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Evaluation of the attenuation, immunogenicity, and efficacy of a live virus vaccine generated by codon-pair bias de-optimization of the 2009 pandemic H1N1 influenza virus, in ferrets

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Abstract

Codon-pair bias de-optimization (CPBD) of viruses involves re-writing viral genes using statistically underrepresented codon pairs, without any changes to the amino acid sequence or codon usage. Previously, this technology has been used to attenuate the influenza A/Puerto Rico/8/34 (H1N1) virus. The de-optimized virus was immunogenic and protected inbred mice from challenge. In order to assess whether CPBD could be used to produce a live vaccine against a clinically relevant influenza virus, we generated an influenza A/California/07/2009 pandemic H1N1 (2009 pH1N1) virus with de-optimized HA and NA gene segments (2009 pH1N1-(HA+NA)^{Min}), and evaluated viral replication and protein expression in MDCK cells, and attenuation, immunogenicity, and efficacy in outbred ferrets. The 2009 pH1N1-(HA+NA)^{Min} virus grew to a similar titer as the 2009 pH1N1 wild type (*wt*) virus in MDCK cells (~10⁶ TCID₅₀/ml), despite reduced HA and NA protein expression on western blot. In ferrets, intranasal inoculation of 2009 pH1N1-(HA+NA)^{Min} virus at doses ranging from 10³ to 10⁵ TCID₅₀ led to seroconversion in all animals and protection from challenge with the 2009 pH1N1 *wt* virus 28 days later. The 2009 pH1N1-(HA+NA)^{Min} virus did not cause clinical illness in ferrets, but replicated to a similar titer as the *wt* virus in the upper and lower respiratory tract, suggesting that de-optimization of additional gene segments may be warranted for improved attenuation. Taken together, our data demonstrate the potential of using CPBD technology for the development of a live influenza virus vaccine if the level of attenuation is optimized.

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Conflict of interest:

Steffen Mueller is an employee of Codagenix Inc.

Keywords

influenza; vaccine; codon pair bias de-optimization

Introduction

Influenza continues to be a global threat to public health: seasonal influenza causes significant morbidity and mortality worldwide each year, highly pathogenic avian influenza viruses are a zoonotic threat, and there is a continued risk that an influenza virus will emerge or evolve with the potential to cause a pandemic. Vaccination remains the primary strategy for the preventing severe disease [1]. Owing to antigenic drift in circulating viruses, a vaccine from one season may not be effective in subsequent seasons, so annual immunization is needed to maintain immunity. Each year, the strains that are to be included in the vaccine for the next influenza season are chosen by the WHO collaborating centers. In the US, quadrivalent vaccines are available for the winter of 2015/2016, comprised of the following influenza virus strains: an influenza A/California/7/2009 (H1N1)-like virus, an influenza A/Switzerland/9715293/2013 (H3N2)-like virus, an influenza B/Phuket/3073/2013 (Yamagata lineage)-like virus, and an influenza B/Brisbane/60/2008 (Victoria lineage)-like virus [2].

Three vaccine platforms are currently licensed for influenza: inactivated, live attenuated, and recombinant hemagglutinin (HA) protein. For inactivated influenza A vaccines, reference strains are produced with the HA and neuraminidase (NA) genes derived from the vaccine target and the internal protein genes from a laboratory strain of influenza virus adapted to grow well in eggs (A/Puerto Rico/8/34; PR8) [3]. Live attenuated influenza A vaccines (LAIV) are generated with the HA and NA genes from the vaccine target and the internal protein genes from a temperature sensitive (*ts*), cold-adapted (*ca*), attenuated (*att*) master donor virus, either influenza A/Ann Arbor/6/60 *ca* (AA *ca*) [4] or influenza A/Leningrad/47/57 *ca* [5]. Recombinant HA vaccines are generated from baculovirus expressed HA [6]. At present, it takes several months from identifying a target strain to distributing a vaccine. This time-line can be problematic in the face of a pandemic. In 2009 an H1N1 pandemic virus emerged in April; despite a rapid response, vaccines were not available for widespread use until after the autumn/winter wave had nearly peaked in the Northern Hemisphere [7, 8].

Inactivated vaccines are administered intramuscularly (i.m.), and primarily induce serum antibodies against the influenza HA glycoprotein. They have an efficacy ranging from 60–100% in preventing influenza in healthy adolescents and adults [9], although the efficacy is lower in the elderly [10]. LAIV are administered intranasally (i.n.), and virus replication is limited to the cooler upper respiratory tract (URT), with no replication in the lower respiratory tract (LRT). LAIVs induce systemic and mucosal immune responses, however a reliable correlate of protection has not yet been found [11]. In children, the efficacy of LAIV is equivalent or superior to inactivated vaccines [11], whereas in adults inactivated vaccines are more efficacious than LAIV [9, 12–14]. In the military, inactivated vaccines were also found to be more effective than LAIV, except in new recruits [15, 16], possibly due to prior

experience with influenza viruses and/or vaccines blunting the replication of the attenuated vaccine virus.

With the view of improving LAIV efficacy, reducing the time of vaccine production, and limiting the interference of pre-existing immunity to the vaccine backbone, novel approaches to vaccine development are being investigated. The technique of codon-pair bias de-optimization (CPBD) has been used to generate attenuated polio, respiratory syncytial, vesicular stomatitis, and influenza viruses [17–21]. Codon pairs do not appear in the host-cell genome at a frequency that would be predicted based on the frequency of the individual codons. Certain codon pairs are statistically overrepresented, while others are underrepresented. Re-writing the influenza PR8 HA and NA gene segments with a large number of underrepresented codon pairs reduced the expression of the encoded proteins compared to proteins of the wild-type (*wt*) virus, and resulted in attenuation of the PR8-(HA+NA)^{Min} virus for mice [21]. Morbidity was not observed in mice inoculated with the PR8-(HA+NA)^{Min} virus at doses up to 10⁵ PFU, and mortality was not observed even following inoculation with a dose as high as 10⁶ PFU. In contrast, all of the mice that were inoculated with the *wt* PR8 virus died at doses of 10² PFU and above. Moreover, mice inoculated with the PR8-(HA+NA)^{Min} virus were protected from homologous virus challenge, even at a dose as low as 10 PFU [21], suggesting CPBD can be used to generate live virus vaccine candidates.

Live virus vaccines produced by CPBD have several advantages over conventional LAIV based on a temperature sensitive, attenuated backbone. When a gene is de-optimized, the amino acid sequence remains unchanged. Therefore, all of the gene segments of a CPBD-based vaccine virus share the protein coding sequence with the *wt* virus. As such, immune responses against a de-optimized virus are likely to be more similar to responses induced by *wt* infection. In addition, vaccine efficacy will not be influenced by immune responses directed against the vaccine backbone resulting from repeated vaccination. CPBD-based vaccine viruses can also be designed and produced *de novo* rapidly, and it should be possible to produce a CPBD-based vaccine with de-optimized HA and NA genes in a matter of weeks following vaccine composition recommendations by the WHO or national public health authority for seasonal influenza or in the face of a new pandemic [21].

Building on the success of CPBD in attenuating the influenza PR8 virus, we applied the technology to a clinically relevant influenza virus, influenza A/California/7/2009 pandemic H1N1 (2009 pH1N1), and evaluated the attenuation, immunogenicity and efficacy of a 2009 pH1N1 virus with de-optimized NA and HA genes (2009 pH1N1-(HA+NA)^{Min}) in outbred ferrets. In addition, we investigated the mechanism of attenuation by comparing the expression of HA and NA proteins and mRNA in infected MDCK cells.

Materials and Methods

Plasmids and cells

Madin-Darby canine kidney (MDCK) and human embryonic kidney (293T) cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained in Eagle's Minimum Essential Medium (E-MEM, Life Technologies, Grand Island, NY) and

Dulbecco's Modified Eagle's Medium (D-MEM, Life Technologies), respectively. The media were supplemented with 10% heat-inactivated fetal bovine serum (FBS, HyClone Laboratories Inc., Logan, UT) and 40mM L-glutamine (Life Technologies). The eight-plasmid reverse genetics systems for generating the 2009 pH1N1 virus have been described previously [22]. The HA and NA gene segments of the 2009 pH1N1 virus were designed to be codon-pair de-optimized (HA^{Min} and NA^{Min}) according to an algorithm described previously [17–19]. Each re-coded gene segment was synthesized *de novo* with a polI promoter and terminator at the termini, in order to facilitate the expression of vRNA upon transfection of susceptible cells [23, 24].

Virus

The *wt* 2009 pH1N1 virus, A/California/07/2009, was kindly provided by the Influenza Division, Centers for Disease Control and Prevention. The virus was propagated in the allanotic cavity of 9–11 day-old embryonated chicken eggs as previously described [25], and the 50% tissue culture infectious dose (TCID₅₀) was determined by 10-fold serial titration in MDCK cells, and calculated by the method developed by Reed and Muench [26].

Animals

Six-month old male ferrets were obtained from Triple F Farms (Sayre, PA). Ferret sera were pre-screened for the presence of antibodies against circulating H1N1, H3N2, and influenza B viruses by hemagglutination inhibition assay (HAI), and only seronegative animals were included in the study. All ferret experiments were performed at the NIH with the approval of and in compliance with the guidelines of the NIAID/NIH Institutional Animal Care and Use Committee.

Generation and propagation of recombinant viruses

Recombinant *wt* 2009 pH1N1 and 2009 pH1N1-(HA+NA)^{Min} viruses were generated by transfecting a co-culture of MDCK and 293T cells with a mixture of the eight relevant reverse genetics plasmids with either the transfection reagent TransIt (Mirus Bio Corp., Madison, WI), or lipofectamine 2000 (Life Technologies) in Opti-MEM (Life Technologies). The viruses were propagated in flasks of MDCK cells in E-MEM supplemented with 40mM L-glutamine, 2% antibiotic-antimycotic (Life Technologies), and L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK) trypsin (Worthington Biochemical Corp., Lakewood, NJ). The supernatant was harvested at 48 hours post-infection, tested for hemagglutinating activity, and the virus titer was determined as described previously [26].

To confirm the identity of the viruses, RNA was extracted from an aliquot of each MDCK cell supernatant with a QIAmp Viral Mini kit (QIAGEN), and subjected to RT-PCR with a QIAGEN OneStep RT-PCR kit (QIAGEN). The sequence of the resulting cDNA was determined with a BigDye sequencing kit and 3730 DNA Analyzer.

Assessment of viral replication in MDCK cells

Confluent monolayers of MDCK cells were washed twice in E-MEM and infected with each virus at room temperature for 1 hour. The inoculum was removed, and cells were incubated

in E-MEM supplemented with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK) trypsin (Worthington Biochemical Corp., Lakewood, NJ), 40mM L-glutamine, and 2% antibiotic-antimycotic (Life Technologies). Cultures were incubated at the indicated temperatures, and supernatant samples were harvested at the indicated times post-infection for quantification of virus by 10-fold serial titration in MDCK cells. Plates were incubated at 37°C for 4 days, whereupon the presence or absence of cytopathic effect (CPE) in each well was determined. The titer was computed as previously described [26], and expressed as $\log_{10}$ TCID₅₀/ml. A Student's *t*-test was used to compare the viral replication.

The ability of each virus to agglutinate turkey red blood cells was quantified by incubating 2-fold serially diluted virus with an equal volume of 0.5% turkey red blood cells for 30 minutes. The presence or absence of agglutination was determined for each viral dilution and expressed in hemagglutination units (HAU).

Assessment of viral protein expression in infected MDCK cells

MDCK cells were infected with the recombinant 2009 pH1N1 *wt* and the 2009 pH1N1-(HA+NA)^{Min} viruses at an MOI of 0.1. Protein was extracted from infected MDCK cells at the indicated times post-infection and 30µg subjected to SDS-PAGE gel electrophoresis and western blot analysis with antibodies against NA (ProSci inc., Poway, CA), HA (kindly supplied by Dr. Johnathan Yewdell), NP (Millipore, Billerica, MA), and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA).

Quantification of messenger RNA in infected MDCK cells

MDCK cells were infected with the recombinant 2009 pH1N1 *wt* and the 2009 pH1N1-(HA+NA)^{Min} viruses at an MOI of 0.1. RNA was extracted from infected MDCK cells with an RNeasy Mini Kit (QIAGEN) at the indicated times post-infection. RNA was reverse transcribed according to the hot-start protocol described by [27], and the resulting cDNA was amplified by qPCR with an ABI PRISM 7500 qPCR machine (Applied Biosystems, Life Technologies) and a SYBR GreenER qPCR Supermix for ABI PRISM (Life Technologies). For each virus, we amplified a single region of the NA, HA, and M gene segments that was conserved between the 2009 pH1N1 *wt* and the 2009 pH1N1-(HA+NA)^{Min} viruses. The RT primers were tagged with an additional 18–20 nucleotides unrelated to the influenza virus, in order to distinguish viral mRNA from complementary (c)RNA according to the protocol by Kawakami *et al.*, [27]. Primer sequences are available on request. The Ct values were normalized to GAPDH and the fold-change in mRNA expression from baseline (0 hour post-infection) was determined. The ratio of NA:M mRNA, and HA:M mRNA were determined for the 2009 pH1N1-(HA+NA)^{Min} virus, and normalized to the corresponding ratios of the 2009 pH1N1 *wt* virus. A Student's *t*-test was used to compare the changes in gene expression between different viruses.

RNA secondary structure analysis

The predicted RNA secondary structures of the de-optimized and *wt* HA and NA genes were compared using the RNAstructure Web Server for RNA Structure Prediction (University of Rochester Medical Center, Rochester, NY).

Assessment of viral replication in ferret tissues

Groups of three lightly anesthetized ferrets were inoculated with the indicated dose of the 2009 pH1N1 *wt* virus, or the 2009 pH1N1-(HA+NA)^{Min} virus in 500 µl L-15 media (Life Technologies) intranasally (i.n.), 250 µl per nostril. Ferrets were euthanized at the indicated times post-infection, and the nasal turbinates and portions of the left and right lung were harvested for virus titration. A 10% (weight/volume) tissue homogenate was made in L-15 media, and the amount of virus determined by serial dilution on MDCK cells. Plates were incubated at 37°C for 4 days, after which the presence or absence of CPE was noted. The virus titer was computed using the Reed and Muench method [26], and titers were expressed as log₁₀ TCID₅₀/g tissue. A Student's *t*-test was used to compare the viral replication.

Assessment of serum antibody titer by hemagglutination inhibition (HAI) assay

Groups of three ferrets were inoculated with the indicated dose of the 2009 pH1N1 *wt* virus, or the 2009 pH1N1-(HA+NA)^{Min} virus and bled for serum on day 28 post-infection. The antibody titer against the biological *wt* 2009 pH1N1 virus was determined by hemagglutination inhibition assay using turkey red blood cells.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism (Version 6). Mean values were compared using a two-sample *t*-test for independent samples (Student's *t*-test). *P*-values of less than or equal to 0.05 were considered significant and denoted with a single asterisk (*), and *p*-values of less than or equal to 0.01 were denoted with a double asterisk (**).

Results

The growth and phenotype of the 2009 pH1N1-(HA+NA)^{Min} virus is similar to the recombinant *wt* virus in MDCK cells

The codon pair bias of the HA and NA genes of the 2009 pH1N1 virus were de-optimized according to computer algorithms and chemically synthesized as previously described [17–19]. The de-optimized HA gene contained 346 synonymous mutations in 1465 nucleotides, and the de-optimized NA gene contained 293 synonymous mutations in 1196 nucleotides (Figure 1A). The 5'-terminal 178, and 3'-terminal 134 nucleotides of the HA^{Min} segment, and the 5'-terminal 132, and 3'-terminal 130 nucleotides of the NA^{Min} segment were not subjected to any sequence modification. The 2009 pH1N1-(HA+NA)^{Min} virus had a significantly reduced level of replication compared to the *wt* virus *in vitro* at early time-points: The mean titers of the de-optimized and *wt* viruses were 10^{2.6} and 10^{4.1} TCID₅₀/ml respectively (*p*<0.05) at 7 hours post infection, and 10^{5.2} and 10^{6.6} TCID₅₀/ml respectively (*p*<0.01) at 18 hours post-infection. However, by 24 hours post-infection, there was no significant difference in the mean titer of the 2009 pH1N1-(HA+NA)^{Min} and *wt* viruses in MDCK cells at 33°C, 37°C, or 39°C (Figure 1C, D and E, respectively), demonstrating that the 2009 pH1N1-(HA+NA)^{Min} virus replicates efficiently in MDCK cells and does not exhibit a *ts* phenotype. As a control, we included a 6:2 reassortant virus with the HA and NA from the 2009 pH1N1 virus and six internal protein genes from the AA *ca* virus that confer the *ts* phenotype. This virus was restricted in replication at 39°C.

HA^{Min} and NA^{Min} expression is reduced in MDCK cells and the reduction of HA^{Min} expression persists

The concept of CPBD is to reduce protein expression of essential viral genes, which in turn leads to virus attenuation. We therefore assessed HA and NA protein expression by western blot analysis in MDCK cells infected with the 2009 pH1N1-(HA+NA)^{Min} virus or the recombinant 2009 pH1N1 *wt* virus. Expression of the HA protein was reduced in cells infected with the 2009 pH1N1-(HA+NA)^{Min} virus compared to the *wt* virus at 8, 16 and 24 hours post-infection (Figure 2A), demonstrating that CPBD reduces protein expression of the target gene. The expression of the NA protein was also reduced at 8 and 16 hours post-infection compared to cells infected with the *wt* virus, but expression was comparable at 24 hours post-infection (Figure 2A), suggesting the kinetics of NA protein expression was delayed but not abrogated by CPBD. In order to further investigate the mechanism of reduced protein expression, the mRNA of HA and NA was quantified by a modification of the protocol described by Kawakami *et al.*, [27]. The ratio of HA:M mRNA was significantly reduced at 8, 16 and 24 hours post infection in cells infected with the 2009 pH1N1-(HA+NA)^{Min} virus compared to the *wt* virus ($p < 0.01$) (Figure 2B). In contrast, the ratio of NA:M mRNA increased over time, and was not significantly different from the *wt* virus at 24 hours post-infection ($p = 0.21$) (Figure 2C). Therefore, CPBD reduced gene expression, however the extent of reduction differed between the gene segments.

At 24 hours post-infection at 37°C, despite similar infectious titers of the 2009 pH1N1-(HA+NA)^{Min} and *wt* viruses (Figures 1B and D), the ability of the 2009 pH1N1-(HA+NA)^{Min} virus to agglutinate turkey red blood cells was reduced. The average ratio of hemagglutination activity per ml: infectious titer per ml (HAU/ml:log₁₀ TCID₅₀/ml) was 1.24 for the 2009 pH1N1-(HA+NA)^{Min} virus and 2.27 for the 2009 pH1N1 *wt* virus (data not shown). This is consistent with the reduced HA expression (Figure 2A).

The 2009 pH1N1-(HA+NA)^{Min} virus is clinically attenuated in ferrets, though virus replication is efficient in the upper and lower respiratory tract

In order to evaluate the attenuation of the 2009 pH1N1-(HA+NA)^{Min} virus *in vivo*, groups of 3 ferrets were inoculated with 10³ TCID₅₀ of either the 2009 pH1N1-(HA+NA)^{Min} virus or the recombinant 2009 pH1N1 *wt* virus, and weighed daily for 10 days post-infection (Figure 3A). Ferrets inoculated with the *wt* virus lost an average of 6.0% of their body weight by day 7 post-infection. This was a significantly greater weight loss than ferrets inoculated with the 2009 pH1N1-(HA+NA)^{Min} virus ($p < 0.01$), where one animal gained 1.7% by day 7 post-infection, and two lost only 1.7 and 2.6% respectively (Figure 3A).

In order to evaluate the replication of the viruses in the respiratory tract of ferrets, groups of 3 animals were inoculated with similar doses of virus and animals were euthanized at 1 and 3 days post infection. At day 1 post-infection, the recombinant *wt* virus replicated in the nasal turbinates to a significantly higher titer (10^{5.8} TCID₅₀/g) than the 2009 pH1N1-(HA+NA)^{Min} virus (10^{3.7} TCID₅₀/g), $p < 0.01$. However, by day 3, there was no significant difference in the titers of the viruses ($p = 0.54$) (Figure 3B). Furthermore, virus replication in the lungs did not significantly differ on days 1 ($p = 0.77$) or 3 post-infection ($p = 0.16$), though neither virus replicated efficiently (Figure 3C). Taken together, these data suggest

that although the 2009 pH1N1-(HA+NA)^{Min} virus is clinically attenuated in ferrets and the kinetics of virus replication are delayed in the URT, the replication of the virus is not restricted in the respiratory tract of ferrets.

The 2009 pH1N1-(HA+NA)^{Min} virus protects ferrets from homologous challenge

To determine whether the 2009 pH1N1-(HA+NA)^{Min} virus induces serum antibody and protects ferrets against homologous challenge, groups of three animals were infected with doses of 10^3 , 10^4 , and 10^5 TCID₅₀ of either the recombinant 2009 pH1N1 *wt* virus, or the 2009 pH1N1-(HA+NA)^{Min} virus, and a group of 3 animals were mock-infected as controls. The animals were bled and challenged with 10^6 TCID₅₀ of the biological *wt* 2009 pH1N1 virus 28 days later and two days post-challenge, ferrets were euthanized and virus titers in the URT and LRT were determined.

All infected animals seroconverted, with serum hemagglutination inhibition (HI) antibody titers to the biological *wt* 2009 pH1N1 virus ranging from 320 to 1280 in ferrets inoculated with either the recombinant 2009 pH1N1 *wt* virus, or the 2009 pH1N1-(HA+NA)^{Min} virus (Figure 4A). Challenge virus was not detected in the nasal turbinates or lungs of any animal that received 10^4 or 10^5 TCID₅₀ of the 2009 pH1N1-(HA+NA)^{Min} virus (Figures 4B and C). In ferrets that received 10^3 TCID₅₀ of the 2009 pH1N1-(HA+NA)^{Min} virus, there was a significant reduction in challenge virus replication in the nasal turbinates ($10^{3.3}$ TCID₅₀/g) compared to mock-vaccinated animals ($10^{7.4}$ TCID₅₀/g), $p < 0.01$ (Figure 4B), and challenge virus was not detected in the lungs (Figure 4C). Taken together, these data demonstrate that doses of 10^3 TCID₅₀ and above of the 2009 pH1N1-(HA+NA)^{Min} virus protects ferrets against homologous challenge.

Discussion

Previously, CPBD has been used to attenuate the laboratory adapted influenza virus, PR8 [19, 21]. When the HA and NA genes of PR8 were de-optimized, the resulting virus (PR8-(HA+NA)^{Min}) protected mice against homologous and heterologous challenge, indicating the potential of this technology for producing novel vaccine viruses [21]. Our primary aim in this study was to extend these observations in two ways: First, by applying CPBD to a clinically relevant influenza virus, 2009 pH1N1; and second, by evaluating viral attenuation, immunogenicity and efficacy in outbred animals (ferrets) rather than in inbred mice. In addition, we further investigated the mechanism of CPBD-mediated attenuation *in vitro*.

We found that the 2009 pH1N1-(HA+NA)^{Min} virus was clinically attenuated in ferrets, with animals losing significantly less weight than those inoculated with the 2009 pH1N1 *wt* virus. Moreover, the 2009 pH1N1-(HA+NA)^{Min} virus induced robust serum antibody responses in ferrets, and protected animals against homologous challenge, even when a low dose of 10^3 TCID₅₀ was administered. Although the virus was not restricted in replication in ferrets, an advantage of CPBD technology over currently licensed LAIV platforms is that the extent of attenuation can be controlled by altering the proportion of the viral genome that is deoptimized. It should, therefore, be possible to generate a more attenuated 2009 pH1N1^{Min} virus by de-optimizing additional gene segments, for example NP or NS-1, or by de-optimizing a greater proportion of the HA or NA gene segments.

The clinical attenuation observed in ferrets is likely due to the delayed replication kinetics of the 2009 pH1N1-(HA+NA)^{Min} virus. It is possible that the slower replication observed in the URT led to less severe inflammation and symptoms. We did not observe a significant difference in the replication kinetics between the *wt* and 2009 pH1N1-(HA+NA)^{Min} viruses in the LRT of ferrets; the mean titers of the *wt* and 2009 pH1N1-(HA+NA)^{Min} viruses were 10^{2.2} TCID₅₀/g and 10^{2.6} TCID₅₀/g respectively 3 days post-infection.

In order to further investigate the mechanism of CPBD-mediated attenuation, we infected MDCK cells with the 2009 pH1N1-(HA+NA)^{Min} virus and the 2009 pH1N1_{wt} virus, and determined the kinetics of protein expression. Compared to the *wt* virus, the expression of both the HA^{Min} and the NA^{Min} proteins was reduced at 8 hours post-infection. This is consistent with observations with the PR8-HA^{Min}, PR8-NP^{Min}, PR8-PB1^{Min}, and PR8-(HA+NA)^{Min} viruses [19, 21]. The reduction in HA and NA expression likely contribute to the delayed replication kinetics. One could speculate that as less HA is expressed during the replication cycle, the binding of progeny virions to host cells is less efficient, and as less NA is expressed, budding and viral spread is reduced. However, we found that while HA expression remained reduced at 16 and 24 hours post-infection, NA expression increased over time. In previous studies, influenza protein expression had only been investigated up to 6 hours post-infection, and the results from this present study suggest the effect of CPBD differs between gene segments. The reason for this is not well understood. Both the HA^{Min} and NA^{Min} genes had approximately the same percentage of the gene segment de-optimized (~24%), with at least 130 nt at the 5' and 3' end left unchanged from the *wt* sequence. Moreover, codon usage and the % GC content remained unchanged from *wt* for both de-optimized gene segments. Given these observations, de-optimizing a greater proportion of the NA gene may lead to a greater or more sustained reduction in viral replication and a more attenuated virus.

Despite the absence of detectable HA protein by western blot, viral titers in culture supernatants reached the equivalent of *wt in vitro*. We found that for a given infectious titer, the ability of the 2009 pH1N1-(HA+NA)^{Min} virus to agglutinate turkey red blood cells was reduced compared to the *wt* virus, but was not abrogated, suggesting that the amount of HA in the supernatant and presumably on virions, is reduced but not absent. These results imply that a relatively small amount of HA is sufficient for viral replication. In addition, infection of ferrets with the 2009 pH1N1-(HA+NA)^{Min} virus induced robust HI antibody titers, demonstrating that a relatively small amount of HA is sufficient to induce a strong anti-HA antibody response.

We found a correlation between protein levels determined by western blot and mRNA levels determined by RTqPCR: The ratio of HA:M mRNA was reduced at 8, 16 and 24 hours post-infection with the 2009 pH1N1-(HA+NA)^{Min} virus compared to the *wt* virus, while the ratio of NA:M mRNA was reduced at 8 hours post-infection, but increased at later time-points. These results are in partial agreement with previous reports with the PR8-(HA+NA)^{Min} virus, where the quantity of NA^{Min} mRNA determined by northern blot was reduced compared to the *wt* virus at 6 and 9 hours post-infection, but there was no reduction in HA^{Min} mRNA despite reduced HA^{Min} protein expression [21]. The reason for this discrepancy remains unknown.

Codon pair bias is thought to be driven by the differences in how two adjacent tRNAs in the ribosome A-site and P-site interact with one another [28]. It appears that CPBD generates an mRNA sequence that is suboptimal for protein translation that may result in increased mistranslation, stalled translation or premature termination of translation. The reduction in mRNA levels we observed might be due to a decrease in transcription or an increase in the degradation of mRNA that failed to be efficiently translated, in a situation analogous to ‘no-go decay’ in yeast [29], but we cannot comment on the effect of CPBD on translation efficiency.

Due to the greatly altered primary nucleotide sequence, the predicted viral (v)RNA secondary structure of the de-optimized genes are different from the predicted vRNA secondary structure of the *wt* genes (data not shown). Recently, it has been suggested that vRNA secondary structure influences viral packaging [30] and it is therefore possible that the packaging efficiency of de-optimized gene segments might differ from that of the *wt* virus. The CPBD HA^{Min} and NA^{Min} gene segments retained at least 130 nucleotides at either termini as the original *wt* sequence, which contains the cis-acting packaging regions, however vRNA-vRNA interactions that involve sequences not previously recognized as packaging signals have recently been identified, which are generally more centrally located within the gene [30]. In addition, CPBD is also known to increase the frequency of CpG and UpA dinucleotides, which may make the RNA more ‘visible’ to the innate antiviral response of the host cell, reducing viral replication [31]. Thus, CPBD may attenuate a virus by multiple mechanisms, and characterizing these remains an active area of research.

In summary, we found that CPBD delayed or reduced the expression of the target genes HA and NA in MDCK cells. In the URT of ferrets, the replication kinetics of the 2009 pH1N1-(HA+NA)^{Min} virus was delayed compared to the 2009 pH1N1 *wt* virus, and the virus was clinically attenuated, yet animals mounted immune responses that protected against homologous challenge, even following a low dose of virus. Taken together, these data demonstrate the proof of concept that a live influenza virus vaccine can be generated from a clinical isolate using CPBD technology. De-optimization of additional gene segments or a greater proportion of the HA and NA genes is likely to attenuate the virus further and may lead to a promising platform that can be applied to pandemic and seasonal influenza vaccines.

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Highlights

- We generated a 2009 pH1N1 vaccine virus with codon pair bias de-optimized HA & NA genes
- The vaccine virus was clinically attenuated and immunogenic in a ferret model
- The vaccine virus protected ferrets from challenge with *wt* 2009 pH1N1 virus
- Additional de-optimization is needed to reduce vaccine virus replication in ferrets

Figure 1. The 2009 pH1N1-(HA+NA)^{Min} virus replicates to a similar titer as the recombinant *wt* virus in MDCK cells, and does not have a temperature sensitive phenotype

The replication of the 2009 pH1N1-(HA+NA)^{Min} virus, which contains codon-pair bias de-optimized HA and NA genes (A) was characterized in MDCK cells and compared to the recombinant *wt* virus. Cells were either infected with an MOI of 1 and the replication kinetics determined over 24 hours at 37°C (B), or an MOI of 0.01 and replication kinetics determined over 4 days at 33°C (C), 37°C (D), and 39°C (E). A 6:2 reassortant virus was included as a control in experiments C, D and E, with the HA and NA from the 2009 pH1N1 virus and six internal protein genes from the AA *ca* virus that confer temperature sensitivity. Titers were expressed as log₁₀ TCID₅₀/ml, and the mean and standard error plotted. An asterisk denotes a statistically significant difference between the replication of the de-optimized and *wt* viruses, which was determined by a Student's t-test (* *p*<0.05, ** *p*<0.01). The limit of detection for this assay was 0.5 log₁₀ TCID₅₀/ml, and is indicated by the broken line in each graph. Individual experiments were performed in triplicate, and the figures shown are representative of two independent experiments.

Figure 2. There is reduction in HA^{Min} and NA^{Min} expression compared to wt in infected MDCK cells and HA^{Min} expression remains reduced over 24 hours but NA^{Min} expression increases over time to similar levels as wt

MDCK cells were infected with either the 2009 pH1N1-(HA+NA)^{Min} virus or the recombinant 2009 pH1N1 wt virus at an MOI of 0.1 and the expression of influenza NA, HA, and NP proteins determined at 8, 16, and 24 hours post-infection by western blot analysis (A). The expression of the cellular protein GAPDH was determined as a loading control. The blot is a representative of three experimental repeats. The Ct values of influenza NA, HA and M genes were determined by RTqPCR, normalized to GAPDH, and the fold change in mRNA expression from baseline (0 hour post-infection) determined. The ratio of NA:M mRNA, and HA:M mRNA were determined for the 2009 pH1N1-(HA+NA)^{Min} virus, and normalized to the corresponding ratios of the 2009 pH1N1 wt virus (B). Mean values were compared by a Student's *t*-test (** *p* < 0.01, ns = not significant). Individual experiments were performed in triplicate, and the figures shown are representative of two independent experiments.

Figure 3. The 2009 pH1N1-(HA+NA)^{Min} virus is clinically attenuated in ferrets, but replicates as efficiently as the recombinant *wt* virus in the upper and lower respiratory tracts

Ferrets were inoculated intranasally with 10^3 TCID₅₀ of either the recombinant 2009 pH1N1 *wt* or 2009 pH1N1-(HA+NA)^{Min} viruses, and weighed daily for 10 days. The percentage weight change from baseline (day 0) was determined and plotted against time (A). Each line represents the percentage weight change from baseline of one animal inoculated with either the 2009 pH1N1-(HA+NA)^{Min} virus (open circles) or the *wt* virus (closed circles). The replication of the 2009 pH1N1-(HA+NA)^{Min} virus was determined in the upper and lower respiratory tracts of ferrets compared to the *wt* virus. The nasal turbinates (B) and lungs (C) from three ferrets per virus were harvested at one and three days post-infection for titration.

At necropsy, portions of the left and right lung were pooled. Each data point represents the titer from an individual animal, with the mean titer indicated by a bar. Titers were expressed as $\log_{10}$ TCID₅₀/g tissue, and the limit of detection for this assay was $10^{1.5}$ TCID₅₀/g tissue, as indicated by the broken line in each graph. Mean values were compared by a Student's *t*-test (** $p < 0.01$, ns = not significant).

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Figure 4. At doses of 10^3 to 10^5 TCID₅₀ the 2009 pH1N1-(HA+NA)^{Min} virus protects ferrets from homologous challenge

Ferrets were inoculated intranasally with 10^3 , 10^4 , or 10^5 TCID₅₀ of either the recombinant 2009 pH1N1 *wt* or 2009 pH1N1-(HA+NA)^{Min} viruses. Twenty-eight days post-inoculation, the antibody titer against the 2009 pH1N1 *wt* virus was determined by hemagglutination inhibition assay (A), compared to mock-vaccinated animals inoculated with L-15 media alone. Animals were challenged with 10^6 TCID₅₀ of the biological *wt* 2009 pH1N1 virus, and viral replication was determined in the upper (B) and lower (C) respiratory tracts of ferrets two-days post-challenge. Each data point represents the titer from an individual animal, with the mean titer indicated by a bar. Titers were expressed as $\log_{10}$ TCID₅₀/g

tissue, and the limit of detection for this assay was $10^{1.5}$ TCID₅₀/g tissue, as indicated by the broken line in each graph. Mean values were compared by a Student's *t*-test (** $p < 0.01$).

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