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Protective effect of a polyvalent influenza DNA vaccine in pigs

Ingrid Karlsson, Marie Borggren, Maiken Worsøe Rosenstierne, Ramona Trebbien, James A. Williams, Enric Vidal, Júlia Vergara-Alert, David Solanes Foz, Ayub Darji, Marta Sisteré-Oró, Joaquim Segalés, Jens Nielsen, Anders Fomsgaard

1. Introduction

Influenza A virus infections in swine herds constitute a well-known challenge to the swine industry. Reproductive problems together with weight loss and aggravation of secondary infections are characteristic of swine influenza and result in serious animal welfare problems and economic losses (Bennett et al., 1999; Olsen et al., 2006). The influenza infection in pigs resembles the infection in humans. The virus replicates in the epithelium of the entire respiratory tract but rarely infects other tissues (van der Laan et al., 2008). The disease lasts for 7–10 days seldom results in death of the animals (van der Laan et al., 2008). In addition, the tremendous genetic plasticity of the virus can result in transmission between animals as well as zoonotic transmission and adaptation to human hosts, resulting in novel pandemic influenza strains such as the pandemic 2009 H1N1 strain (Ito et al., 1998; Nelson and Vincent, 2015; Smith et al., 2009). A successful, more broadly
A quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assay was utilized to monitor viral loads in nasal swab samples (day 0, 3, 5, 7, 9 and 13 pc). RNA was extracted with a MagNA Pure LC Instrument applying the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche diagnostics). Primers and probes for the neuraminidase (NA) gene of challenge virus A/California/7/09 (H1N1)pdm09 were used to detect the challenge virus. The beta-actin housekeeping gene was used as a control for correct sampling that should contain pig derived cell material in the swabs. Quantification of virus was performed by using a standard curve developed by serial dilutions of H1N1pdm09 virus with known TCD50/mL concentration,
2.4. Enzyme-linked immunosorbent assay (ELISA)

An ELISA was conducted to measure influenza-specific IgG responses in the sera as previously described (Borggren et al., 2015). The influenza virus protein used for coating was hemagglutinin (HA) from A/California/04/09 (H1N1)pdm09 (Sino Biological Inc.). A horseradish peroxidase-conjugated anti-pig-IgG antibody (AbD Serotec) followed by TMB substrate (Kem-En-Tec Diagnostics) was used for detection. The binding antibody titers are expressed as the reciprocal of the sample dilution giving an optical density (OD) value of 1.0 (Bragsstad et al., 2011).

2.5. Hemagglutination inhibition (HI) assay

The HI assay was performed according to the protocols of the WHO as previously described (Borggren et al., 2015) against virus strain A/California/07/09 (H1N1pdm).

2.6. Microneutralization assay (MN)

Development of neutralizing antibodies was determined according to the protocols of the WHO (Who, 2011). Viruses used were A/California/07/09 (H1N1pdm), A/swine/DK/102586/2007 (H1N1) and A/swine/DK/10496/2008 (H1N1), with 100 TCID50 as the inoculum. A/California/07/09 (H1N1pdm) and A/swine/DK/10496/2008 (H1N1) both have an avian-like H1. The MN titers is defined as the reciprocal dilution giving 50% infection inhibition and is calculated as stated in the WHO protocol (Fenyö et al., 2009; Who, 2011) and Linear interpolation by Reed and Muench (Reed and Muench, 1938) is used to estimate titers if falling between two adjacent serum dilutions.

2.7. Neuraminidase inhibition (NAI) assay

The neuraminidase inhibition activity was determined using the NA-Fluor Influenza Neuraminidase Assay kit (Life technologies) according to the manufactures protocol, where serum and virus was allowed to incubate 30 min at 37 °C before adding the NA-Fluor™ Substrate. The two virus strains, A/California/07/09 (H1N1)pdm09 and A/swine/DK/10496/2008 (H1N1), were used at 1:10 dilution, determined to give a relative fluorescence unit (RFU) within the linear range. Serum from day – 36, day 0 and day 13 pc were tested at 4-fold dilutions starting at 1:10 up to 1:1040. Non-linear regression, with log-transformed serum dilutions as the independent variable, was performed to calculate the serum dilution that would inhibit 50% NA activity (IC50) (Graph-Pad Prism software).

2.8. Statistical analysis

Differences between the groups were calculated using two-way ANOVA and Dunnett’s multiple comparison test, and correlations between different humoral immunity assays was performed using Spearman rank correlation (GraphPad Prism v.6, GraphPad software).

3. Results

3.1. Clinical evaluation

None of the pigs showed any signs of clinical disease during the experiment, and elevated body temperatures were not observed in any individual animal.

3.2. Pathological evaluation

Few pigs displayed mild pulmonary cranio-ventral consolidation (Table 1), potentially compatible with pulmonary collapse (non-virus related) or broncho-interstitial pneumonia (virus related). Histopathological evaluation revealed that pigs receiving the highest dose of DNA

Table 1
Pathological findings.

| Group                | Pig no. | Gross-pathological lesions                                                                 | Scores-lungs | Swine influenza virus-like lesions               |
|----------------------|---------|---------------------------------------------------------------------------------------------|--------------|--------------------------------------------------|
| 800 μg DNA/vacc.     | 1       | No apparent lesions                                                                         | 0            | None                                             |
|                      | 2       | Small consolidated areas (multifocal) in left median lobe                                    | 0            | None                                             |
|                      | 3       | Minimal consolidation foci in the left lobe                                                  | 0            | None                                             |
|                      | 4       | No apparent lesions                                                                         | 0            | None                                             |
|                      | 5       | No apparent lesions                                                                         | 0            | None                                             |
| 500 μg DNA/vacc.     | 5       | Very minimal consolidation foci right cranial lobe and left median lobe                      | 0.5          | Very mild broncho-interstitial pneumonia          |
|                      | 7       | Small consolidated areas in right median lobe and cranial portion of right diaphragmatic lobe | 0.5          | Very mild broncho-interstitial pneumonia          |
|                      | 8       | No apparent lesions                                                                         | 0            | None                                             |
|                      | 9       | Minimal consolidation foci in the left medium and right cranial lobes                        | 0.5          | Very mild broncho-interstitial pneumonia          |
| No vaccine           | 10      | No apparent lesions                                                                         | 0            | None                                             |
|                      | 11      | Lung consolidation in right median lobe and more areas in both cranial lobes                 | 1.5          | Mild-to-moderate broncho-interstitial pneumonia  |
|                      | 12      | Small consolidation areas in both median lobes                                               | 2            | Moderate broncho-interstitial pneumonia           |
|                      | 13      | Consolidation areas in right median and left median lobes and cranial areas of left diaphragmatic lobe | 0            | None                                             |
|                      | 14      | Small areas of consolidation in the right apical lobe                                        | 0.5          | Very mild broncho-interstitial pneumonia          |
|                      | 15      | Very small areas of multifocal consolidation in the right apical lobe and moderate extensive areas in the left middle lobe | 1.5          | Mild-to-moderate broncho-interstitial pneumonia  |

* Scoring system of lung lesions by influenza in pigs according to Detmer et al. (2013) 0, No airsrides affected; 0.5, Only a few isolated airsrides affected; 1, Localized cluster of affected airsrides in (1 or 2 lobs); 1.5, Several airsrides affected throughout section plus minimal interstitial infiltrates; 2, Several airsrides affected throughout section plus mild to moderate interstitial infiltrates; 2.5, Several airsrides affected, often severely, plus moderate interstitial and alveolar infiltrates; 3, Many airsrides affected, often severely, plus moderate interstitial and alveolar infiltrates.
(800 μg) showed no lung lesions, thus no evidence of infection (5/5 scored 0). Those receiving the lower dose (500 μg) showed mild lung pathology in 3/5 with a score of 0.5, indicating that only a few isolated airways were affected. Four out of five pigs from the unvaccinated group showed mild to moderate microscopic lung lesions consistent with broncho-interstitial pneumonia (1/5 scored 0; 1/5 scored 0.5; 2/5 scored 1.5 and 1/5 scored 2). The histopathological examination revealed that the lesions observed in three pigs were due to lung collapse, not related to the influenza infection.

3.3. Antibody responses in DNA vaccinated pigs

All vaccinated pigs developed antibodies to HA, NA, M, and NP proteins. Pigs receiving the highest dose of DNA (800 μg) developed influenza H1pdm09-specific binding antibodies by ELISA 14 days after the initial immunization (Fig. 1A). Following the second immunization, the antibody titers increased and pigs receiving the lower dose of DNA (500 μg) also developed antibodies. Control animals, not receiving any immunization, developed a detectable HA-specific antibody response only after viral challenge. Functionality of the HA binding antibodies was confirmed by the HI assay, demonstrating that DNA vaccination elicited an HI antibody response against the H1N1pdm09 virus (Fig. 1B). Both vaccinated groups showed significantly higher HI titers than the control group after the second immunization. After challenge, the vaccinated animals maintained their HI titers and the control animals demonstrated a response to the challenge virus two weeks after challenge. Binding antibody titers (ELISA) correlated with HI titers in the vaccinated animals (P < 0.0001, R = 0.69, Spearman rank correlation test).

Neutralization (Fig. 2) and neuraminidase inhibition (NAI) (Fig. 3) assays supported the ELISA antibody response findings. Pigs given the highest dose of DNA (800 μg) showed robust levels of neutralizing antibodies against A/California/07/09 (H1N1)pdm09, A/swine/DK/102586/2007 (H1N1) and A/swine/DK/10496/2008 (H1N1). Both the high and low dose DNA vaccinations induced NAI antibodies against both A/California/07/09 (H1N1)pdm09 and A/swine/DK/10496/2008 (H1N1). After challenge, the unvaccinated pigs developed neutralizing antibodies as well as NAI antibodies, while the levels of the vaccinated animals remained unchanged. The ELISA IgG titers correlated with the neutralizing antibody titers in the vaccinated animals (P < 0.0001 R = 0.62, Spearman rank correlation test). The background values (day −36) in the MN assay differed in Fig. 2A–C, with a higher background pre-vaccination to the human H1pdm09. This could be due to the variation between the three different virus isolates in this assay. The higher neutralizing antibody titer against the human H1N1, compared to the two swine-virus isolates may be explained if H1N1pdm09 is easier to neutralize in vitro settings. Alternatively, it is possible that some cross-reacting antibodies reacting with H1N1pdm09 in the MN assay were already present in the pigs. However, all pig sera were negative pre-vaccination in ELISA detecting binding antibodies to the H1N1pdm09. Thus, the background phenomenon in the MN assay was specific for the neutralization assay. Significant increase from this background value could be detected in Fig. 2A to monitor the effect of the vaccinations.

3.4. Detection of virus in nasal swabs

Influenza virus RNA was detected in nasal swabs in two out of five non-vaccinated pigs three days after challenge. Viral RNA could not be detected in any of the 10 vaccinated pigs (< 15 TCID50/ml) (Fig. 4). The median peak viral load was 1.95 log TCID50/ml (range 1.61–2.29 log TCID50/ml; N = 2) for non-vaccinated control pigs, and they cleared the virus to undetectable levels at day 7 pc. Same results were obtained using qRT-PCR for the NA, PB1 and M viral genes (data not shown).

4. Discussion

Previously, we have shown that an optimized polyvalent influenza DNA vaccine induced cross-reactive humoral and cellular immune responses in growing pigs after needle-less intradermal application (Borggren et al., 2016). In the present study, we demonstrated that this DNA vaccine provided protection against influenza challenge in pigs. In the present study, we challenged our vaccine applying the commonly used animal model where each individual animal is infected with a standardized dose of challenge virus (Bragstad et al., 2013) as the alternative to larger studies using contact infection models. This enabled us to consider animal welfare recommendations by using a limited number of experimental animals and provided the opportunity to compare with the results of similar studies, e.g. (Bragstad et al., 2013; Busquets et al., 2010; Gorres et al., 2011). None of the vaccinated pigs shed virus. In addition, there was a total lack (800 μg group) or only
evaluated by the capacity of the sera to prevent the infection of MDCK cells by (A) H1N1pdm09 and (B) swine 2008 H1N1 virus isolates. The MN titer was determined as the reciprocal dilution providing 50% infection inhibition, calculated with a linear interpolation method (Reed and Muench, 1938). Serum samples with a titer below the detectable limit of the assay (lowest serum dilution tested was 1:20) were assigned a value of 10 for graphical representation and statistical analyses. Error bars indicate the mean ± SEM, and significant differences from the no-vaccine control group are indicated by **: p < 0.01; *: p < 0.05.

Fig. 2. Cross-reactive anti-H1N1 neutralizing antibody responses in vaccinated pig sera. Pigs were vaccinated twice (arrows) i.d. with needle-free delivery with 800 μg (n = 5) or 500 μg (n = 5), or not DNA vaccinated at all (n = 5). The pre- and post-challenge pig sera were tested in a micro-neutralization (MN) assay. Neutralizing antibody titers, MN titers, were calculated as the serum dilution providing 50% infection inhibition. Neutralizing antibody titers were determined as the reciprocal dilution providing 50% infection inhibition, calculated with a linear interpolation method (Reed and Muench, 1938). Serum samples with a titer below the detectable limit of the assay (lowest serum dilution tested was 1:20) were assigned a value of 10 for graphical representation and statistical analyses. Error bars indicate the mean ± SEM, and significant differences from the no-vaccine control group are indicated by **: p < 0.01; *: p < 0.05.

Fig. 3. Cross-reactive anti-H1N1 neuraminidase inhibition (NAI) in vaccinated pig sera. Pigs were vaccinated twice (arrows) i.d. with needle-free delivery with 800 μg (n = 5) or 500 μg (n = 5), or not DNA vaccinated at all (n = 5). The pre- and post-challenge pig sera were tested in an NAI assay. NAI titers, or the serum dilutions that would inhibit 50% of neuraminidase activity (IC50), were tested against (A) H1N1pdm09 and (B) swine 2008 H1N1 virus isolates. Error bars indicate the mean ± SEM.

Minimal amounts (500 μg group) of microscopic lung lesions seen in the groups receiving the DNA immunization. The humoral response after viral challenge supported the capability of the vaccine to provide protection since the two vaccinated groups had no increase in antibody titers after challenge, indicating the presence of functional antibodies developed after vaccination. As observed in other influenza challenge studies (Busquets et al., 2010; Vergara-Alert et al., 2012), the challenged pigs did not show signs of clinical disease related to influenza infection. Gross and histopathological findings of broncho-interstitial pneumonia, indicative of influenza virus infection, were mainly observed in the unvaccinated pigs compared to the vaccinated pigs upon challenge, further supporting the protective capabilities of the DNA vaccine tested. The challenge virus H1N1pdm2009 turned out to constitute a relatively mild challenge when looking at the relatively low pathology scores. Yet, this virus is one of the major challenges in production pigs in Europe, and transmission between human and pigs were documented during the 2009 pandemic period (Nelson and Vincent, 2015; Rambaut and Holmes, 2009). Although it is the primary contemporary circulating influenza pathogen in industrialized pig production there are limited vaccine possibilities available for it. Therefore, we believe it represents a highly relevant virus for a novel vaccine study. Importantly, the absence of clinical signs of disease during the experiment supports previous findings (Borggren et al., 2016; Bragstad et al., 2013) which indicated that the vaccine did not show adverse effects in vaccinated animals.

Virus shedding was detected in two out of five unvaccinated control pigs, only. However, the development of antibody responses in all individual control pigs after challenge, demonstrated that all five control pigs were successfully infected. The amount of virus used for inoculation in the present study is comparable to that used successfully to induce effective infection in previous studies that also utilized intranasal inoculation (Bragstad et al., 2013; Trebbien et al., 2013). However, individual pig characteristics may have an effect on viral shedding, as seen also in humans (Lloyd-Smith et al., 2005; Skene et al., 2014). In addition, it remains possible that viral shedding may have occurred on days when nasal swab samples were not collected. Measurements of the housekeeping gene beta-actin in parallel to H1N1pdm09 virus in the qRT-PCR assay excluded differences in the sampled nasal swab material and/or RNA extraction (data not shown) as a source of variations in the nasal swab analyses.

All (5/5) non-vaccinated animals became infected, as evaluated by several or all criteria’s: lung pathology indicative of influenza infection in all but pig 13 (Table 1), that did however shed virus (Fig. 4 and/or viral shedding (Fig. 4, pig 12, 13), and importantly the development of specific antibodies in response to the challenge in all non-vaccinated animals (5/5). This was observed in all antibody tests applied, i.e. ELISA (Fig. 1A) and neutralizing antibody test (Fig. 2A) and HI test (Fig. 1B) and NA inhibition test (Fig. 3). In contrast, for the high vaccine dose (800 μg), none (0/5) got infected, as judged by all of the same measured criteria’s: no shedding (Fig. 4), no increase in specific binding
antibodies (Fig. 1A (800 μg)), no increase in functional HAI antibodies (Fig. 1B (800 μg)), no increase in MN titers (Fig. 2A (800 μg)), no increase in NAI (Fig. 3A (800 μg)), no viral shedding (Fig. 4) and no local lung pathology and no evidence of infection in the lung (Table 1). The titers in the vaccinated pigs increased due to the vaccinations from day −36 and −15 and until the day of challenge (day 0). However, from challenge to day 7 and 13 post challenge antibody titers remained either constant or decreased slightly (Fig. 1A + B) but in contrast to the non-vaccinated pigs there was no significant increase in the antibody titers. The slight drop in titers day 7 post challenge (Fig. 2A) could be due to antigen-antibody complexing or assay variation, but is not significant (Fig. 2A) and there was no change in titers from day of challenge (day 0). However, from challenge to day 7 and 13 post challenge antibody titers remained either constant or decreased slightly (Fig. 1A + B) but in contrast to the non-vaccinated pigs there was no significant increase in the antibody titers. The slight drop in titers day 7 post challenge (Fig. 2A) could be due to antigen-antibody complexing or assay variation, but is not significant (Fig. 2A) and there was no change in titers from day of challenge (day 0) to day 13 post challenge, especially no significant increase from day of challenge as seen in the non-vaccinated pigs. Thus, 5/5 became infected in the non-vaccinated group versus 0/5 in the high dose (800 μg) vaccine group. This was statistically significant (p = 0.0079, Fisher’s exact test).

For the low vaccine group (500 μg), we did also not find any shedding of virus, no increase in specific binding antibodies (ELISA Fig. 1A (500 μg)), no increase in HI titers (Fig. 1B (500 μg)), no increase in NAI (Fig. 3A) (500 μg), no shedding (Table 1). However, we did see some local lung pathology in 3/5 pigs described as “very mild”, and a slight increase in Nab (Fig. 2A) although not statistically significant. Thus, the lower dose vaccine seemed to provide only partial protection. This could support a dose-related protection.

Recently, we reported an in-depth analysis of the immune response induced by this influenza DNA vaccine in a dose-response titration experiment, which included cross-reactive humoral and cellular dose-dependent vaccine responses (Borggren et al., 2016). Based on results from that study, the DNA vaccine doses used in the present study were chosen. The current results confirm and extend the prior results by demonstrating protection against influenza challenge by the induced response. Both doses of DNA vaccine given, 800 μg (133 μg per gene/plasmid) and 500 μg (83 μg per gene/plasmid), induced protection when pigs where challenged with pandemic H1N1. However, the induced humoral response in the higher dose 800 μg DNA group was broader in the in vitro neutralization analysis than the lower dose DNA group, which neutralized a virus homologous to the vaccine only. This observation indicates that a higher dose of vaccine is required to gain a broader cross-reactive humoral response. Thus, it is possible that a challenge with a more heterologous virus strain to the DNA vaccine would differentiate protection between the two DNA doses. However, it is promising for the DNA technology that even a low dose of 500 μg DNA, had a protective effect on a homologous virus strain, since the general low immunogenicity of DNA vaccines in larger animals (Ferraro et al., 2011) has previously been overcome with much larger doses of DNA (Enama et al., 2014). Previous challenge studies by us (Borggren et al., 2015) and others (Gorres et al., 2011) have demonstrated protective effects with DNA vaccine doses of 2000 μg and up. It is tempting to speculate that the optimizations conducted for the DNA vaccine used herein (Borggren et al., 2015) have improved the immunogenicity in such a way that the potential to deliver substantially lower doses of DNA in the future seems realistic. This would be a central aspect for the practical use of a DNA vaccine in swine herds where vaccine costs are of utmost importance.

The demonstrated DNA dose-dependence was also reflected in the induced humoral response after immunizations. The higher DNA dose already demonstrated ELISA binding and HI titers after the first immunization, which were boosted after the second one. In contrast, the lower dose of 500 μg DNA seems to require two immunizations to induce a measurable humoral response. As for the commercial protein-based influenza vaccines the protective effect of the DNA most likely includes the vaccine-induced H1N1pdm09 IgG antibodies (Kutzler and Weiner, 2008; Pica and Palese, 2013; Van Reeth and Ma, 2013). In addition to the antibodies to HA, there were also antibodies to the NA surface protein, which neutralized the NA activity in the NAI assay. The relative or combined role of neutralizing anti-HA and anti-NA antibodies may be important (Pica and Palese, 2013) but will require further experiments to be defined.

Other potential mechanisms may be involved using DNA vaccines instead of protein vaccines, such as cellular immunity e.g. towards the more conserved antigens nucleoprotein (NP) and matrix (M) (Bragstad et al., 2011; Gorres et al., 2011; Liu, 2011; Sridhar et al., 2013). The cellular immunity was not assessed in this study, since our recent evaluation of vaccine-induced immunity already clearly demonstrated a strong cell-mediated immune response after DNA immunization (Borggren et al., 2016), including both IFN-γ-producing T cells against several different influenza specific proteins and functional proliferation following influenza stimulation. In addition, we have previously demonstrated protection against influenza induced by NP and M genes of 1918 origin alone in ferrets (Bragstad et al., 2011). However, in vivo expression of NP, M1 and M2 was confirmed by measuring antibody induction to these proteins in ELISA (data not shown).

Future studies including larger experiments using also contact-infected pigs are needed to address the potential impact of cross-reactive protection against infection of also other influenza strains. We have previously reported DNA-induced protective cross-reactive antibodies between different H3N2 influenza strains in pigs (Bragstad et al., 2013)

**Fig. 4.** Protective efficacy of the influenza DNA vaccine. Vaccinated and control animals were challenged with pandemic H1N1 A/California/07/2009. Post-challenge viral loads were assessed for up to 13 days in nasal swabs. Data are expressed as the mean log_{10} virus titer ± SEM.
and thus speculate that the present optimized DNA vaccine has the potential to induce broad protection.

Conflicts of interest
James Williams has an equity interest in Nature Technology Corporation. The other authors declare that there are no other conflicts of interest.

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