(–)
7	Contact	–	(–)	–	–	+	+	+	–	–	(–)	–	+	–	–	–	–	–	–	–	–	(+)
8	Contact	–	(–)	–	–	+	+	+	+	–	(–)	–	+	–	–	–	–	–	–	–	–	(+)
9	Contact	–	(–)	–	–	+	+	+	–	–	(–)	–	+	–	–	–	–	–	–	–	–	(–)

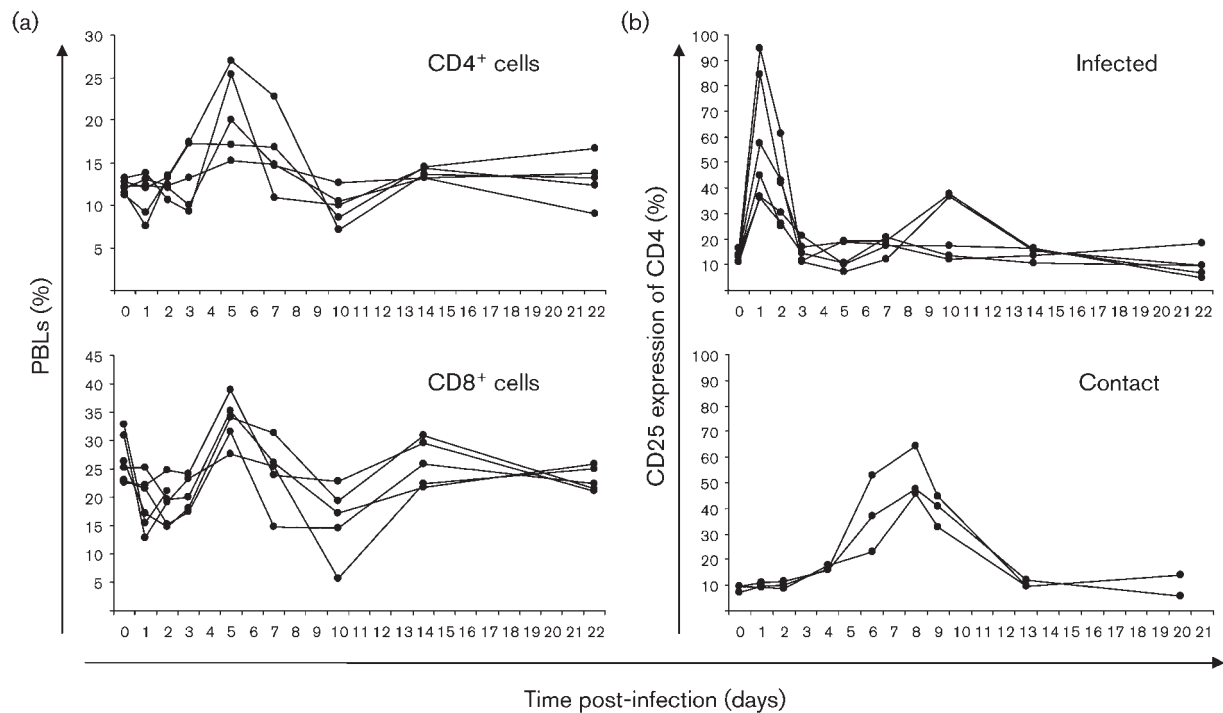


Fig. 1. Analysis of CD4⁺ and CD8⁺ T cells in the course of novel influenza virus A/H1N1 infection. CD4⁺ and CD8⁺ T cells as a percentage of total peripheral blood leukocytes (PBLs) (a) and the percentage expression of CD25 (α -chain of the interleukin-2 receptor) on CD4⁺ T cells in infected and contact pigs (b) are shown.

Competition ELISA (ID-Vet). Three of the five infected pigs were anti-NP antibody-positive at day 7 p.i. By day 10 p.i., all infected animals had developed anti-NP antibodies. The first contact animals became positive at day 10 p.i. The anti-H1 immune response was investigated by using a HerdChek Swine Influenza Virus (H1N1) Antibody Test kit (IDEXX). One contact animal tested positive at day 7 of the experiment. By day 14, five of eight animals had become positive in the H1 ELISA, compared with seven of eight animals in the NP ELISA (Table 1). Obviously, the H1 ELISA detected antibodies against the novel influenza virus A/H1N1, but the sensitivity was lower than that of the NP ELISA.

Immunological analysis revealed a transient increase of CD4⁺ and CD8⁺ T cells with peak levels between days 5 and 6 p.i. (Fig. 1a), which coincided with the major clinical symptoms in the animals. A similar increase was also observed for B cells in the peripheral blood (data not shown). Further characterization of the CD4⁺ T cells revealed an early activation of these cells within 24–48 h p.i. (Fig. 1b), characterized by a significant increase in the expression of CD25 in all infected animals. The three contact animals also showed CD4⁺ T-cell activation with peak levels 7–8 days p.i.

In a second set of experiments, pigs were infected similarly by the intranasal route with 10^6 TCID₅₀ of the same virus

for pathomorphological examinations on days 2, 4 and 6 p.i. On day 2 p.i., animals did not show any lung lesions, except a very slight hyperaemia of the nasal turbinates. However, gross lesions in the lungs were observed at 6 days p.i. They were rubbery in texture and characterized by a diffusely non-collapsed parenchyma. Within the cranial lobes, there were multifocal areas of bronchiopneumonia also scattered through the accessory lobes, as well as the cranial parts of the caudal lobes (Fig. 2a). Few of these pneumonic areas were associated with bronchi. The nasal conchae were diffusely bright red and covered by opaque mucus (Fig. 2b).

In contrast to transmission of the infection to contact pigs, no infection of contact chickens occurred in our experiment. The five contact chickens did not develop clinical signs, did not excrete virus and also did not develop anti-influenza virus antibodies. Obviously, the high transmissibility of the virus observed in humans also applies to pigs, but not to the transmission of the virus to chickens, as all animals were housed together without cages in one room. This is supported by direct infection experiments of chickens with the novel influenza virus A/H1N1, which did not result in clinical signs or infection (data not shown).

The current investigation showed that intranasal infection of pigs with 10^6 TCID₅₀ of the novel influenza A/H1N1 virus results in virus excretion, clinical signs, activation of the cellular and humoral immune response and transmis-

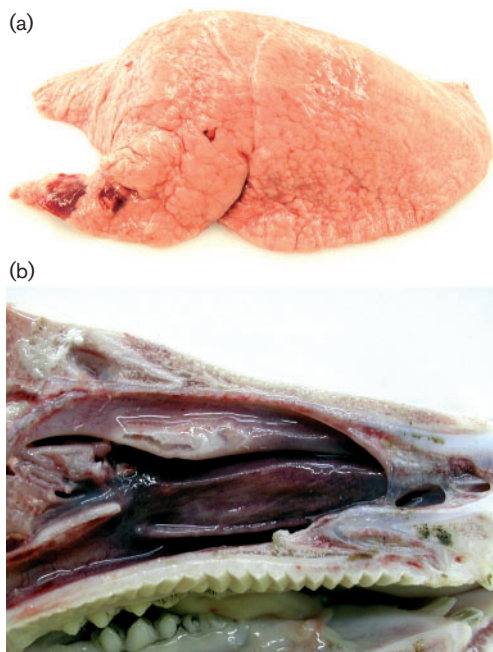


Fig. 2. (a) Pig lung at day 6 p.i. Within the left middle lobe, there are multifocal areas of dark red discoloration and consolidation. The diffusely emphysematous and rubbery lung parenchyma did not collapse. (b) Pig nasal cavity at day 6 p.i. The mucosa of the nasal turbinates and the nasopharynx are diffusely hyperaemic and show increased mucous secretion.

sion of the virus to contact pigs. Typical influenza-like symptoms, such as sneezing and nasal discharge, were observed between days 4 and 5 p.i., which is somewhat delayed compared with previous infection experiments using avian influenza virus-like porcine influenza A/H1N1 viruses currently circulating in the European pig population. In addition, we observed diarrhoea associated with the infection, probably due to the compromised general condition of the pigs during the acute phase of the infection. Despite the fact that diarrhoea was also seen in 30% of human patients infected in recent years with triple-reassortant swine influenza A (H1) viruses in the USA (Shinde *et al.*, 2009), no diarrhoea was observed in another study of experimental influenza virus A/H1N1 infection of pigs (Brookes *et al.*, 2009). It remains to be determined whether diarrhoea is more commonly associated with the novel influenza virus A/H1N1 infection in pigs. In the present study, virus transmission to contact pigs occurred rapidly. Even 3 days after contact, all naïve contact pigs had already started to shed virus. It can be concluded that pigs are susceptible to the novel influenza virus A/H1N1 and it must be assumed that this virus will spread fast and efficiently if introduced into swine farms, possibly establishing endemic infections. Case reports from Canada (http://www.oie.int/wahis/public.php?page=single_report&pop=1&reportid=8065) and Argentina (http://www.oie.int/wahis/public.php?page=single_report&pop=1&reportid=

8238) concerning putative human-to-pig transmissions and also experimental studies of sequential passages of the virus in pigs (Brookes *et al.*, 2009) support this observation. So far, pigs or other animals have not been demonstrated to be involved in the epidemiology or spread of the novel influenza virus A/H1N1. However, with the increasing numbers of human infections, a spillover of this virus to pigs is becoming more likely. The prevention of human-to-pig transmissions should have high priority in order to avoid involvement of pigs in the epidemiology of this pandemic. As recommended by the OIE, national veterinary services must monitor animal populations effectively for clinical signs of the disease and farmers must follow their veterinary hygiene regulations strictly. Persons suspected of infection should not be allowed to be in contact with pigs. This might be difficult to ensure, especially in backyard holdings. Therefore, appropriate restriction measures for novel influenza virus A/H1N1-infected swine holdings must be agreed on. In addition, vaccination experiments in pigs with currently licensed vaccines against different influenza H1 viruses and with novel influenza virus A/H1N1-specific vaccines should give conclusive information about whether available vaccines are able to induce immunity, protect from clinical signs and/or inhibit virus shedding in pigs. These experiments help to direct infectious disease-control programmes and to improve our understanding of the factors that determine virus pathogenicity and transmissibility in pigs and at the animal-human interface.

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