

Pandemic influenza 1918 H1N1 and 1968 H3N2 DNA vaccines induce cross-reactive immunity in ferrets against infection with viruses drifted for decades

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Background Alternative influenza vaccines and vaccine production forms are needed as the conventional protein vaccines do not induce broad cross-reactivity against drifted strains. Furthermore, fast vaccine production is especially important in a pandemic situation, and broader vaccine reactivity would diminish the need for frequent change in the vaccine formulations.

Objective In this study, we compared the ability of pandemic influenza DNA vaccines to induce immunity against distantly related strains within a subtype with the immunity induced by conventional trivalent protein vaccines against homologous virus challenge.

Methods Ferrets were immunised by particle-mediated epidermal delivery (gene gun) with DNA vaccines based on the haemagglutinin (HA) and neuraminidase (NA) and/or the matrix (M) and nucleoprotein genes of the 1918 H1N1 Spanish influenza pandemic virus or the 1968 H3N2 Hong Kong influenza pandemic virus. The animals were challenged with contemporary H1N1 or H3N2 viruses.

Results We demonstrated that DNA vaccines encoding proteins of the original 1918 H1N1 pandemic virus induced protective cross-reactive immune responses in ferrets against infection with a 1947 H1N1 virus and a recent 1999 H1N1 virus. Similarly, a DNA vaccine, based on the HA and NA of the 1968 H3N2 pandemic virus, induced cross-reactive immune responses against a recent 2005 H3N2 virus challenge.

Conclusions DNA vaccines based on pandemic or recent seasonal influenza genes induced cross-reactive immunity against contemporary virus challenge as good as or superior to contemporary conventional trivalent protein vaccines. This suggests a unique ability of influenza DNA to induce cross-protective immunity against both contemporary and long-time drifted viruses.

Keywords Cross-reactive immunity, DNA vaccine, influenza, pandemic influenza.

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Introduction

Influenza vaccines inducing cross-reactive immune responses would be of great advantage against seasonal and emerging influenza viruses. The humoral immunity raised by the commercial protein vaccine against seasonal influenza confers variable and sometimes poor cross-reactivity against drifted strains. Thus, the current influenza vaccines have to be evaluated every season as the influenza viruses are continuously changing their antigenicity. Protein vaccine efficiency in otherwise healthy individuals can be as low as 24% when there is a mismatch between the vaccine

strain and the circulating strain.^{1,2} Thus, the current influenza protein vaccine is less effective against drifted variants. In addition, production in fertilised hens' eggs is costly and time-consuming. These are major drawbacks in a pandemic situation as the current human infections with the novel swine H1N1 pandemic virus.

DNA vaccines provide an alternative to conventional influenza protein vaccines. The immune responses obtained by DNA vaccines mimic the protective responses after a natural infection inducing both humoral and cellular immunity.^{3,4} Therefore, DNA vaccines have the ability to induce a broader and more long-lived protection and

contribute to a dose-sparing strategy. Several DNA vaccines are now licensed in veterinary medicine⁵ or are in clinical trials, illustrating the commercial potential and validation of the improved new generation DNA vaccines. The first generation of DNA vaccines produced good results against homologous virus infection in mice, but poorer results in higher mammals and humans.^{3,4} However, the new generation DNA vaccines against influenza in humans have now been shown to be immunogenic,⁶ and its efficiency have been evaluated in humans.⁷ DNA vaccines, either alone or in combination with other vaccines, show great promise for future human vaccines.⁸

The most severe influenza to date was the 1918 H1N1 'Spanish flu', which killed at least 50 million people worldwide during 1918 and 1919.⁹ Based on preserved specimens, all genes have been genetically characterised and the entire virus has been reconstructed.¹⁰ This provides a unique opportunity to elucidate the mechanisms of pathogenesis, but also any unique immunogenic properties of this first case of the pandemic strain. Recently, a lifelong specific immunity to the 1918 H1N1 virus was shown in some individuals born in or before 1915.¹¹ We hypothesise that employing the original pandemic 1918 H1N1 and 1968 H3N2 strains as DNA vaccines may induce similar long-time protection, but also cross-immune protection against long-time drifted viruses within the same subtype.

DNA vaccination based on the first appearing (pandemic) H1N1 and H3N2 viruses and challenge with recent strains provides the possibility to test cross-reactive immunity to viruses drifted for many years. We demonstrate that DNA vaccines are able to induce cross-reactive humoral immunity against long-time drifted viruses and efficiently decrease shedding of virus.

Methods

Construction of the DNA vaccines

The 1918 pandemic H1N1 genes were designed from nucleotide sequences published in GenBank (HA A/South Carolina/1/18 AF117241, neuraminidase (NA), nucleoprotein (NP) and matrix (M) A/Brevig Mission/1/18 AF250356, AY744035 and AY130766, respectively) (referred to as 1918 H1N1 virus or 1918 H1N1 DNA). The A/New Caledonia/20/99(H1N1) genes were designed from sequences of the MDCK-cultivated challenge virus (referred to as 1999 H1N1 virus or 1999 H1N1 DNA). The 1968 pandemic H3N2 genes were designed from the GenBank accessions of A/Aichi/2/1968(H3N2), HA AB295605 and NA AB295606, respectively (referred to as 1968 H3N2 virus or 1968 H3N2 DNA). The A/Wisconsin/67/05(H3N2) genes were designed from sequences of the MDCK-cultivated challenge virus (referred to as 2005 virus or 2005 H3N2 DNA).

The genes were synthesised and codon-optimised for efficient expression in ferrets and humans by GeneArt (Regensburg, Germany) and cloned into a modified pWRG7079 (PowderJect, Middleton, Wisconsin, USA) DNA vaccine vector.¹² H3N2 genes were cloned into a clinical pKCMV standard expression vector kindly provided by Britta Wahren, Karolinska Institute, Sweden.¹³ Key elements in the expression vectors are the Kozak ribosomal signal sequence, a kanamycin resistance gene, strong constitutive CMV-IE promoter, polyadenylation signals and an intron A sequence in the pWRG7079 vector. Endotoxin-free DNA purifications of the vaccine clones were prepared by EndoFree Plasmid Giga Kit (Qiagen, Hilden, Germany). All vaccines were control sequenced as described previously.¹⁴ Expression of influenza genes *in vitro* was validated by radio immunoprecipitation assay,¹⁵ and functionality of expressed HA proteins was validated by haemadsorption assay.¹⁶

Animal studies

Seven-month-old outbred ferrets (*Mustela putorius furo*) (Ole Olesen, Møldrup, Denmark) were chip-tagged (pet-id; E-vet, Haderslev, Denmark). The animals were fed a standard diet with food and water *ad libitum* and housed according to the Danish Animal Experimentation Act, based on the Council of Europe Convention ETS 123, on a license granted by the Ministry of Justice. The animals were kept at level II biosecurity facilities at the Faculty of Life Sciences, Copenhagen, Denmark. The ferrets were gene gun (Helios; Bio-Rad, Hercules, CA, USA) inoculated (400 psi compressed helium) on shaved abdominal skin, using a total of 2 µg DNA/mg-coated 1.6 µm-sized gold particles and 0.5 mg gold/shot giving 1 µg DNA/shot with an 80–95% coating efficiency. Each ferret was gene gun immunised three times at 2-week intervals with the DNA vaccine. The control groups were vaccinated twice, 3 weeks apart, with the human dose (15 µg HA of each component in 0.5 ml) of the trivalent protein vaccine, Influvac 2006/2007 [subunit, Solvay Pharmaceuticals, Olst, the Netherlands components: A/New Caledonia/20/99 (H1N1), A/Wisconsin/67/2005 (H3N2), B/Malaysia/2506/2004] or Vaxigrip 2007/08 [split, Sanofi Pasteur, Lyon, France. Components: A/Solomon Island/3/2006(H1N1), A/Wisconsin/67/2005(H3N2), B/Malaysia/2506/2004]. Ferrets were challenged 10–14 days after the last immunisation with 5.5×10^5 – 1×10^7 50% tissue culture infectious dose (TCID₅₀) of A/Fort Monmouth/1/47(H1N1) (ATCC VR-97, MDCK P2), A/New Caledonia/20/99(H1N1) (WHO, MDCK P3) or A/Wisconsin/67/05(H3N2) (WHO, MDCK P3) virus (referred to as 1947 H1N1 virus, 1999 H1N1 virus and 2005 H3N2 virus, respectively) in 1000 µl PBS administered into the nostrils with a pipette. Blood was collected from the cranial vena cava of anaesthetised (tiletamine/zolazepam) ferrets before and sequentially after challenge.

Animals were terminated at day 10 or 12 post-challenge with pentobarbital.

Vaccination and challenge details on individual experiments

Experiment involving the 1947 H1N1 virus challenge (Fig. 1)
Three groups of six female ferrets were vaccinated as follows: HA and NA 1918 H1N1 DNA, HA, NA, NP and M 1918 H1N1 DNA and empty plasmid (negative DNA vaccine control). All ferrets received four shots. Haemagglutinin/neuraminidase-mixed DNA vaccines were given in two shots, and NP/M DNA-mixed vaccines were given in two shots. Group 1 receiving only HA and NA DNA vaccine were additionally shot twice with empty plasmid DNA, ensuring that all animals received the same amount of

DNA and the same number of shots for each vaccination. Ferrets were DNA vaccinated three times, 2 weeks apart and challenged intranasally with 5.5×10^5 TCID₅₀ of the A/Fort Monmouth/1/1947 (H1N1) virus 10 days after the last immunisation.

Experiment involving the 1999 H1N1 virus challenge (Fig. 2)
Five groups of five female ferrets were vaccinated as follows: HA and NA 1918 H1N1 DNA, HA and NA A/New Caledonia/20/99(H1N1) DNA, NP and M 1918 H1N1 DNA, conventional trivalent protein vaccine (Influvac 2006/07; Solvay Pharmaceuticals) and empty plasmid (negative DNA vaccine control). DNA-vaccinated ferrets received two shots of DNA, three times, 2 weeks apart. Ferrets receiving conventional protein vaccine were immunised twice, 3 weeks apart. All groups were challenged with

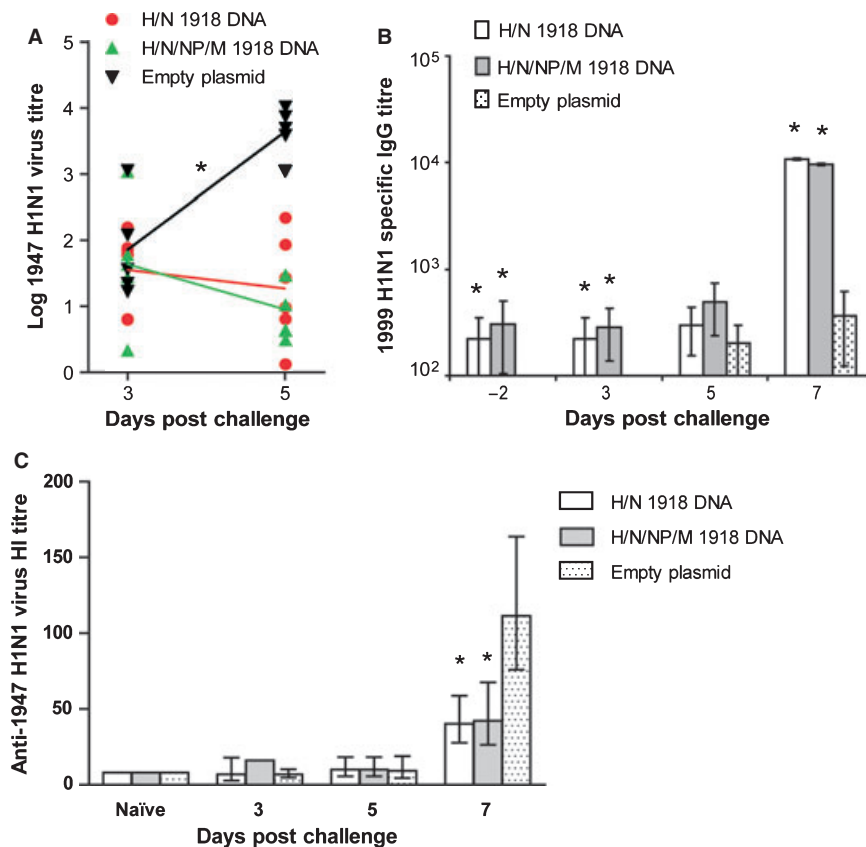


Figure 1. H1N1 1918 DNA vaccines induce cross-protective immunity against a 1947 H1N1 influenza virus challenge. 1947 H1N1 virus load in nasal washings (A) was measured by real-time RT-PCR on the M gene and expressed as individual log-virus copy number. Data is presented as individual log-virus titre with linear regression lines for each vaccination group; H/N 1918 H1N1 DNA (red), H/N/NP/M 1918 H1N1 DNA (green) and control group, empty plasmid (black). A slope significantly none-zero is indicated with an asterisk. A/New Caledonia/20/99(H1N1)-specific serum IgG antibodies were measured by ELISA (B) and presented as mean end-point titre with standard deviation for each vaccination group; H/N 1918 H1N1 DNA (white), H/N/NP/M 1918 H1N1 DNA (grey) and control group, empty plasmid (dotted). An asterisk indicates DNA-vaccinated groups significantly ($P < 0.05$) different from the control group, empty plasmid. Haemagglutination inhibition (HI) antibodies against the 1947 H1N1 virus (C) were measured by a HI assay with 0.75% guinea pig red blood cells. Haemagglutination inhibition titres are given as geometric mean titre with 95% confidence interval. H/N 1918 H1N1 DNA (white), H/N/NP/M 1918 H1N1 DNA (grey) and control group, empty plasmid (dotted). An asterisk indicates DNA-vaccinated groups significantly ($P < 0.05$) different from the control group.

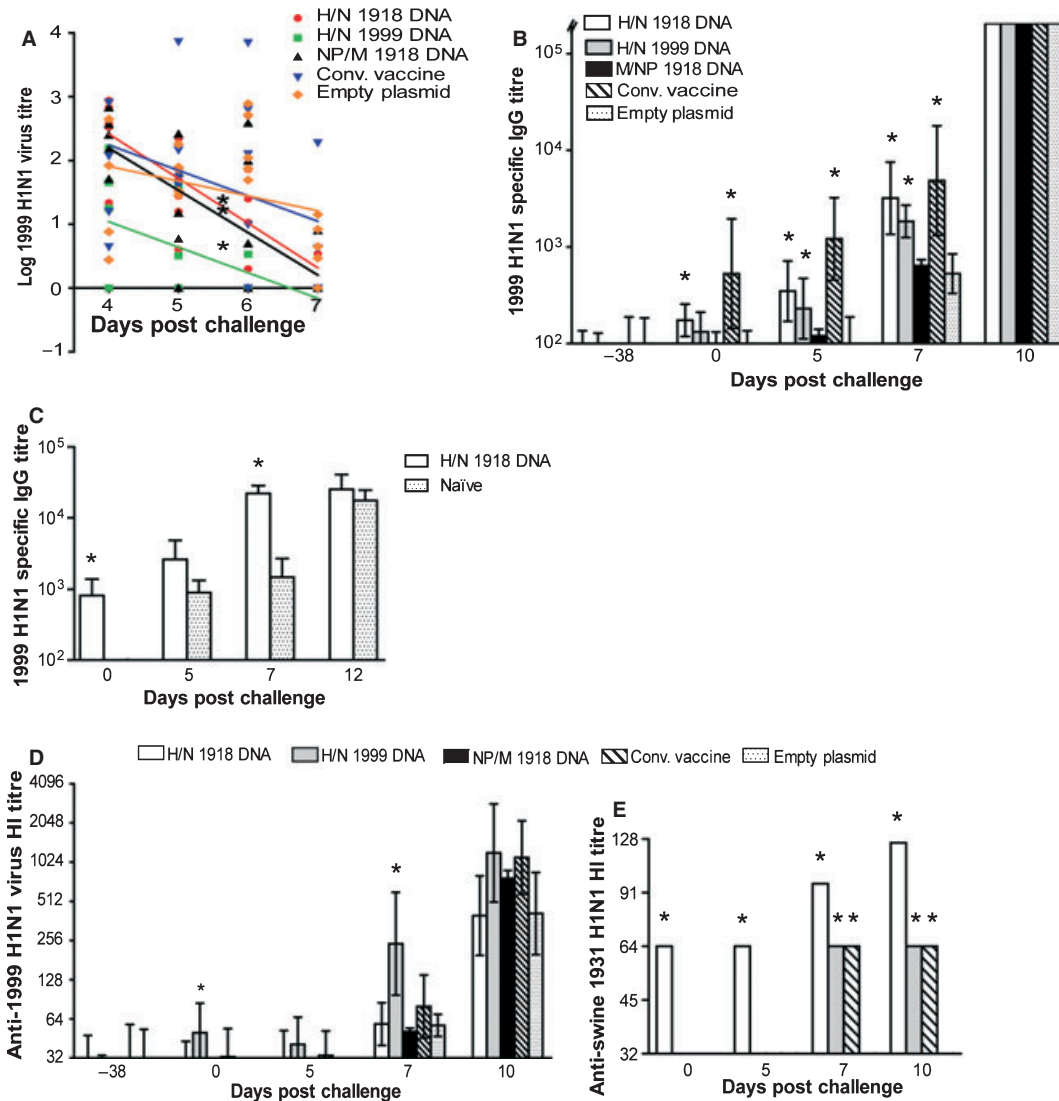


Figure 2. 1918 H1N1 DNA vaccines induce cross-reactive immunity against a contemporary 1999 H1N1 influenza virus challenge. 1999 H1N1 virus load in nasal washings (A) was measured by real-time RT-PCR on the M gene and expressed as individual log-virus copy number. Data is presented as individual log-virus titre with linear regression lines for each vaccination group; H/N 1918 H1N1 DNA (red), H/N 1999 H1N1 DNA (green), NP/M 1918 H1N1 DNA (black) and the control groups, conventional trivalent protein vaccine (blue) and empty plasmid (orange). A slope significantly non-zero is indicated with an asterisk. Anti-A/New Caledonia/20/99(H1N1)-specific IgG antibodies were measured by ELISA (B) and presented as geometric mean end-point titres with 95% confidence interval. Vaccine groups: H/N 1918 H1N1 DNA (white), H/N 1999 H1N1 DNA (grey), NP/M 1918 H1N1 DNA (black), conventional trivalent protein vaccine (striped) and empty plasmid (dotted). Day 38 pre-challenge (day 0) was the day of the first vaccine dose. Anti-A/New Caledonia/20/99(H1N1)-specific IgG antibodies induced by the H/N-1918 DNA-vaccinated ferrets (white) compared to naïve ferrets (dotted) are presented as geometric mean end-point titre with 95% confidence interval (C). Haemagglutination inhibition (HI) antibodies against eight haemagglutination units of A/New Caledonia/20/99(H1N1) virus (D) were measured by HI assays with 0.75% guinea pig red blood cells. Haemagglutination inhibition titres are given as geometric mean titre with 95% confidence interval. Day 38 pre-challenge (day 0) was the day of the first vaccine dose. Pools of sera from each group were tested in duplicates against 1918 H1N1-like swine 1931 virus (E). An asterisk indicates vaccine groups significantly ($P < 0.05$) different from the empty plasmid control group.

1×10^6 TCID₅₀ A/New Caledonia/20/99(H1N1) virus 10 days after the last immunisation.

Experiment involving the 2005 H3N2 virus challenge (Fig. 3)
Four groups of six male ferrets were vaccinated as follows: HA and NA 1968 H3N2 DNA, HA and NA A/Wisconsin/

67/05(H3N2) DNA, conventional trivalent protein vaccine (Vaxigrip 2007/08; Sanofi Pasteur) and unvaccinated naïve ferrets. DNA-vaccinated ferrets received four shots of DNA, three times, 2 weeks apart. Ferrets receiving conventional protein vaccine were immunised twice, 2 weeks apart. All groups were challenged with 1×10^7 TCID₅₀

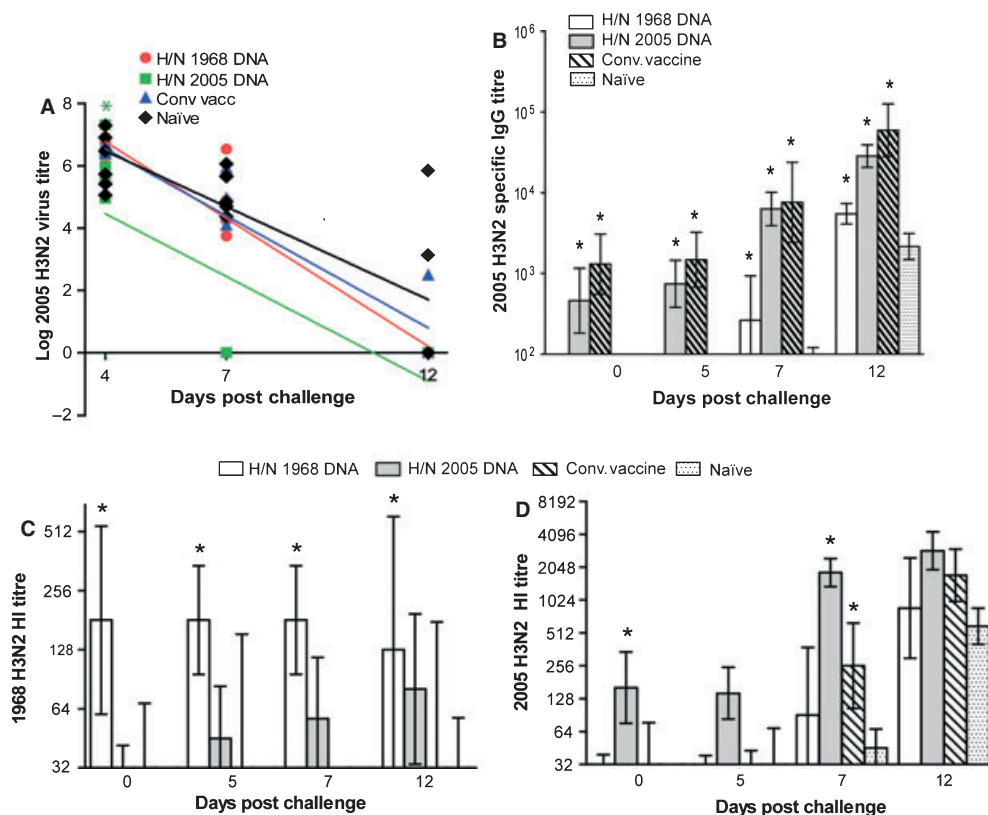


Figure 3. 1968 H3N2 DNA vaccine induces cross-reactivity against a contemporary 2005 H3N2 influenza virus infection. 2005 H3N2 virus load in nasal washings (A) was measured by real-time RT-PCR on the M gene and expressed as individual log-virus copy number. Data is presented as individual log-virus titre with linear regression lines for each vaccination group; H/N 1968 H3N2 DNA (red), H/N 2005 H3N2 DNA (green), conventional trivalent protein vaccine (blue) and the naïve, unvaccinated control group (black). Significant reduction ($P < 0.05$) in median virus titre from day 4 to 7 in the H/N 2005 DNA-vaccinated group is indicated with an asterisk. Anti-A/Wisconsin/67/05(H3N2)-specific IgG serum antibodies were measured by ELISA (B) and presented as the reciprocal geometric mean titre with 95% confidence interval of the dilution correlating with an OD of one read from a standard curve included on each plate. An asterisk indicates vaccine groups significantly ($P < 0.05$) different from the naïve control group. Haemagglutination inhibition (HI) antibodies were measured against eight haemagglutination units of A/Aichi/2/68(H3N2) virus (C) or A/Wisconsin/67/05(H3N2) virus (D) with 0.75% guinea pig red blood cells. Haemagglutination inhibition titres are given as geometric mean titre with 95% confidence interval of the reciprocal value of the last dilution of sera completely inhibiting haemagglutination. The vaccine groups were as follows: H/N 1968 H3N2 DNA (white), H/N 2005 H3N2 DNA (grey), conventional trivalent protein vaccine (striped) and naïve unvaccinated ferrets (dotted). An asterisk indicates vaccine groups significantly ($P < 0.05$) different from the naïve control group.

A/Wisconsin/67/2005(H3N2) virus 14 days after the last immunisation.

Virus titration

The determination of 50% tissue culture infectious dose (TCID₅₀/ml) was carried out in 96-well plates containing a 90–95% confluent MDCK cell monolayer. The MDCK cells were incubated with serial 10-fold dilutions of virus culture supernatant in cell medium (Eagles minimal essential medium supplemented with 2 mm l-glutamine, 1% gentamycin and trypsin; without foetal calf serum) at 37°C, 5% CO₂. Fifty microlitre of each dilution was added to five wells. After 2 hours of incubation, 150 µl cell medium with trypsin was added, and the plates were incubated for 2 days. The TCID₅₀ of the virus stock was calculated by the method of Reed and Muench.¹⁷

Real-time RT-PCR assay for quantification of influenza A virus

The nostrils of each ferret were flushed with 1 ml PBS, and the flushings were kept at –80°C for real-time RT-PCR analysis. RNA from the nasal washings (200 µl) was extracted on a MagNA Pure LC Instrument applying the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche diagnostics, Basel, Switzerland). The RT-PCR was performed with oligonucleotide primer and probe sequences as described elsewhere.¹⁸ Extracted RNA (5 µl) was added to 20 µl of master mix consisting of 10 µm of each primer and 2 µm of the Taqman probe labelled with FAM in the 5' end and black hole quencher 1 in the 3' end together with reagents from the OneStep[®] RT-PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Target sequences were amplified on the MX3005

cycler system (Stratagene): 20 minutes at 50°C, 15 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 60 seconds at 55°C. The quantification of virus was performed by using a standard curve developed by serial dilutions of H1N1 or H3N2 virus with known virus copy number.

Influenza-specific serum antibodies determined by ELISA

Antibody levels were measured by two similar ELISA procedures. Detection of antibodies from the H1N1 experiments was performed as follows: ELISA plates (Immuno 96 MicroWell™ Plates, MaxiSorp; Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 µl/well of 1 µg HA/ml, influenza protein vaccine (Influvac 2006–2007) diluted in carbonate buffer pH 9.6. Wells were blocked with 2% BSA/PBS for 2 hours at room temperature. Plates were washed with 0.05% Tween-20/PBS (PBST). Sera were serially diluted in dilution buffer (0.1% BSA/PBST pH 7.2) and incubated for 1 hour at room temperature. Following three washing steps, plates were incubated for 1 hour with biotinylated rabbit anti-mink IgG antibody¹⁹ diluted 1:500, washed and further incubated for half an hour at room temperature with 1:1000 dilution of horseradish peroxidase streptavidin (DakoCytomation, Glostrup, Denmark). The reaction was developed with OPD (DakoCytomation) and stopped after 15 minutes by adding 50 µl/well 0.5 M H₂SO₄ and read at 492 nm with a reference at 650 nm. End-point titres were calculated as the reciprocal sera dilution corresponding to the last positive signal, four times above background.

For the H3N2 experiments, the ELISA plates were coated with 1 µg HA/ml, influenza protein vaccine (Vaxigrip 2007–2008), blocked for 1 hour and washed in 0.1% Triton-X100. The biotinylated rabbit anti-mink IgG antibody (150 µl/well) was diluted 1:250. The reaction was developed with TMB One substrate (Kem-En-Tec, Taastrup, Denmark) and stopped by adding 50 µl 0.2 M H₂SO₄ to each well and read at 450 nm with a reference at 650 nm. A positive run-control (a positive ferret serum) was included on each plate together with a standard curve prepared from pooled ferret sera collected on day 12 post-challenge. Titres were calculated as the mean reciprocal dilution correlating with to an OD of one on the standard curve of each plate. Sera from two ferrets in the 1968 H3N2 DNA-vaccinated group and two from the conventional protein-vaccinated group had to be excluded from all the analysis as ELISA revealed pre-immunity against H3N2 influenza virus.

Haemagglutination inhibitory antibodies

Ferret sera were treated with receptor-destroying enzyme (RDE(II); Seiken, Tokyo, Japan) as described by the

manufacturer. One part of ferret serum was blood-absorbed by 20 parts of packed guinea pig red blood cells and incubated for 1 hour at 4°C followed by centrifugation. Viruses were titrated by a haemagglutination assay according to the protocols of the WHO²⁰ with 0.75% guinea pig red blood cells in U-bottom plates (U96 MicroWell Plates; Nunc) and incubated for 1 hour. Virus was standardised to 100% haemagglutination end-point titre of eight haemagglutination units. The haemagglutination inhibition (HI) assay was performed according to the protocols of WHO²⁰ with 0.75% guinea pig red blood cells in U-bottom 96-well plates (U96 MicroWell Plates; Nunc) and the HI titres read as the reciprocal of the last dilution of sera that completely inhibits haemagglutination.

Prediction of glycosylation sites

Potential N-linked glycosylation sites in the HA1 region of the HA protein were predicted using nine artificial neural networks with the NetNGlyc 1.0 Server²¹ CBS, DTU, Lyngby, Denmark. A threshold value of >0.5 average potential score was set to predict glycosylated sites.

Statistical analysis

Statistical analyses were performed in GraphPad Prism v. 5.0, GraphPad Software, Inc., La Jolla, California, USA. Vaccine groups were evaluated against empty plasmid or naïve groups by two-tailed *t*-test (unless stated otherwise). *P*-values <0.05 were considered statistically significant.

Results

1918 H1N1 DNA vaccines induce protective immunity against a 1947 H1N1 virus challenge

Ferrets DNA vaccinated with genes from the pandemic 1918 H1N1 virus were challenged with the heterologous A/Fort Monmouth/1/47 (H1N1) virus, to investigate possible cross-protection. Ferrets vaccinated with codon-optimised HA + NA (H/N) 1918 or HA + NA + NP + M (H/N/NP/M) 1918 H1N1 genes did reduce virus shedding in nasal washings after challenge with the 1947 H1N1 virus (Fig. 1A). Both the H/N 1918 and H/N/NP/M 1918 DNA vaccines significantly improved virus clearance compared to the empty plasmid DNA vaccine (Fig. 1A). In contrast, virus titres increased >100-fold from day 3 to 5 in ferrets vaccinated with the empty plasmid backbone. Thus, the pandemic 1918 H1N1 DNA vaccines were able to efficiently reduce the virus shedding after the 1947 H1N1 virus challenge. The efficiency was not improved further by adding the internal NP and M genes in the DNA vaccine.

Sera from the 1918 H1N1 DNA-vaccinated ferrets, challenged with the 1947 virus, were further tested for broadly cross-reactive antibodies in an ELISA specific for an A/New Caledonia/20/99 (H1N1) virus (Fig. 1B). Surprisingly, sig-

nificantly high levels of antibodies cross-reacting with the 1999 H1N1 antigens were found in sera after 1918 H1N1 DNA vaccination and 7 days post-1947-virus challenge (Fig. 1B). Thus, the 1918 H1N1 DNA vaccines induced cross-reactive antibody response against the more than 80-year drifted 1999 H1N1 virus after a 1947 H1N1 virus challenge. Haemagglutination inhibition antibodies against the 1999 H1N1 virus were not measurable (not shown). Haemagglutination inhibition antibodies against the 1947 H1N1 virus challenge reflected the virus titre measurements in the nasal washings (Fig. 1C); the 1918 DNA-vaccinated ferrets were less infected than the control group. The 1918 DNA vaccines did not induce measurable HI antibodies cross-reacting with the 1947 challenge strain. However, these results may indicate the presence of antigenic sites shared by the three different H1N1 strains, not necessarily measurable by an HI assay.

1918 H1N1 DNA vaccine induces cross-reactive immunity against a 1999 H1N1 virus infection

We further investigated the extent of cross-reactivity of the 1918 DNA vaccine by testing its ability to protect against the more than 80-year drifted recent virus, A/New Caledonia/20/99 (H1N1). Virus levels measured in the DNA-vaccinated groups decreased significantly during the first week post-infection (Fig. 2A). Interestingly, no such decline was observed in the groups vaccinated with the conventional homologous protein vaccine or the empty plasmid (Fig. 2A) suggesting a specific effect related to the vaccine model. The NP/M DNA vaccine only based on the two more conserved internal proteins, NP and M, also improved virus clearance, indicating that immune mechanisms other than neutralising antibodies against the surface proteins may contribute to the protection. As expected, ferrets vaccinated with H/N 1999 DNA cleared the homologous 1999 H1N1 virus challenge infection efficiently by day 5 (Fig. 2A).

1918 H1N1 DNA vaccine induces influenza-specific antibody responses against a 1999 H1N1 virus

Ferrets vaccinated with H/N 1918 H1N1 DNA displayed significantly higher levels of anti-1999 H1N1 virus IgG titre compared to the negative control group at the day of the 1999 H1N1 virus challenge (day 38 after the first immunisation), indicating the presence of cross-reacting 1999 virus antibodies induced by the pandemic 1918 H1N1 DNA vaccination (Fig. 2B). As expected, antibodies were also generated after conventional protein vaccination. At day 5 post-1999 virus challenge, both the H/N H1N1 1918 and H/N H1N1 1999 DNA-vaccinated ferrets had significantly elevated vaccine-induced 1999 H1N1 influenza-specific recall antibodies compared to the empty plasmid group (Fig. 2B). These results indicate that the pandemic 1918 DNA vaccine and

the 1999 H1N1 DNA vaccine induce comparable levels of humoral immune responses against the contemporary H1N1 virus. Interestingly, recall antibody titres against the 1999 virus was comparable in the groups vaccinated with H/N 1918 DNA vaccine and the conventional vaccine (Fig. 2B).

In a repeat experiment, H/N 1918 DNA-vaccinated ferrets and non-vaccinated controls were challenged with 1999 H1N1 virus (Fig. 2C). Again, 1999 H1N1 virus-specific antibody levels were significantly higher in the H/N 1918 DNA-vaccinated ferrets.

Induction of HI antibodies after H1N1 DNA vaccination

Only ferrets vaccinated with the H/N 1999 H1N1 DNA vaccine had high inhibitory HI titres against the A/New Caledonia/20/99 (H1N1) virus at day 0, the day of the 1999 H1N1 virus challenge (Fig. 2D), indicating HI antibodies activated by the DNA vaccination. The ferrets vaccinated with H/N 1918 H1N1 DNA were not expected to give HI titres against the 1999 H1N1 virus before challenge because of the narrow specificity of the HI antibodies. At the day of challenge (experiment day 38), three of four ferrets in the H/N 1999 H1N1 DNA vaccine group were seroprotected (HI titre ≥ 40) whereas one of five ferrets in the conventional vaccine and H/N 1918 DNA vaccine groups were seroprotected.

Human antibodies against the 1918 H1N1 virus react well with the 1918-like A/Swine/Iowa/15/31 (H1N1) (ATCC-VR-333) virus (as shown by others⁶ H/N 1918 DNA vaccine-induced antibodies were therefore measured in an HI assay against the swine 1931 H1N1 virus (Fig. 2E). The H/N 1918 DNA vaccine induced significant 1931 H1N1 HI antibodies after vaccination pre-challenge. This antibody response increased upon challenge with the human 1999 H1N1 virus. Also, the 1999 H1N1 DNA vaccine and conventional vaccine-induced antibodies able to cross-react with the swine 1931 H1N1 virus, but only 7 days after a 1999 H1N1 virus infection (Fig. 2E), indicating that virus challenge triggers broad cross-reacting HI antibodies induced by DNA vaccination.

1968 H3N2 DNA vaccinations followed by a 2005 H3N2 virus challenge

To explore the generality of the DNA vaccines based on pandemic strains, we based the new DNA vaccine on the HA and NA genes of another pandemic virus, namely the H3N2 virus from 1968 (H/N 1968 DNA). Immunised animals were challenged with the contemporary H3N2 virus, A/Wisconsin/67/05. None of the vaccines completely prevented the initial detection of high-challenge virus titres in the nasal washings (Fig. 3A). The 1968 H3N2 DNA vaccine and the conventional trivalent protein vaccine did not

reduce viral load efficiently. However, the 2005 H3N2 DNA-vaccinated ferrets cleared the infection completely by day 7, and the 1968 H3N2 DNA-vaccinated ferrets had no detectable viral load after day 7 (Fig. 3A). In contrast, virus could be detected as long as 12- days post-challenge for one ferret in the conventional protein vaccine group (titre>100 TCID₅₀/ml) and two ferrets in the unvaccinated group (titre>1000 TCID₅₀/ml).

1968 H3N2 DNA vaccine induce cross-reactive recall antibodies

The H/N 1968 H3N2 DNA vaccine induced specific antibodies that cross-reacted with the 2005 H3N2 virus at day 7 post-infection (Fig. 3B). H/N 2005 H3N2 DNA vaccine and conventional trivalent protein vaccine induced comparable high levels of antibodies after vaccination (Fig. 3B).

Induction of HI antibodies after H3N2 DNA vaccination

The H/N 1968 H3N2 DNA vaccine was able to induce HI antibodies towards homologous virus (Fig. 3C). Seroconversion, defined as >fourfold increase between pre- and post-vaccination HI titre, was observed in three of four ferrets, and all ferrets were seroprotected (HI titre >40) at the day of challenge. At day 5 post-challenge, all ferrets had seroconverted. Ferrets vaccinated with H/N 2005 H3N2 DNA and challenged with 2005 H3N2 virus showed low but increasing cross-reactivity against the 1968 virus upon challenge (Fig. 3C).

At the day of challenge (day 42), all ferrets vaccinated with H/N 2005 H3N2 DNA had seroconverted and were HI seroprotected against 2005 H3N2 virus compared to only one ferret in the conventional vaccinated group. Also, the recall response at day 7 post-infection was significantly higher for ferrets vaccinated with H/N 2005 H3N2 DNA than for ferrets vaccinated with the conventional protein vaccine (Fig. 3D). These results indicate that the influenza DNA vaccines induce higher levels of specific HI antibodies than the conventional trivalent protein vaccine.

Table 1. Predicted *N*-glycosylation sites in the H1N1 HA1 region*

	Amino acid									
	11	23	54	87	125	127	155	160	269	287
A/South Carolina/1/18	x	x		x						x
A/Fort Monmouth/1/47	x	x		x		x	x		x	x
A/New Caledonia/20/99	x	x	x	x	x			x		x

*H1 numbering based on the A/South Carolina/1/18 strain.

Table 2. Predicted *N*-glycosylation sites in the H3N2 HA1 region*

	Amino acid									
	8	22	38	63	81	133	144	165	246	285
A/Aichi/2/1968	x	x	x		x			x		x
A/Wisconsin/67/05	x	x		x		x	x	x	x	x

*H3 numbering.

The pandemic HA genes possess minimal N-linked glycosylation sequons

The N-linked glycosylation sequons of the HA vaccine genes and the HAs of the different challenge viruses were compared (Tables 1 and 2). The pandemic HA genes of the pandemic H1N1 and H3N2 viruses possess the least number of sequons. These sequons, located in the stalk region of HA1, have become conserved in preceding strains. There has been an accumulation of sequons, mainly in the globular head of HA, in strains preceding the first occurring pandemic viruses.^{22–24}

Discussion

There is an urgent need for alternative influenza vaccines with broader coverage and faster production forms. One approach is new DNA vaccines with improved immunogenicity in humans^{6,7,25} providing cross-reactive cellular and humoral immune responses.²⁶

When studying the extent of cross-protection, we conducted a number of vaccination experiments in ferrets, using genes from the pandemic 1918 H1N1 or 1968 H3N2 viruses. We found that the H/N 1918 H1N1 DNA vaccine efficiently limited shedding of a 1947 H1N1 virus. Furthermore, H/N 1918 DNA-vaccinated animals were even able to clear infection with a contemporary 1999 H1N1 virus. The viruses used as challenge circulated approximately 30 and 80 years later than the vaccine components. As expected, DNA vaccines based on genes from the homologous challenge viruses induced the highest protection against the homologous virus challenges in this study. However, the 1918 H1N1 and 1968 H3N2 DNA vaccines induced as good as or better protective immunity to 1999 H1N1 and 2005 H3N2 challenge, respectively, when compared to the commercially available homologous trivalent protein vaccines. The DNA vaccine based on the 1968 H3N2 virus did not reduce virus shedding as efficiently as the DNA vaccine based on the 1918 H1N1 virus, despite good antibody induction. This could be because of higher infectivity of the H3N2 challenge virus compared to the H1N1 challenge virus. The H1N1 viruses have also changed

less during time compared to the H3N2 viruses,²⁷ and therefore, better cross-reactivity between H1N1 viruses may be expected.

The surprising cross-reactive immunity observed after DNA vaccination with genes from pandemic 1918 H1N1 and 1968 H3N2 may partly be explained by the non-adapted genes themselves²⁸ and/or by the intrinsic ability of the optimised DNA vaccines to induce relevant B-cell and T-cell immune responses.²⁹ In this study, we have only investigated the humoral immune response. The contribution of the cellular immunity will require further studies. The DNA vaccine encoding only the internal proteins NP and M from 1918 enabled ferrets to clear a challenge infection by the extensively drifted 1999 H1N1 virus more efficiently than the conventional trivalent protein vaccine homologous to the H1N1 challenge virus. Also, the 1918 H1N1 DNA vaccine was able to clear the contemporary virus infection despite no measurable cross-reactive HI titres at the day of challenge. These results demonstrate that cross-reactive immunity is mediated by mechanisms beyond neutralising antibodies. T-cell immunity by the NP/M DNA vaccine may play an important role in this cross-reactive immunity as the M and NP proteins are highly conserved. Therefore, the addition of NP and M genes in the DNA vaccines may improve cross-protection by different immunological mechanisms similar to a natural infection.^{30,31}

The HA gene was included in the DNA vaccines to prevent and neutralise the infection whereas antibodies against the NA should prevent release of newly synthesised virus particles, as demonstrated by others.^{32,33} The cross-reactivity of NA antibodies towards drifted viruses may be explained by the high identity within the NA subtypes.^{34,35} An NA DNA vaccine based on the Aichi 1968 (H3N2) virus has previously induced complete protection against homologous and heterologous virus challenge in mice.³⁶ Also, humans with immunity against human N1 virus are able to respond against the highly pathogenic avian H5N1 virus.³⁷ Cross-reactivity within subtypes of influenza viruses is well known. Studies have shown that a vaccine prepared from the A/New Caledonia/20/99 (H1N1) virus was able to provide some degree of protection against a lethal 1918 recombinant virus challenge in mice, which could not be explained by either HI or neutralising antibodies.²⁸ People exposed to H1N1 viruses in the late 1940s had detectable antibodies against H1N1 in the 1978 outbreak.³⁸ Also, some people naturally infected with the 1918 H1N1 virus between 1928 and 1933 still have antibody titres against the 1918 viruses.³⁹ Therefore, a vaccine inducing cross-reactivity would be of great value in preventing influenza.

The A/South Carolina/1/18 and A/New Caledonia/20/99 H1N1 viruses in this study are 18.4% different

in the HA1 protein and possess eight substitutions at residues involved in the antigenic sites (defined by Caton, *et al.*,⁴⁰) while the NAs differ by 13%. The A/Aichi/2/68 and A/Wisconsin/67/05 H3N2 viruses differ by 18.2% in the HA1 protein and by 13% in the NA protein. It is striking that the pandemic H3N2 DNA vaccines are able to induce cross-reactivity against a strain that has discrepancy in 49 of a total of 129 residues involved in the HA antigenic sites. Cross-protection and cross-reactivity by DNA vaccines against viruses differing by 11–13% in the HA1 region have been demonstrated by others.^{41–43} Altogether, the overall difference between the glycoproteins may play a minor role compared to the location of the discrepancies.²² We speculate that the pandemic antigens may possess the ability to induce broad cross-reacting recall antibody responses as these have not yet accumulated glycosylations camouflaging epitopes, and therefore, more conserved epitopes may be available for immune induction.^{44,45} A recent paper by Rechert *et al.*,²³ explains the protection against the novel 2009 H1N1 in elderly as pre-exposure to a H1N1 virus with similar glycosylation patterns as the novel H1N1 virus. Glycosylation sequons exceeding the conserved ones might mask immunogenicity. We have shown that the pandemic HA genes in our DNA vaccines possess the least amount of glycosylations. These sequons have become conserved since then, while preceding strains have gained additional sequons, mainly in the globular head.^{22–24} Therefore, the cross-reactivity of the pandemic DNA vaccine genes with more recent strains could be related to the limited number of glycosylation sites.

Influenza broad spectrum neutralising antibodies⁴⁶ as well as common antigenic sites^{47,48} have recently been identified. Our results indicate the presence of common epitopes in the 1918, 1947 and 1999 H1N1 viruses. Thus, the 1918 H1N1 DNA vaccination, followed by 1947 H1N1 virus challenge, induced ELISA IgG antibodies cross-reacting strongly with the 1999 H1N1 antigens. Unexpectedly, influenza 1999 H1N1-specific IgG antibodies were also induced after 1918 H1N1 DNA vaccination, and in fact, the recall response towards the 1999 virus challenge was comparable to the response observed after vaccination with the conventional trivalent homologous protein vaccine. Likewise, vaccination with 1968 H3N2 DNA induced cross-reactive recall IgG antibody responses towards contemporary 2005 H3N2 influenza antigen after infection with the 2005 H3N2 virus. Haemagglutinin inhibitory antibodies neutralising the influenza virus only bind a few specific epitopes on the HA protein³² while total ELISA IgG antibodies have a broader range of binding sites, both on HA and NA. In this study, the DNA vaccines were more effective at inducing HI titres after vaccination than the conventional trivalent protein vaccine.

To our surprise, post-challenge HI antibodies from ferrets vaccinated with 2005 H3N2 DNA were able to gradually cross-react with the 1968 pandemic H3N2 virus. Post-challenge sera from ferrets vaccinated with the conventional protein vaccine did not show this ability. The same trend was observed in the H1N1 experiment. Post-1999 virus-challenged ferrets vaccinated with both 1999 DNA and conventional protein vaccines developed cross-reaction against the 1918-like H1N1 swine influenza virus from 1931. The reason why the antibodies induced by the conventional trivalent protein vaccine are able to recognise the swine 1931 H1N1 virus and not the H3N2 from 1968 may be that the H1 protein has changed less over time than the H3 protein.²² This could also explain the better virus clearance observed in the H1N1 experiments.

The immunised ferrets in these studies were naïve to the challenge viruses. Most young children will not have pre-existing immunity to influenza. Adults are expected not to be naïve and might therefore respond even better to the DNA vaccination. Pre-exposure by influenza might trigger a broader cross-protection by DNA vaccination.

DNA vaccines mimic live virus infection by their intracellular antigen expression activating both the humoral and cell-mediated immunity by balanced Th1 and Th2 immune responses.^{29,49} In contrast, the conventional inactivated influenza protein vaccines mainly induce a biased Th2 response, directed against the influenza surface glycoproteins. The DNA vaccine production of antigens in their native conformational and glycosylated form in highly immune competent skin, may improve the presentation of conformational relevant antigens to the host immune system. It is likely that the response after DNA vaccination could be even more effective than the natural infection as the influenza virus possesses mechanisms to abrogate the host cell immune defences. Such regulatory elements can be avoided in the DNA vaccine.

The mechanisms behind broad cross-protection warrant further investigation as they could be the key to improved influenza vaccines with broader protection. The DNA vaccines investigated in this study did limit virus shedding and mediated faster recovery in ferrets after both drifted and homologous influenza virus infections far better than the current trivalent protein vaccine. We propose that DNA vaccines will induce improved protection also in humans against homologous as well as drifted influenza virus infections.

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Author contributions

Conceived and designed the experiments: KB, AFO, BA, LPN and CM. Performed the experiments: KB, CM, BA, KJ, AFO and JT. Analysed the data. KB, CM, AFO. Wrote the paper: KB and AFO.

Competing interests

The authors declare no competing interests.

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