Vaccine 29 (2011) 1545–1550

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Vaccination with a soluble recombinant hemagglutinin trimer protects pigs against a challenge with pandemic (H1N1) 2009 influenza virus

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ARTICLE INFO

Article history:
Received 14 October 2010
Received in revised form
15 December 2010
Accepted 22 December 2010
Available online 8 January 2011

Keywords:
Influenza
H1N1
Hemagglutinin
Soluble trimer
Swine
Pandemic

ABSTRACT

In 2009 a new influenza A/H1N1 virus strain ("pandemic (H1N1) 2009", H1N1v) emerged that rapidly spread around the world. The virus is suspected to have originated in swine through reassortment and to have subsequently crossed the species-barrier towards humans. Several cases of reintroduction into pigs have since been reported, which could possibly create a reservoir for human exposure or ultimately become endemic in the pig population with similar clinical disease problems as current swine influenza strains. A soluble trimer of hemagglutinin (HA), derived from the H1N1v, was used as a vaccine in pigs to investigate the extent to which this vaccine would be able to protect pigs against infection with the H1N1v influenza strain, especially with respect to reducing virus replication and excretion. In a group of unvaccinated control pigs, no clinical symptoms were observed, but (histo)pathological changes consistent with an influenza infection were found on days 1 and 3 after inoculation. Live virus was isolated from the upper and lower respiratory tract, with titres up to 10^6 $TCID_{50}$ per gram of tissue. Furthermore, live virus was detected in brain samples. Control pigs were shedding live virus for up to 6 days after infection, with titres of up to 10^5 $TCID_{50}$ per nasal or oropharyngeal swab. The soluble H1N1v HA trimer diminished virus replication and excretion after a double vaccination and subsequent challenge. Live virus could not be detected in any of the samples taken from the vaccinated pigs. Vaccines based on soluble HA trimers provide an attractive alternative to the current inactivated vaccines.

1. Introduction

In April 2009 a new influenza A/H1N1 virus strain was detected in two children in Southern California, both suffering from respiratory disease [1]. Full sequence analysis showed that this new influenza strain, currently named "pandemic (H1N1) 2009" (H1N1v), is likely a reassortant between North American and Eurasian swine influenza strains [2,3]. Unlike most other introductions of swine influenza strains in the human population, this strain was successful in human-to-human transmission. The virus spread quickly to other countries and continents and finally, on the 11th June 2009, the WHO declared this outbreak to be a pandemic, the first one since 1968 (Hong Kong flu).

On 28 April 2009, the Canadian Food Inspection Agency became involved in the first field infection of swine with this H1N1v [4]. Introduction of the virus through an infected human was suspected, but could not be proven. On the 25th June, a second swine herd, in Argentina, was reported to the World Organization for Animal Health (OIE) as being infected [5]. Also in this case, introduction through infected humans was suspected, but could not be confirmed. In both cases the clinical symptoms in the pigs were rather mild and recovery of the pigs was uneventful. Many more such cases in swine herds have since been detected, in countries all over the world.

The susceptibility of pigs to this particular virus strain has been confirmed in several experimental studies [6–8]. Clinical symptoms in pigs were shown to be similar to those caused by endemic swine influenza strains. It was also shown that virus transmission to susceptible pigs, at least those naïve for antibodies against any swine influenza viruses, readily occurs. Whether the H1N1v is able to outcompete endemic H1N1 and/or H1N2 strains, or whether it would be able to co-exist with these endemic strains in swine, is as yet unknown. In such cases pigs may become a reservoir from which repeated introductions into the human population could occur.

It can be expected that the more widespread the infection becomes in humans, the more often the virus will be introduced in swine. Infected pigs may therefore become a source of infection for humans, even if the virus would not succeed in becoming endemic

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doi:10.1016/j.vaccine.2010.12.096

in the pig population. Humans in contact with high concentrations of infected pigs may be exposed to much higher amounts of virus than when exposed to infected humans. This could result in much more severe clinical symptoms, even in a higher mortality. Possible contact persons are not just the farmers and their family, but also include veterinarians, pig consultants, traders, transporters, visitors of pig markets and slaughterhouse personnel.

A way to decrease the risk for people involved may be vaccination of pigs, with the primary aim of reducing virus excretion and therefore exposure of humans to the virus. Conventional vaccines consist of whole viruses propagated in either embryonated chicken eggs or cell cultures, which are subsequently inactivated and adjuvanted. In case new such vaccines, based on new influenza subtypes, are needed, the development, registration and subsequent production takes a relatively long time, taking care of safety, efficacy and production issues. As an alternative a recombinant purified hemagglutinin (HA) could be used as a vaccine. One such recombinant, a secretable, soluble trimer of the HA ectodomain from the H1N1v influenza strain, was constructed and formulated as a vaccine to be tested in swine. The aim of this study was to determine to what extent this vaccine is able to protect against infection with the H1N1v influenza strain, especially with respect to reducing virus replication and excretion. It was shown that the HA trimer was almost complete able to prevent virus replication and excretion after a double vaccination.

2. Materials and methods

2.1. Study design

The study was carried out with 18 pigs, divided into two groups of 9. In one group the pigs were vaccinated twice, with a four week interval. At the age of 10 weeks they were vaccinated for the first time. The other group was an unvaccinated control group. Three weeks after the second vaccination the animals in both groups were challenged, resp. inoculated with the H1N1v virus. At days 1 and 3 post inoculation (p.i.) 3 pigs from each group were euthanized. The remaining 3 pigs in each group were euthanized at day 21 p.i., the end of the experiment.

The design of the experiment was evaluated and approved by the Ethical Committee for Animal Experiments of the Animal Sciences Group.

2.2. Animals

Nine-week-old piglets were purchased from a high-health breeding herd in which no seroconversions against any influenza subtype had been observed for more than 2 years. Before purchasing the pigs, all were tested individually with an NP-ELISA (IDEXX) and in hemagglutination inhibition assays against H1N1, H1N2 and H3N2 influenza virus strains that are endemic in the swine population.

2.3. Vaccine and vaccination

Based on H3 numbering, a cDNA clone corresponding to residues 16–524 of the HA from A/California/04/2009(H1N1) (Genbank accession no. ABW90137.1) was synthesized using human-preferred codons by GenScript USA Inc. The HA ectodomain-encoding cDNA was cloned into the pCD5 expression vector for efficient expression in mammalian cells [9]. The pCD5-Cal/04/09 vector had been modified such that the HA-encoding cDNA was cloned in frame with DNA sequences coding for a signal sequence, a GCN4 isoleucine zipper trimerization motif (KRMKQIEDKIEEIESKQKKIENEIARIKK) [10] and the Strep-tagII (WSHPQFEK; IBA, Germany). The HA ectodomain was expressed in HEK293T as previously described [11]. HA protein expression and secretion was confirmed by sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) followed by western blotting using a mouse anti-Strep-tag antibody (IBA, Germany). Secreted HA proteins were purified using Strep-tactin sepharose beads according to the manufacturer's instructions (IBA, Germany). The concentration of purified protein was determined by using a Nanodrop 1000 spectrophotometer (Isogen Life Sciences) according the manufacturer's instructions. Oligomeric status of the HA protein was determined by analyzing the elution profile using a Superdex200GL 10–300 column and by blue-native gel-electrophoresis. The vaccine was formulated with Specol [12,13] as an adjuvant, at 25 μg HA per dose of 2 ml. Pigs were vaccinated intramuscularly.

2.4. Virus and inoculation

Influenza virus A/Netherlands/602/2009 (H1N1)v was isolated from the first confirmed case in the Netherlands [14]. The patient was a 3-year old boy, developing a fever and symptoms of respiratory disease after returning from Mexico with his family. A nasal swab was taken before the patient was treated with oseltamivir. Virus was initially grown on embryonated eggs, and subsequently passaged on Madin–Darby canine kidney (MDCK) cells before it was used to inoculate the pigs. This virus differs by 8 amino acids from the A/California/4/2009 (H1N1)v strain [14]. Because it is, however, closer to the consensus sequence, it is considered representative of the circulating H1N1v influenza strains.

Pigs were inoculated with a dose of $10^{7.5}$ $TCID_{50}$, suspended in 2 ml PBS, of which 1 ml was nebulised within each nostril.

2.5. Observations and sampling

Clinical symptoms and body temperature were recorded daily from day 3 before inoculation until the end of the experiment. At days 1–3 p.i. clinical symptoms and body-temperature were recorded twice per day with a 12 h interval.

Serum samples were collected during both times of vaccination, at the time of inoculation, and 7, 10, 14 and 21 days p.i.

Oropharyngeal and nasal swabs were collected daily from all animals still alive from day 0 to 11 p.i., and on days 14, 17 and 21 p.i. For oropharyngeal swabs multi-layered gauze dressings in a pair of tweezers were used to scrape the palatine tonsils at the dorsal pharyngeal wall, behind the soft palate. Nasal swabs were collected using sterile rayon swabs (Medical Wire & Equipment, Corsham, United Kingdom). The swabs were suspended in 4 ml (oropharyngeal swabs) or 2 ml (nasal swabs) medium (Eagle minimum essential medium (EMEM) (Gibco, Invitrogen, Breda, The Netherlands) with 5% fetal bovine serum (FBS), and 10% antibiotics). After centrifugation (1800 × *g* for 15 min) the samples were stored at −70 °C until analysis.

At days 1 and 3 p.i. 3 pigs from each group were euthanized and a gross pathological examination was performed. Thirteen different tissue samples were collected from each of these pigs for histological and/or virological examinations: nasal mucosa from the turbinates, tonsils, trachea, tracheobronchial lymph nodes (TBLN), six pieces of lung, brainstem, cerebrum and cerebellum. The lung pieces originated from the right apical lobe (lung 1), the right cardiac lobe (lung 2), the right diaphragmatic lobe (lung 3), the left diaphragmatic lobe (lung 4), the left cardiac lobe (lung 5), and the left apical lobe (lung 6).

For (immuno)histology, tissue samples were fixed in 10% neutral buffered formalin for a maximum of 48 h, embedded in paraffin and tissue slides were stained with hematoxylin and eosin. For immunohistological evaluation tissue slides were mounted on silicon coated glass slides, deparaffinised and exposed to 1%

H_2O_2 to block endogenous peroxidase. After washing, the slides were treated with protease type XXIV (0.1 mg/ml, diluted in PBS, Sigma®, order nr. P8038) for 10 min. Samples were incubated with 10% normal goat serum and thereafter incubated with a murine monoclonal antibody, directed against the Influenza A virus nucleoprotein (HB65 MCA) for 45 min. After rinsing, slides were incubated with a HRP labelled polymer conjugated to an anti-murine IgG antibody (DAKO Envision™+ System) and to visualize the immunohistochemical signal followed by treatment with diaminobenzidine tetrahydrochloride and counterstaining with hematoxylin eosin.

For virological examination, 0.1 g from each tissue sample was added to 0.6 ml of medium (same as used for the swabs), and homogenized using the MagNaLyser (Roche Applied Science) for 30 s at 3500 × *g*. After centrifugation (9500 × *g* for 5 min), 0.4 ml of the supernatant was added to a further 1.2 ml of medium and stored at −70 °C until analysis.

At day 21 p.i. the remaining pigs where euthanized. Lungs were collected for a broncho-alveolar lavage, using 50 ml of cold (4 °C) phosphate-buffered saline (PBS). The broncho-alveolar lavage fluid (BALF) obtained was centrifuged (9500 × *g* for 5 min) and stored at −70 °C until analysis.

2.6. PCR

Nasal swabs, oropharyngeal swabs, tissue homogenates and BALF were all tested with a quantitative real time RT-PCR (qRT-PCR). A one-tube qRT-PCR was performed to detect the matrix gene of the influenza virus. The Qiagen one-step RT-PCR kit was used with a 25 μl reaction mixture containing 1 μl of kit-supplied enzyme mixture, 1 μl dNTP mix, 4 U of RNase inhibitor (Promega, Madison, WI), 0.5 μM of each primer M-Fw (5′-CTTCTAACCGAGGTCGAAACGTA-3′), M-Rev (5′-CACTGGGCACGGTGAGC-3′), and 0.3 μM of probe M (5′-6FAM-TCAGGCCCCCTCAAAGCCGA-X-ph). The qRT-PCR was performed with the MX4000 (Stratagene®, Texas). The RT-PCR program consisted of 30 min at 50 °C and 15 min at 95 °C. A three-step cycling protocol was used as follows: 95 °C for 5 s, 58 °C for 15 s, and of 72 °C for 20 s for 45 cycles. In each PCR run a standard curve was included with a known virus concentration. Results of the PCR are expressed as $TCID_{50}$-equivalents per swab or per gram of tissue. $TCID_{50}$-equivalents are a relative measure and not necessarily represent live virus.

2.7. Virus isolation

Nasal swabs, oropharyngeal swabs, tissue homogenates and BALF were all tested in a virus isolation with end-titration on MDCK-I-BD5 cells [15]. Samples were initially diluted with the same amount of GMEM/EMEM medium containing 1% bovine serum albumin and antibiotics (twofold dilution). This initial dilution was serially diluted tenfold in the same medium. The diluted samples (100 μl/well) were mixed with 150 μl of 2×10^5 MDCK-I-BD5 cells/ml and incubated for 48 h at 37 °C and 5% CO_2. The monolayers were subsequently washed with PBS, frozen at −20 °C and fixed with 4% cold (4 °C) paraformaldehyde for 10 min. After washing, viral NP-protein-containing cells were stained using HRPO-conjugated monoclonal antibody HB65 and 3-amino-9-ethyl-carbozole (AEC; Sigma–Aldrich, The Netherlands) as a substrate for HRPO. Samples were tested in eightfold and titres were calculated according to the method of Spearman-Kärber [16]. Virus titres are expressed as $TCID_{50}$ per swab or per gram of tissue.

2.8. Hemagglutination inhibition test

The hemagglutination inhibition (HI) test was carried out as described before [17]. Before testing, samples were inactivated for 30 min at 56 °C. Subsequently they were pre-treated with receptor destroying enzyme (RDE) and chicken red blood cells to remove non-specific agglutinins and hemagglutination inhibitors. Starting at an initial dilution of 1:10, sample were tested in two-fold dilution series. Samples were incubated for 60 min after adding antigen and another 45 min after adding chicken red blood cells and subsequently read. The antigens used in the test were the A/Netherlands/602/2009 (H1N1)v and, for swine influenza, the A/Swine/Best/96 (H1N1) [18] and A/Swine/Gent/7625/99 (H1N2) [19]. All were standardised at 4 hemagglutinating units per 25 μl.

2.9. Virus neutralisation test

The virus neutralisation tests were performed on MDCK-I-BD5 cells [15]. Sera were heat inactivated for 30 min at 56 °C before testing. Twofold serial dilutions of the sera were made in GMEM/EMEM medium containing 1% bovine serum albumin and antibiotics in 96-well plates. The diluted sera (50 μl/well) were mixed with 100 $TCID_{50}$ of the influenza viruses (50 μl) and incubated at 37 °C and 5% CO_2 for 1 h. Thereafter 150 μl of 2×10^5 MDCK-I-BD5 cells/ml were added to each well. The plates were incubated at 37 °C and 5% CO_2 for 48 h. The monolayers were washed with PBS, frozen at −20 °C and fixed with 4% cold (4 °C) paraformaldehyde for 10 min. After washing, viral NP-protein-containing cells were stained using HRPO-conjugated monoclonal antibody HB65 and 3-amino-9-ethyl-carbozole (AEC; Sigma–Aldrich, The Netherlands) as a substrate for HRPO. A complete lack of staining was scored as positive neutralisation. VN-antibody titres were expressed as the reciprocal of the highest serum dilution giving positive neutralisation.

3. Results

3.1. Clinical symptoms

No clinical symptoms were observed in any of the inoculated animals, neither in the control group, nor in the vaccinated group. Body temperatures of all animals remained within normal range during the whole animal experiment. One of the pigs from the vaccinated group died between the first and second vaccination of unrelated causes (Mulberry heart disease) and could not be replaced. In this group therefore only 2 pigs were left after day 3 p.i. until the end of the experiment at day 21 p.i.

3.2. Pathological and histological findings

At day 1 p.i. some reduced retraction of the lungs was observed in one of the control pigs, and some moderate hyperaemia of the nasal mucosa in one of the vaccinated pigs. Histology of the lungs revealed a slight to mild focal interstitial pneumonia in all control pigs, accompanied with a mild catarrhal bronchiolitis in one of them. A slight focal interstitial pneumonia was present in one of the vaccinated pigs. Immunohistochemistry showed the presence of virus in lungs and nasal mucosa of all control pigs, and in some individual cases also in the trachea, tonsil and tracheobronchial lymph node. Vaccinated pigs were all negative in the immunohistochemistry.

Gross pathology revealed at 3 days p.i. a mild to moderate focal or multifocal pneumonia in all control pigs. In two of the vaccinated pigs a mild reduced retraction of the lungs was observed, with some moderate hyperaemia of the trachea in one of these cases, and some moderate hyperaemia of the nasal mucosa in the other. Histology revealed a mild to moderate interstitial pneumonia in all three control pigs, with a moderate catarrhal bronchitis/bronchiolitis with focal epithelial necrosis and intra luminal cell debris in two of these

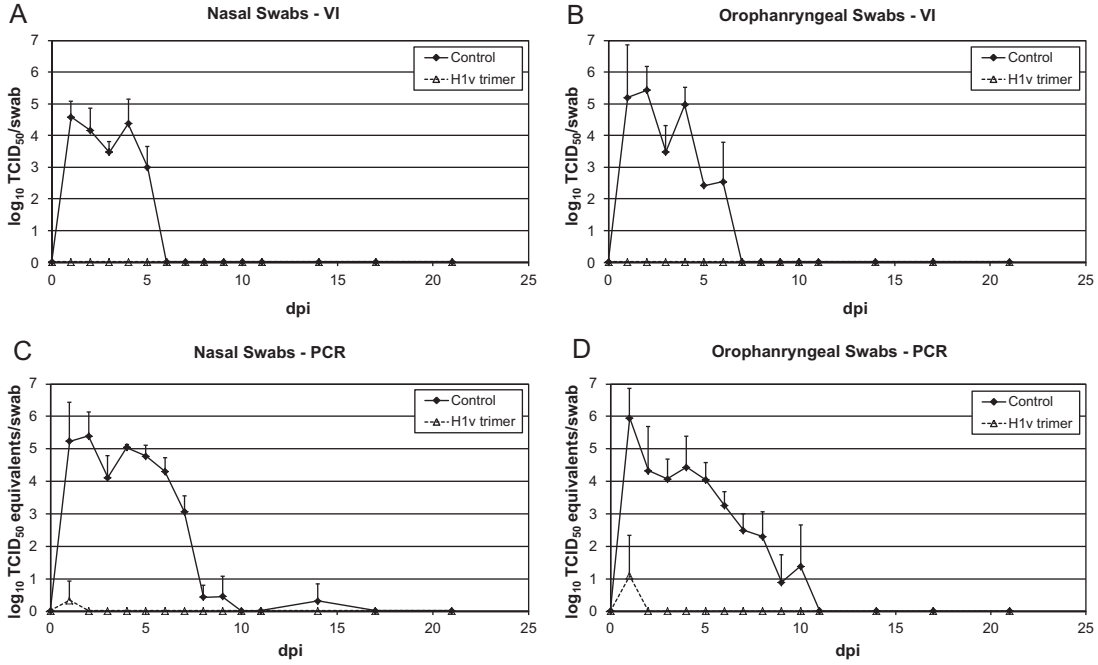

Fig. 1. Average virus isolation (VI; in log $TCID_{50}$) and PCR (in log $TCID_{50}$ equivalents) titres in nasal and oropharyngeal swabs (n = 9/8 on days 0 and 1 p.i. for the control and vaccinated group respectively, n = 6/5 on days 2 and 3 p.i. and n = 3/2 on days 4–21 p.i). Standard deviations are shown.

pigs. Two of the three vaccinated pigs showed some slight interstitial pneumonia. Immunohistochemistry of the lungs was again positive in all three control pigs, with 2 of them also positive in the nasal mucosa and trachea. Vaccinated pigs were all negative in the immunohistochemistry.

3.3. Antigen detection

From all control pigs, live virus could already be isolated at day 1 p.i. from nasal and oropharyngeal swabs, at titres ranging from $10^{2.4}$ to $10^{6.4}$ $TCID_{50}$ per swab. Comparable virus titres were observed until day 4 p.i., declining thereafter. No live virus could be isolated from day 6 p.i. (nasal swabs) or day 7 p.i. (oropharyngeal swabs) onward, respectively. Virus titres seemed overall slightly higher in oropharyngeal swabs than in nasal swabs. From none of the vaccinated pigs live virus could be isolated from nasal or oropharyngeal swabs at any time (Fig. 1A and B).

Viral genome titres peaked on the same days as live virus, but could be detected somewhat longer, until day 10 p.i. in oropharyngeal swabs and day 9 p.i. in nasal swabs from control animals (although one further single weak positive nasal swab was found on day 14 p.i.). From the vaccinated pigs, only on day 1 p.i. genome was detected from multiple animals, but at low amounts (Fig. 1C and D).

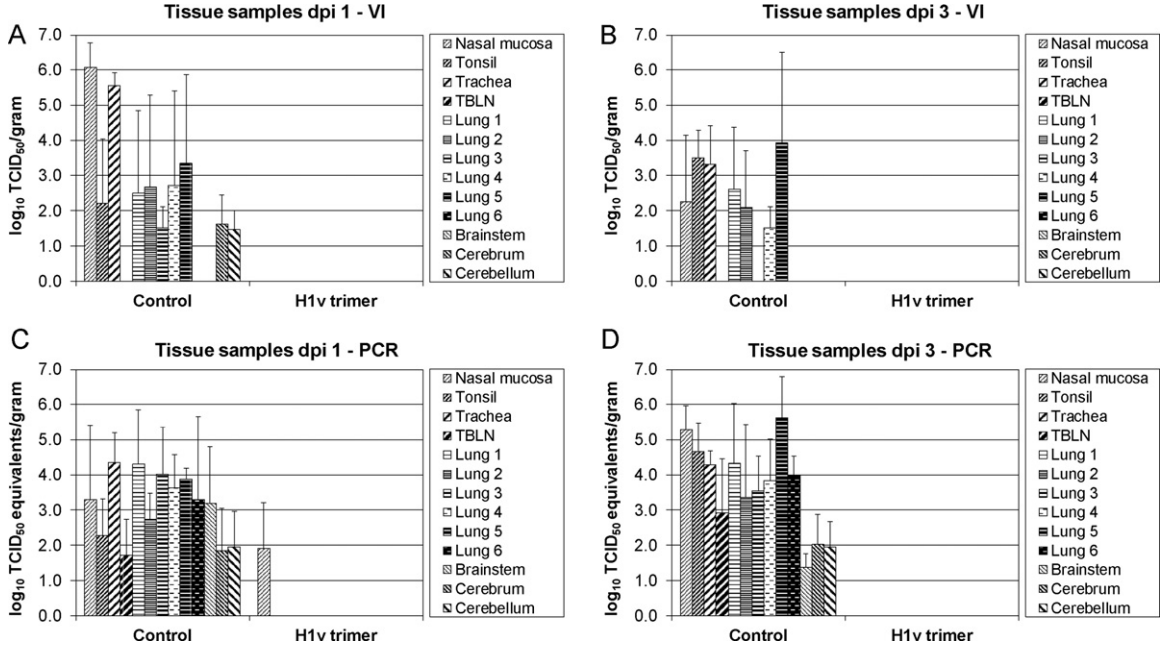

Fig. 2. Average virus isolation (VI; in log $TCID_{50}$) and PCR (in log $TCID_{50}$ equivalents) titres in 13 different pieces of tissue (3 pigs per day and per group). Standard deviations are shown.

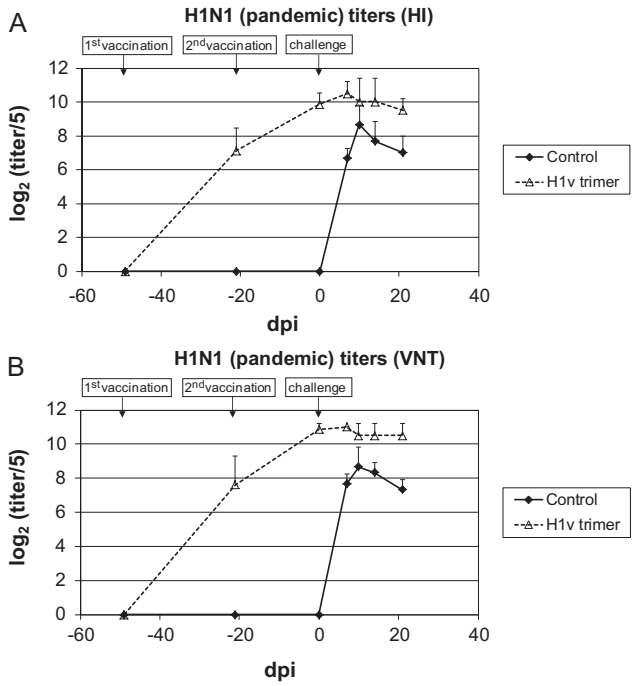

Fig. 3. Average hemagglutination inhibition (HI) and virus neutralisation (VNT) titres against H1N1v after vaccination and subsequent challenge ($n = 9/8$ before and on the day of challenge for the control and vaccinated group respectively, $n = 3/2$ after the challenge). Standard deviations are shown.

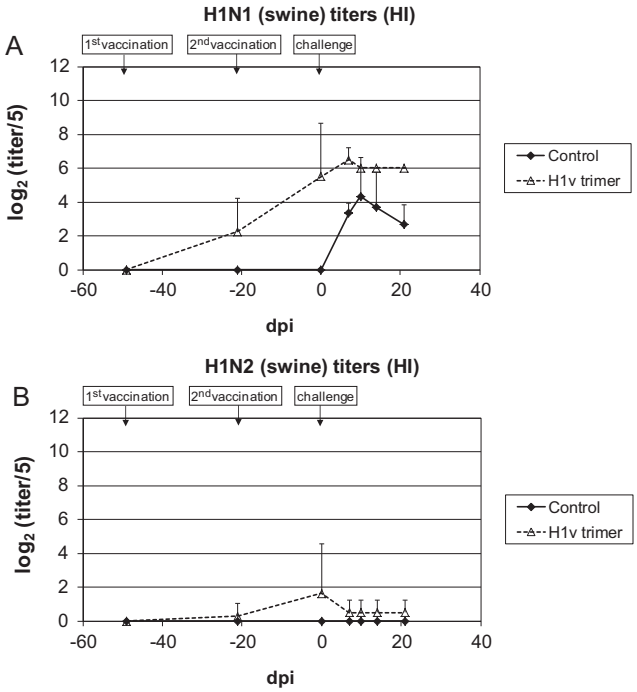

Fig. 4. Average hemagglutination inhibition (HI) titres when using H1N1 and H1N2 subtypes of swine origin after vaccination and challenge ($n = 9/8$ before and on the day of challenge for the control and vaccinated group respectively, $n = 3/2$ after the challenge). Standard deviations are shown.

On day 1 p.i. live virus could be isolated from the control animals from the upper and lower respiratory tract, with the highest titres in the nasal mucosa and trachea. Low amounts of live virus were also detected in the cerebrum and cerebellum. No live virus was isolated from TBLN (Fig. 2A). On day 3 p.i. live virus was only detected from the upper and lower respiratory tract, but no longer from parts of the central nervous system and still not from the TBLN (Fig. 2B). From the vaccinated animals no live virus could be isolated from any of the tissue samples at either time point. (Fig. 2A and B)

On days 1 and 3 p.i. virus genome could be detected by PCR from all tissue samples from the control pigs, including from the TBLN and central nervous system. In only one of the vaccinated animals, viral genome was detected in nasal mucosa at day 1 p.i. (Fig. 2C and D).

BALF from pigs euthanized at day 21 p.i. was negative in the PCR.

3.4. Serology

Already after the first vaccination, at the time of the second vaccination, high antibody titres against the homologous H1N1v strain were seen, both in the HI-test (Fig. 3A) and in a VNT (Fig. 3B). The second vaccination resulted in a further rise of these antibody titres to levels >10,000.

After inoculation with the challenge virus, the non-vaccinated animals responded with titres up to 2560, peaking at 10 days p.i. and then decreasing again. In the vaccinated animals almost no changes were seen in the levels of the titres after the challenge (Fig. 3A and B).

Cross-reactivity, both after vaccination and after inoculation/challenge, was seen in HI-tests and VNT when a swine influenza strain of subtype H1N1 was used in the test, but not when an H1N2 strain of swine origin was used. Results for the HI-tests are shown in Fig. 4. VNT results are not shown as they were almost identical to the HI-results.

4. Discussion

The soluble H1N1v HA trimer was almost completely able to prevent virus replication and excretion after a double vaccination and subsequent homologues challenge. Live virus could not be detected in any of the samples taken from the vaccinated pigs. Viral genome was only detected at day 1 p.i. in nasal and oropharyngeal swabs and at day 1 p.i. in the nasal mucosa from one of the euthanized pigs. The amount of genome detected from the swabs was very low, but genome could be detected in multiple animals. This viral genome may very well represent residual challenge virus. However, some very limited virus replication in the upper respiratory tract in the vaccinated groups can not be excluded, as high levels of virus replication were already observed at day 1 p.i. in the control group.

A recombinant purified HA has several advantages compared to whole inactivated vaccines. Most importantly, the development of a HA-based vaccine has a relatively short lead-time, allowing for a fast response to a potential emergency situation. It can be produced using safe and scalable conditions, without the need of growing live viruses and the disadvantages related to that. HA vaccines also allow for the use as marker vaccines, although this will depend also on other circulating influenza strains in the target population. Marker vaccines make it possible to serologically detect and monitor infections in a vaccinated population, allowing for the collection of invaluable epidemiological data.

The advantage of recombinant HA trimers over recombinant HA monomers is that the former induce higher levels of neutralising antibodies [20]. In part this is likely due to the fact that trimers mimic the natural membrane-bound structure, including the relevant epitopes to induce neutralising antibodies against. Trimeric HA preparations therefore seem more promising vaccine candidates than previously used HA monomers.

Vaccination of pigs reduces the exposure of humans to the influenza virus almost completely. In case pigs are deemed a poten-

tial source of infection for humans, vaccination of herds at risk, or even the entire pig population, therefore seems a realistic option. The vaccine could however also be used for humans themselves. Similar results with an HA trimer based on H5N1 in poultry and mice [21], but also ferrets [22], suggest that the use of these recombinant HA trimers is promising in general.

In this experiment we used a rather high dose of HA as proof of principle for the soluble trimer. Further studies would need to determine the efficacy of the vaccine at lower doses. The lower the dose, the easier it would be to produce sufficient quantities of vaccine in a short time, which is one of the most crucial issues during a pandemic or other emergency situation. Furthermore, it would make the vaccine more cost-affordable, which is especially relevant for continuous use of the vaccine in pig herds, for instance for use of this kind of vaccines against swine influenza strains that are endemic.

Contrary to previous inoculation studies with the H1N1v influenza virus [6–8], no clinical symptoms were seen in the inoculated control animals. Nevertheless, virus titres from nasal and oropharyngeal swabs were higher than published before [7], and also relatively high virus titres were found in all parts of the lungs, providing sufficient evidence that the inoculation itself was successful. Furthermore, pathological changes, both macroscopic and microscopic, were abundantly present in the unvaccinated controls, while only some minor changes were seen in some of the vaccinated pigs. In our study the pigs were much older than in the other published studies. Whether this explains the lack of clinical symptoms, remains to be seen. In a previous study with swine influenza virus in naïve pigs, clinical symptoms seemed to be even more severe in older pigs [23].

Antibodies against the H1N1v influenza virus are readily detected by an HI assay in which a current European swine strain of the H1N1 subtype is used. This cross-reactivity may result in difficulties to correctly identify infections in swine with H1N1v influenza strains by serology. Infections with swine H1N1 influenza strains are very common in many European countries, with seroprevalences in sows up to 80%, and herd prevalences up to more than 95% [24]. On the other hand, due to this high prevalence of H1N1 antibodies, it may be more difficult for the H1N1v influenza virus to become endemic in the swine population. Currently no reports can be found that suggest a wide spread of H1N1v influenza virus in swine populations where other H1N1 strains are endemic. It remains to be seen how the epidemiology of H1N1v will develop, whether it will be able to co-circulate with current H1N1 strains or whether one strains will eventually predominating the other. Furthermore, recombination with current swine strains in Europe could occur, as happened before with the European swine H3N2 [25] and H1N2 strain [26]. This could increase the potential of the H1N1v influenza strain to become endemic in the swine population.

Acknowledgements

The authors thank the animal caretakers who took care of the animals and collected all the samples. Ralph Kok, Rob Zwart, Eline Verheij and Sjaak Quak are thanked for their outstanding assistance in the pathology, histopathology and other laboratory tests.

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