

Rapid generation of pandemic influenza virus vaccine candidate strains using synthetic DNA

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Background Vaccination is considered the most effective means of reducing influenza burden. The emergence of H5N1 and pandemic spread of novel H1N1/2009 viruses reinforces the need to have strategies in place to rapidly develop seed viruses for vaccine manufacture.

Methods Candidate pandemic vaccine strains consisting of the circulating strain haemagglutinin (HA) and neuraminidase (NA) in an A/PR/8/34 backbone were generated using alternative synthetic DNA approaches, including site-directed mutagenesis of DNA encoding related virus strains, and rapid generation of virus using synthetic DNA cloned into plasmid vectors.

Results Firstly, synthetic A/Bar Headed Goose/Qinghai/1A/2005 (H5N1) virus was generated from an A/Vietnam/1194/2004

template using site-directed mutagenesis. Secondly, A/Whooper Swan/Mongolia/244/2005 (H5N1) and A/California/04/09 (H1N1) viruses were generated using synthetic DNA encoding the viral HA and NA genes. Replication and antigenicity of the synthetic viruses were comparable to that of the corresponding non-synthetic viruses.

Conclusions In the event of an influenza pandemic, the use of these approaches may significantly reduce the time required to generate and distribute the vaccine seed virus and vaccine manufacture. These approaches also offer the advantage of not needing to handle wild-type virus, potentially diminishing biocontainment requirements.

Keywords Influenza vaccine, pathogenic virus, synthetic vaccines.

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Background

The spread of novel influenza viruses amongst susceptible bird and animal populations presents an increased risk for a potentially devastating human pandemic. This has been demonstrated by the recent H1N1 pandemic^{1,2} and the continuing spread of the more pathogenic H5N1 influenza strains.³ Surveillance of H5N1 viruses isolated from infected humans or circulating in bird populations has revealed at present 10 genetically distinct clades.⁴ Outbreaks of H7N2, H7N3, H7N7 and H9N2 influenza viruses in bird populations have demonstrated transmissibility to humans, in some cases with fatal results,⁵ emphasising the unpredictable nature of future influenza pandemics.

With the ongoing evolution and diversification of influenza viruses, it is not possible to predict which viruses will be antigenically closest to an eventual pandemic strain. In response to a potential H5N1 pandemic, a number of representative candidate vaccine viruses have been

recommended for pre-pandemic vaccine manufacture and stockpiling.⁴

The time taken to develop appropriate seed viruses is of key importance for the efficient development of pandemic vaccines. As the majority of influenza vaccines are manufactured in embryonated chicken eggs, influenza A vaccine candidate viruses are often egg-adapted and reassorted with high-growth parents to optimise growth in eggs. To facilitate growth in eggs and to improve the safety profile of potentially pathogenic vaccine candidates, mutations such as removal of the polybasic region in the haemagglutinin (HA) of H5N1 viruses must be introduced to remove regions of the viral genome that increase pathogenicity.^{6–9} The vaccine candidate strain is produced using reverse genetics, using the mutated HA and neuraminidase (NA) of the candidate strain with the backbone genes of the egg-adapted high-growth strain A/Puerto Rico/8/34.^{10–13} These reverse genetics-derived viruses are distributed to influenza vaccine manufacturers who prepare seed stocks of these

viruses. Vaccine can then be produced as required for distribution or stockpiling.

To optimise the protection of populations from infection by pandemic influenza, it is desirable to rapidly produce vaccine derived from the relevant pandemic strain. Using egg-based reassortment, preparation of the vaccine candidate strain takes a minimum of approximately 21 working days.¹³ The use of HA and NA DNA cloned from a wild-type isolate to generate a virus using reverse genetics can be significantly faster – for example, a recent study has demonstrated the generation of H1N1 and H3N2 viruses within 9–12 days using multi-segment RT-PCR.¹⁴ However, the time required to produce a seed virus from which to manufacture vaccine may be affected by a number of factors. This includes the requirement to introduce mutations into a candidate virus either to improve its growth in eggs or to increase its safety profile. Distribution of vaccine candidate strains may be adversely affected as a result of transport delays, including any transport restrictions put in place following declaration of a pandemic of a novel influenza virus. This is particularly relevant for geographically isolated locations, such as Australia where our laboratory is situated. Further, legal issues regarding the supply of pandemic vaccine candidate viruses may also hinder the production and distribution of candidate vaccine strains.

In this study, we investigated two methods to synthetically generate H5N1 and pandemic (H1N1) 2009 candidate vaccine strains. Firstly, we used site-directed mutagenesis to generate the HA and NA of the candidate virus A/Bar Headed Goose/Qinghai/1A/2005 (H5N1) from the HA and NA of A/Vietnam/1194/2004 (NIBRG-14). Secondly, we use synthetic DNA encoding the HA of A/Whooper Swan/Mongolia/244/05(H5N1), and the HA and NA of A/California/04/09 (H1N1) to generate these viruses, respectively. We show that the replication and the antigenic profile of the synthetic H5N1 and H1N1 viruses were similar to those of viruses prepared by current methods.

Materials and methods

Cells

293T cells were maintained in DMEM (Invitrogen, Carlsbad, CA, USA) containing 10% foetal bovine serum (SAFC), 2 mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B (SAFC). MDCK cells were maintained in EMEM (Invitrogen) containing 10% foetal bovine serum, 2 mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B. For reverse genetics experiments, six-well plates were seeded with a coculture of 293T

and MDCK cells and were used for transfection when approximately 70% confluent.

Viruses

A/Vietnam/1194/2004 (H5N1; NIBRG-14)¹³ was obtained from NIBSC (Potters Bar, UK). Reverse genetics A/Bar Headed Goose/Qinghai/1A/2005 (St Jude H5N1 influenza seed virus 163222) and A/Whooper Swan/Mongolia/244/2005 (St Jude H5N1 influenza seed virus 163243) were provided by St. Jude's Children's Research Hospital (Memphis, TN, USA). Wild-type A/California/4/09 (H1N1) and A/California/7/09 (H1N1) were supplied by WHO Collaborating Centre for Reference and Research on Influenza. Egg A/California/7/09 reassortants with A/PR/8/34 were generated at CSL Ltd (IVR-153) or at the New York Medical Centre (X-179A). A/mallard/South Korea/12A/2006 and A/chicken/Lao/26/2006 were supplied by the Australian Animal Health Laboratories. Reverse genetics viruses A/Anhui/1/2005 (A/Anhui/1/2005-PR8-IBCDC-RG6) and A/Indonesia/5/2005 (A/Indonesia/05/2005-PR8-IBCDC-RG2) were obtained from CDC (Atlanta, GA, USA). Virus stocks were prepared in 10- to 12-day-old embryonated chicken eggs and stored at –80°C until required.

Site-directed mutagenesis

Viral RNA was extracted from the allantoic fluid of embryonated chicken eggs infected with NIBRG-14, using a QIA-amp viral RNA mini kit (Qiagen, Hilden, Germany). cDNA of NIBRG-14 HA and NA genes was generated by reverse transcription using Superscript II reverse transcriptase (Invitrogen), with 5' TCGAGGTCTCCGCCAGCAAAGCAGGGTTCCAATC 3' (HA) and 5' TCGACGTCTCCGCCAGCAAAGCAGGAGTTTAAA 3' (NA) primers (Geneworks, Thebarton, SA, Australia). For subcloning, cDNA was amplified using Pfx polymerase (Invitrogen), with either 5' TCGAGGTCTCCGGGAGCAAAGCAGGGTTCCAATC 3' and 5' CGATGGTCTCGTATTAGTAGAAACAAGGGTGTTTTTAAC 3' (HA) or 5' TCGACGTCTCCGGGAGCAAAGCAGGAGTTTAAA 3' and 5' CGATCGTCTCGTATTAGTAGAAACAAGGAGTTTTTTTGAAC 3' (NA) primer pairs. The amplified cDNA was digested with *Bsa*I (New England Biolabs, Ipswich, MA, USA) and ligated into the pHW2000 vector¹⁰ using T4 DNA ligase (Promega, Madison, WI, USA).

Site-directed mutagenesis was performed on the NIBRG-14 HA and NA plasmids, to match the amino acid coding regions of A/Bar Headed Goose/Qinghai/1A/2005 (St Jude H5N1 influenza seed virus 163222, GenBank accession numbers DQ659327 and DQ659325, for HA and NA, respectively), using a QuikChange II Site-Directed Mutagenesis kit (Stratagene, Cedar Creek, TX, USA) as described in Table 1a (HA) and b (NA). Plasmid sequences were confirmed by sequencing (Micromon, Vic., Australia).

Table 1 (a) Site-directed mutagenesis of NIBRG-14 HA to produce A/Bar Headed Goose/Qinghai/1A/2005 HA. (b) Site-directed mutagenesis of NIBRG-14 NA to produce A/Bar Headed Goose/Qinghai/1A/2005 NA

Site	Forward primer	Reverse primer
(a)		
F8L	aatagtcttcttctgcaatagtcagtc	gactgactattgcaagaagaagcactatt
I86L	gtgtgacgaattcctcaatgtgccggaatgg	ccattccgacattgaggaattcgacacac
A99I	catagtgagagaagatcaatccagccaatgacctctgttac	gtaacagaggtcattggctggattgattcttccactatg
V102A	catagtgagagaagatcaatccagccaatgacctctgttac	gtaacagaggtcattggctggattgattcttccactatg
D110N	ctctgttaccagggaattcaatgactatg	catagtcattgaaattccctgggtaacagag
S140D	caaaagtcttggctcgatcatgaagcctcatcagggggtgagctc	gagctcaccctgatgaggctcatgatcggaaccaagaactttg
L145S	caaaagtcttggctcgatcatgaagcctcatcagggggtgagctc	gagctcaccctgatgaggctcatgatcggaaccaagaactttg
K155R	ccataccagggaaggtcctctttttc	gaaaaaggaggacctccctggatgg
S171N	caaaaagaacaatgcataccaaca	tgttgggtatgcattgtctttttg
T172A	caaaaagaacaatgcataccaaca	tgttgggtatgcattgtctttttg
K205R	cagagcagacaaggctctatcaaaaacc	gggtttgatagagccttctgctctg
R228K	gattggtaaccaaaaatagctactgatc	gatctagtagctattttgtaccaatc
Y268N	cattgctccagaaaatgcatacaaaattg	caatttgtatgcaatttctggagcaatg
M298I	gtcaaaactccaatagggcgataaac	gtttatcgccctattggagtttgac
R339I	gaaatagccctcaaatgagacgcgaggattatttg	ccaaataatcctcgctcctcaatttgagggtatttc
K489R	gagttctatcatagatgtgataatg	cattatcacatcatgatagaactc
I530T	gaatcaataggaacttaccaaatattg	caatatttggtaagtctcattgattc
(b)		
T17I	caatctgtatggaattggaatagttagctta	taagctaactattccaattaccatacagattg
H39Q	cattcaattcagacaggaatcaacgccaagctgaaccaatc	gattggtcagcttggcgttgattccctgtctgaattgaatg
H44R	cattcaattcagacaggaatcaacgccaagctgaaccaatc	gattggtcagcttggcgttgattccctgtctgaattgaatg
S46A	cattcaattcagacaggaatcaacgccaagctgaaccaatc	gattggtcagcttggcgttgattccctgtctgaattgaatg
N53K	cagcaataactaaatttctactagaaagc	gctttctcagtaagaatttagattgctg
L54F	cagcaataactaaatttctactagaaagc	gctttctcagtaagaatttagattgctg
K64T	ctgtggcttcagtaacattagcgggcaattc	gaattgcccctaagtactgaagccacag
N75S	ctttgccccattagcggatgggctgta	tacagccatccgctaaggggcaaag
K91R	taaggatcggttccagggggatgtgtttg	caaacacatccccctggaaccgatcctta
H233Y	atggtcaggcatcataaagatcttcaaaatg	ccattttgaagatcttatgatgcctgacct
S320P	gtccgggtgccctaacggggcatatggggtaaaaggg	cccttttaccatagccccgttaggggacaccggac
G324Y	gtccgggtgccctaacggggcatatggggtaaaaggg	cccttttaccatagccccgttaggggacaccggac
E362G	ccaaatgggtggactggaacggacagcagc	gctgctgctcgttccagtcacccaattgg
G434S	tagtgacactgtgagttggtcttgccc	ggccaagaccaactcacagtgtcacta

Plasmids

Plasmids encoding each of the 8 genes of A/Puerto Rico/8/34 in the pHW2000 vector, as well as the empty vector, were obtained under licence from MedImmune (MedImmune, MD, USA). For reverse genetics experiments, plasmid DNA was diluted to a concentration of 100 ng/ μ l prior to transfection.

Synthetic DNA

The HA gene of A/Whooper Swan/Mongolia/244/2005 (St Jude H5N1 influenza seed virus 163243, GenBank accession DQ659326) was synthesised and cloned into pMK (Geneart, Regensburg, Germany). The HA gene was synthesised flanked by *BsmB1* restriction sites, lacking a polybasic region, and contained the modification made to the upstream arginine (AGA to CGA) described by Subbarao *et al.*,¹¹ to minimise the likelihood of reversion to a

polybasic site upon passage. Following receipt of the plasmid, pMK-WhooperSwan-HA, the modified HA gene was excised and subcloned into pHW2000 (pHW-WhooperSwan-HA). Integrity of the A/Whooper Swan/Mongolia/244/2005 HA gene was confirmed by sequencing across the HA coding region.

Synthetic genes encoding the HA and NA proteins of A/California/04/2009 (H1N1; GenBank accession numbers FJ966082 and FJ966084, respectively) flanked by *BsmB1* restriction sites were synthesised (DNA2.0, CA, USA) and subcloned into pHW2000 as described above (pHW-Cali/4/09-HA and pHW-Cali/4/09-NA). A Q223R mutation was introduced into pHW-Cali/4/09-HA by PCR amplification with 5' CAATAAGACCCAAAGTGAGGGA-TCGAGAAGGG 3' and 5' CTATTTCCGGCTTGAACCTC-TTGCTGTATCTTGAGT 3' primers followed by blunt-ended ligation.

Reverse genetics

Reverse genetics to produce influenza viruses from an eight-plasmid system has been previously described.¹⁰ Briefly, 18 μl of TransIT-293 (Mirus) was added to 250 μl of OptiMEM medium (Invitrogen) and incubated at room temperature for 15 minutes. Plasmid DNA (consisting of 1 μg each of the appropriate HA and NA cDNA genes, and the six remaining backbone genes of A/PR/8/34) was combined before addition to the diluted TransIT-293, and the mixture was incubated for 20 minutes at room temperature. Transfection complexes were added to a well of a six-well plate containing a coculture of 293T and MDCK cells in transfection medium (OptiMEM containing 2 mM l-glutamine, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and 0.25 $\mu\text{g}/\text{ml}$ amphotericin B) and incubated at 35°C and 5% CO₂. At 24-hour post-transfection, 1 ml of transfection medium containing 2 $\mu\text{g}/\text{ml}$ of TPCK-trypsin (Worthington, Lakewood, NJ, USA) was added. Cells were incubated for a further 2 days before the harvest of transfection supernatants.

Propagation of virus in eggs

For virus rescue, 10- to 12-day-old specific pathogen-free (SPF) eggs were inoculated with 100–200 μl of transfection supernatant and incubated at 35°C and 60% humidity for 48 hours. Eggs were chilled overnight at 4°C, before allantoic fluid was harvested and HA assays were performed by incubation of serial twofold dilutions of virus with 2 volumes of 0.5% chicken red blood cells. For virus propagation, allantoic fluid from infected embryonated eggs was diluted in calcium- and magnesium-free PBS (1 in 10⁵ to 1 in 10⁷, as indicated) and was used to inoculate SPF eggs at 200 $\mu\text{l}/\text{egg}$. Non-SPF eggs were used for virus growth comparisons. For growth comparison of swine H1N1 influenza viruses, for each virus, 36 non-SPF eggs were inoculated with 200 $\mu\text{l}/\text{egg}$ of egg-derived seed virus diluted 1 in 10⁵ in PBS, to mimic the conditions used for manufacture of influenza virus vaccine. Eggs were incubated for 2 days at 35°C, chilled, and the HA titre of allantoic fluid pooled from all viable eggs was measured.

Haemagglutinin inhibition (HAI) assays

Viruses were diluted to 4–8 HA units/25 μl , and 25 μl was added to serial twofold dilutions of receptor destroying enzyme (RDE; Seiken, Japan)-treated ferret antisera or monoclonal antibodies 151, 165, 166 and 168 (CSL) in a 96-well V-bottom plate (starting dilution 1 in 20 for all sera and antibodies). The virus/antiserum mixture was incubated at room temperature for 1 hour, before 25 μl of 1% turkey, horse or chicken red blood cells was added to each well as specified. Plates were incubated at room temperature for 30 minutes, and the haemagglutination inhibi-

tion titre was calculated to be the highest antibody dilution at which haemagglutination was inhibited. For H5N1 assays, the ferret antisera used for HAI assays were collected from ferrets infected with the attenuated H5N1 strains NIBRG-14, A/Anhui/1/2005, A/Indonesia/5/2005 or A/Bar Headed Goose/Qinghai/1A/2005 viruses at 21 days post-infection, and from an uninfected ferret. The H5N1 mouse monoclonal antibodies were raised against NIBRG-14 (mAb 151) or A/Bar Headed Goose/Qinghai/1A/2005 (mAbs 165, 166, 168). For swine H1N1 influenza haemagglutinin assays, antisera were collected from ferrets infected with wild-type A/California/07/2009 (H1N1), A/Auckland/1/2009 (H1N1), A/Brisbane/2013/2009 (H1N1), and the swine-derived human isolate A/Philippines/344/2004 (H1N2).¹⁵ Seronegative control sera were collected from uninfected ferrets.

Pathogenicity testing

Eight healthy 5-week-old SPF chickens were challenged intravenously with 200 μl of allantoic fluid infected with CSLRG63 (synthetic H5N1 A/Whooper Swan/Mongolia/244/2005), diluted 1 in 10 in PBS. Control groups of eight chickens each were challenged with either an HPAI H5N1 strain (A/chicken/Lao/26/2006) or an LPAI H5N2 strain (A/mallard/South Korea/12A/2006). Birds were observed daily for clinical signs, and an intravenous pathogenicity index (IVPI) score was given to determine pathogenicity. The birds were euthanised following display of clear signs of illness, or at 10 days post-infection.

Results

Two different approaches were used to produce vaccine candidate viruses from synthetic DNA. Firstly, the HA and NA of NIBRG-14 (recombinant A/Vietnam/1194/2004 (H5N1)) were mutated to match the amino acid sequences of A/Bar Headed Goose/Qinghai/1A/2005 (H5N1), using site-directed mutagenesis. Secondly, DNA encoding the HA gene of A/Whooper Swan/Mongolia/244/2005 (H5N1) or the HA and NA of A/California/04/09 (H1N1) were synthesised and cloned into the pHW2000 vector for reverse genetics. Both approaches are summarised in Figure 1 and compared to the current approach in which the HA and NA genes are amplified from wild-type strains in a reference laboratory and cloned into the pHW2000 vector. For H5N1 viruses, the HA was mutated to remove the polybasic region, and virus was prepared and analysed.

Synthesis and growth of A/Bar Headed Goose/Qinghai/1A/2005 (H5N1)

Site-directed mutagenesis was used to mutate plasmids encoding the HA and NA genes of NIBRG-14 to match

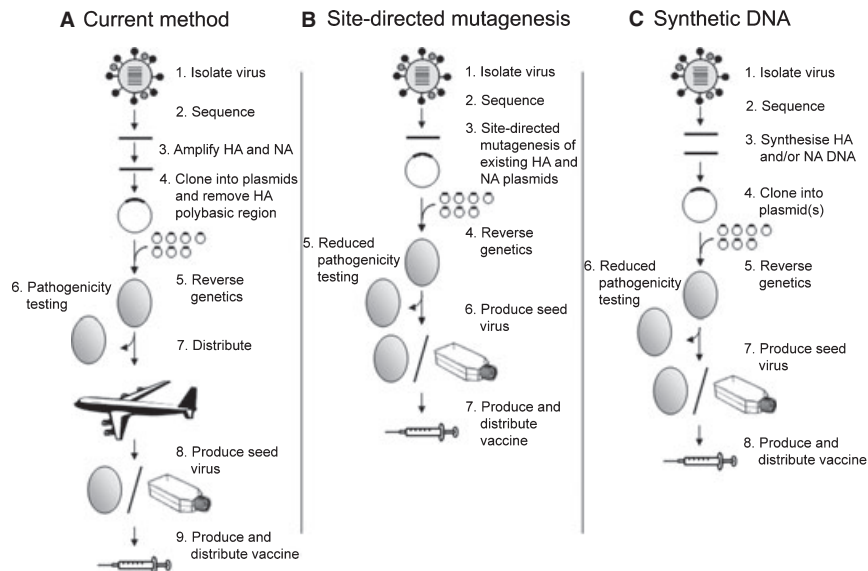


Figure 1. Steps required for the generation of influenza virus vaccine candidates using reverse genetics. In the current method (A), viral HA and neuraminidase (NA) are isolated from wild-type virus, cloned into plasmid DNA for reverse genetics, and the polybasic region is removed before the candidate strain is produced using reverse genetics and distributed to vaccine manufacturers. Alternatively, vaccine manufacturers may produce plasmids for reverse genetics on-site by site-directed mutagenesis of the template DNA of a related strain (B), or using synthetic DNA encoding the HA and NA of the vaccine candidate strain with the polybasic region already removed (C).

the amino acid sequence of A/Bar Headed Goose/Qinghai/1A/2005. A total of 17 changes were made to the HA amino acid sequence (21 nucleotides), and 14 for NA (15 nucleotides). The synthetic HA and NA coding regions differed from those of A/Bar Headed Goose/Qinghai/1A/2005 (St Jude H5N1 influenza seed virus 163222) by 38 nucleotides for HA and 20 nucleotides for NA. The resulting plasmids were then used in reverse genetics to produce a virus, CSLRG29, containing the HA and NA of A/Bar Headed Goose/Qinghai/1A/2005 and the 6 remaining backbone genes of A/PR/8/34. The rescued virus was passaged twice in embryonated SPF chicken eggs. No atypical egg deaths were observed.

The growth of CSLRG29 was then compared to that of the reverse genetics vaccine candidate virus A/Bar Headed Goose/Qinghai/1A/2005. Stocks of each virus were diluted 1 in 10^5 and used to inoculate embryonated eggs (12 eggs per virus, 200 μ l/egg). Viruses were diluted in this manner to mimic the conditions used for the production of influenza virus vaccine. Following the harvest of allantoic fluid, viral titres were measured by HA assay. We demonstrated that replication of CSLRG29 (230 ± 42 HAU) was similar to that of non-synthetic A/Bar Headed Goose/Qinghai/1A/2005 (237 ± 14 HAU) (values represent standard mean \pm SEM).

Antigenicity of CSLRG29 (A/Bar Headed Goose/Qinghai/1A/2005)

The antigenicity of CSLRG29 was compared to that of A/Bar Headed Goose/Qinghai/1A/2005 and other H5N1

viruses using HAI assays. A panel of ferret antisera, raised against a number of reverse genetics H5N1 vaccine candidate viruses, was used as well as monoclonal antibodies raised against NIBRG-14 (mAb 151) and A/Bar Headed Goose/Qinghai/1A/2005 (mAbs 165, 166 and 168). These results showed similar HAI profiles for CSLRG29 and A/Bar Headed Goose/Qinghai/1A/2005, although differences in reactivity were observed for both the NIBRG-14 and A/Indonesia/5/2005 antisera, as well as the mAb 151, which was raised against NIBRG-14 (Table 2). Some differences were also seen for NIBRG-23 (A/turkey/Turkey/1/2005), which is a clade 2.2 virus like A/Bar Headed Goose/Qinghai/1A/2005. It is unknown why CSLRG29 reacted differently to the wild-type virus, as sequencing revealed no changes to the amino acid sequence of the synthetic virus. However, we have also observed such differences on occasions with conventionally derived influenza A reassortants. Overall, CSLRG-29 was antigenically most similar to viruses that were tested from clade 2.2, which includes A/Bar Headed Goose/Qinghai/1A/2005.

Synthesis and growth of synthetic A/Whooper Swan/Mongolia/244/2005 (H5N1)

Synthetic DNA encoding the HA of A/Whooper Swan/Mongolia/244/2005 was cloned into pHW2000 and used to generate a virus, CSLRG63, by reverse genetics. The synthetic A/Bar Headed Goose/Qinghai/1A/2005 NA was used, which differs from the amino acid sequence of A/Whooper Swan/Mongolia/244/2005 NA (GenBank

Table 2. Haemagglutination inhibition analysis of synthetic A/Bar Headed Goose/Qinghai/1A/2005 (CSLRG29)

Virus	H5N1 ferret antisera					H5N1 mouse monoclonal antibodies			
	NIBRG-14	A/BHG	A/Anhui	A/Indo	Seroneg	151	165	166	168
NIBRG-14	640	20	160	40	< 20	> 2560	640	> 2560	20
A/BHG	160	> 2560	80	80	< 20	< 20	1280	> 2560	1280
A/Anhui	80	40	1280	160	< 20	> 2560	< 20	< 20	< 20
A/Indo	40	160	640	1280	< 20	1280	< 20	< 20	< 20
NIBRG-23	80	640	160	640	< 20	160	1280	> 2560	1280
CSLRG29	40	1280	80	320	< 20	640	1280	> 2560	640

H5N1 viruses: NIBRG-14 – A/Vietnam/1194/2004; A/BHG – RG A/Bar Headed Goose/Qinghai/1A/2005 (St Jude H5N1 influenza seed virus 163222); A/Anhui – A/Anhui/1/2005 (IBCDC RG-6); A/Indo – A/Indonesia/5/2005 (IBCDC RG-2); NIBRG-23 – A/turkey/Turkey/1/2005; CSLRG29 – synthetic A/Bar Headed Goose/Qinghai/1A/2005. Seroneg – serum from uninfected ferret. Monoclonal antibody 151 anti-NIBRG-14; 165, 166, and 168 anti-A/Bar Headed Goose/Qinghai/1A/2005. Assay performed using 1% horse RBC. Bold values represent paired virus/antisera raised against the same virus.

accession DQ659324) by 21 nucleotides and 1 amino acid (N228S), as well as the six backbone genes of A/PR/8/34. CSLRG63 was passaged a total of 4 times in SPF eggs, with the HA titre remaining relatively stable and no atypical egg deaths observed (data not shown).

The replication of CSLRG63 was compared to that of non-synthetic A/Whooper Swan/Mongolia/244/2005 by inoculation of 24, 12-day-old embryonated chicken eggs with 200 μ l of allantoic fluid per egg (diluted 1 in 10⁵ or 1 in 10⁶ in PBS). Replication of CSLRG63 (217 \pm 22 HAU) was similar to that of A/Whooper Swan/Mongolia/244/2005 (200 \pm 31 HAU) (values represent standard mean \pm SEM).

Comparison of the antigenicity of CSLRG63 to A/Whooper Swan/Mongolia/244/2005 in HAI assays revealed the strains were essentially identical, when a panel

of ferret antisera and the mouse anti-NIBRG-14 monoclonal antibody 151 were used (Table 3).

Pathogenicity of synthetic CSLRG63 (A/Whooper Swan/Mongolia/244/2005)

Influenza vaccine candidate viruses derived from wild-type viruses have been required to be tested for pathogenicity to ensure they have been sufficiently attenuated for use in vaccine manufacture. We tested CSLRG63 for pathogenicity by obtaining an IVPI score from chickens over 10 days following infection. Chickens challenged with CSLRG63 or the LPAI H5N2 isolate A/Mallard/South Korea/12A/2006 appeared healthy for 10 days post-challenge, after which they were euthanised, recording an IVPI of 0. In contrast, all chickens infected with the HPAI H5N1 isolate A/chicken/Lao/26/2006 either died or were euthanised within

Table 3. Haemagglutination inhibition analysis of synthetic A/Whooper Swan/Mongolia/244/2005 (CSLRG63)

	H5N1 ferret antisera					
	NIBRG-14	A/BHG	A/Anhui	A/Indo	Seroneg	mAb 151
NIBRG-14	320	\leq 20	80	\leq 20	\leq 20	\geq 2560
A/BHG	160	640	80	160	\leq 20	\leq 20
A/Anhui	80	40	320	80	\leq 20	960
A/Indo	\leq 20	80	120	640	\leq 20	640
NIBRG-23	80	480	80	320	< 20	\leq 20
A/Whooper	40	480	60	240	< 20	< 20
CSLRG63	40	480	40	240	< 20	< 20

H5N1 viruses: NIBRG-14 – A/Vietnam/1194/2004; A/BHG – RG A/Bar Headed Goose/Qinghai/1A/2005 (St Jude H5N1 influenza seed virus 163222); A/Anhui – A/Anhui/1/2005 (IBCDC RG-6); A/Indo – A/Indonesia/5/2005 (IBCDC RG-2); NIBRG-23 – A/turkey/Turkey/1/2005; A/Whooper – A/Whooper Swan/Mongolia/244/2005 (St Jude H5N1 influenza seed virus 163243); CSLRG63 – synthetic A/Whooper Swan/Mongolia/244/2005. Seroneg – serum from uninfected ferret. Monoclonal antibody 151 anti-NIBRG-14. Assay performed using 1% turkey RBC. Bold values represent paired virus/antisera raised against the same virus.

24 hours post-challenge, recording an IVPI of 3.0. These results indicate that the synthetic A/Whooper Swan/Mongolia/244/2005 virus can be classified as an LPAI virus according to the clinical criteria cited by the World Organisation for Animal Health.¹⁶

Synthesis of A/California/04/09 and A/California/07/09 (H1N1)

Initial attempts at rescue of synthetic A/California/04/09 and A/California/07/09 viruses with A/PR/8/34 backbone genes using reverse genetics in Vero cells were unsuccessful. Sequence analysis of egg reassortant viruses revealed the presence of a mutation in viral HA, Q223R (personal communication, CDC). Introduction of this mutation into the coding sequence of the synthetic A/California/04/09 and A/California/07/09 HA genes resulted in successful rescue of both viruses (CSLRG101 and CSLRG110B, respectively) in eggs using reverse genetics, following transfection of co-cultured 293T and MDCK cells. Replication of both reverse genetics viruses was decreased compared to the A/California/07/2009 egg reassortant virus X-179A. However, upon introduction of a second mutation observed in X-179A, K209T, into the HA gene of both reverse genetics viruses (CSLRG99 and CSLRG112 for A/California/04/09 and A/California/07/09, respectively), replication was comparable to that of the reassorted virus (Figure 2). For all reverse genetic swine influenza viruses, HA titres remained relatively stable over multiple (3 or 4) passages in eggs.

Analysis of the antigenicity of the A/California/04/2009 and A/California/07/2009 reverse genetics viruses (CSLRG99, CSLRG101, CSLRG110B, CSLRG112) in HAI assays, compared to the A/California/07/2009 reassortants

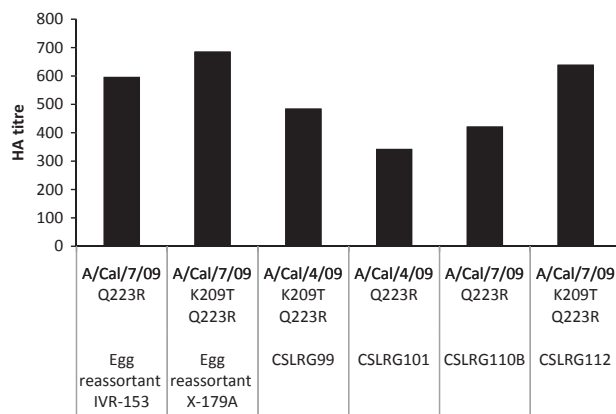


Figure 2. Growth comparison of reassortant and reverse genetics swine influenza viruses in eggs. Bars represent HA activity in allantoic fluid pooled from 36 eggs for each virus. Results are from a single experiment representative of two separate growth comparisons and data accumulated from serial passage of each virus up to 4 times in eggs.

IVR-153 and X-179A, showed very similar antigenic profiles for all viruses (Table 4).

Discussion

In this study, we successfully generated three influenza vaccine candidate strains using two different approaches involving the use of synthetic DNA for the production of virus by reverse genetics. For all three viruses, the replication and antigenicity of the synthetic virus were found to be very similar to the equivalent virus produced using egg-based reassorting or cloning of the HA and NA coding regions into a suitable vector for rescue by reverse genetics.

In the first approach, site-directed mutagenesis is used to produce the HA and NA of the vaccine candidate strain using as a template a strain that is relatively closely related. In this case, the HA and NA of A/Bar Headed Goose/Qinghai/1A/2005 (H5N1 clade 2.2) were produced using NIBRG-14 (A/Vietnam/1194/2004; H5N1 clade 1) as a template, which required considerable changes to HA and NA [17 changes to the amino acid sequence (21 nucleotide changes) of HA and 14 amino acid (15 nucleotide) changes for NA]. This approach requires that the HA and NA of the template strain are first cloned into an appropriate vector, and the polybasic region of HA removed. In this instance, mutations were introduced individually, but the process could be significantly expedited by combining mutagenesis reactions. This approach is ideally suited to produce candidate viruses that are closely related to a pre-existing virus isolate. Under this scenario, the rate-limiting steps for generating a synthetic virus are the number of mutations that need to be generated and the time required to design and manufacture primers required for mutagenesis once the sequence of a vaccine candidate strain is known. However, if several rounds of mutagenesis are required, or if the candidate vaccine strain is not closely related to pre-existing virus isolates, our second synthetic gene approach provides a faster and more efficient method to produce a synthetic candidate virus.

The second approach investigated in this study involves the cloning of synthetic strands of DNA encoding HA and/or NA into a suitable vector, for use in reverse genetics to produce the candidate vaccine strain. In this case, a synthetic strand of DNA encoding the HA of A/Whooper Swan/Mongolia/244/2005 (H5N1) was purchased and subsequently cloned into the pHW2000 vector. The NA of A/Bar Headed Goose/Qinghai/1A/2005 (H5N1), which differs from that of A/Whooper Swan/244/2005 by a single amino acid, was used to generate virus using reverse genetics. In addition, a synthetic A/California/04/09 (H1N1) virus was generated using this method, using the synthetic approach for both HA and NA genes. This approach is suitable for the production of any vaccine

Table 4. Haemagglutinin inhibition analysis of A/California/04/2009 and A/California/07/2009 reverse genetics and reassortant viruses

Virus	Mutations	Ferret antisera				Seroneg	
		A/Cal	A/Auck	A/Bris	A/Phil		
IVR-153	A/Cal/7/09	R	5120	1280	5120	1280	< 20
X-179A	A/Cal/7/09	T, R	5120	1280	10240	1280	< 20
CSLRG99	A/Cal/4/09	T, R	5120	1280	5120	1280	< 20
CSLRG101	A/Cal/4/09	R	5120	1280	5120	1280	< 20
CSLRG110B	A/Cal/7/09	R	2560	640	2560	640	< 20
CSLRG112	A/Cal/7/09	T, R	2560	640	2560	640	< 20

Mutations: T – K209T; R – Q223R. Ferret antisera: A/Cal – A/California/07/2009 (H1N1); A/Auck – A/Auckland/1/2009 (H1N1); A/Bris – A/Brisbane/2013/2009 (H1N1); A/Phil – A/Philippines/344/2004 (H1N2); Seroneg – uninfected. Assay performed using 1% chicken red blood cells.

candidate strain, including H2, H7 and H9 viruses. Here, the rate-limiting step is the time required to manufacture the synthetic DNA. Following receipt of DNA, the synthetic gene can then be rapidly cloned into a vector and used to generate the vaccine candidate strain using reverse genetics. An advantage of this approach to the generation of synthetic vaccine candidate viruses is that mutations such as the removal of the polybasic region of the HA gene can be introduced prior to gene synthesis, thus minimising any mutagenesis steps that may be required. Whilst all of the synthetically derived viruses appeared to be antigenically similar to the reverse genetics viruses produced by classical RG techniques using one-way HAI analysis, if these viruses were to be used to produce a human vaccine, it would be necessary to perform a two-way HAI analysis, to compare reactivity of the test and reference viruses with ferret antisera raised against the synthetic reverse genetics viruses. It would also be necessary to perform vaccination trials in both experimental animals and humans, prior to use as a licensed vaccine. However, these extra analyses were beyond the scope of this study.

In the event of a severe influenza pandemic, it is possible that there may be significant delays in the production and distribution of a vaccine candidate strain. The reassorted and attenuated pandemic strain will need to be produced, possibly using reverse genetics, tested for pathogenicity to ensure safety and distributed to influenza virus vaccine manufacturers worldwide. However, it is possible that distribution of viruses may be significantly impeded by restrictions in travel and transport that are likely to be put in place should such a pandemic occur, in particular for regions outside the US. For regions such as Australia, where our laboratory is situated, it may be the case that the HA and NA sequences will be available to begin DNA synthesis before the wild-type pandemic strain has been received to begin the reassorting process, as was the case for the 2009 swine influenza pandemic. The use of either of the

approaches investigated here for the production of vaccine candidate strains will provide a way of bypassing these delays, as the requirement for transport will be greatly reduced. This approach allows influenza reference laboratories to rapidly produce vaccine candidate strains on-site once the sequence of the pandemic strain is available. Although in the cases demonstrated here we used cell lines not validated for the purpose of vaccine production (293T and MDCK cells), for vaccine manufacture candidate strains must be produced in certified and licensed cells lines, such as Vero cells, which have been shown to be free from adventitious agents and to be suitable for the production of influenza viruses through reverse genetics to be used in human influenza vaccines.^{12,13,17} In addition, because pathogenic wild-type viruses may only be manipulated in a limited number of laboratories worldwide, owing to the high level of biosafety required, our approaches allow for the production of attenuated vaccine candidate viruses in any laboratory approved for the growth of influenza viruses.

For the case of A/California/04/2009, the initial experiments described here comprised part of our early response to the pandemic. Synthetic DNA was ordered on 27 April 2009, one day after WHO selection of vaccine candidate viruses. The synthetic HA gene was received here in Australia 7 days later on May 4, only hours before the arrival of A/California/04/2009 and A/California/07/2009 wild-type viruses. The A/California/04/2009 synthetic NA gene was received on May 7. Cloning of both the synthetic HA, and the HA and NA amplified from RNA extracted from the wild-type viruses ('wild-type' HA and NA), into the pHW2000 plasmid for transfection, as well as the mixed infection of the egg reassorting process, all commenced on May 4. By working extended hours, the synthetic HA plasmid was ready to transfect as a 7:1 reassortant with the A/PR/8/34 backbone genes on May 6; the wild-type HA and NA were transfected on May 7; and the synthetic HA and NA were transfected on May 9. Allantoic fluid was

harvested from eggs inoculated with transfection supernatant on May 11, 12 and 14 for the synthetic 7:1 reassortant, wild-type 6:2 reassortant and synthetic 6:2 reassortant reverse genetics viruses, respectively. A further 2–3 days would have been required for the preparation of a virus seed lot. Unfortunately, in this case, virus generation was not successful owing to the absence of egg adaptation mutations in HA that were observed to occur upon multiple egg passages during reassortment. Seed virus derived from egg reassortment of A/California/07/2009 (IVR-153) was prepared on May 25, 21 days after receipt of wild-type virus.

The initial failure to rescue reverse genetic A/California/04/2009 was rectified upon introduction of the egg adaptation mutations K209T and Q223R that were observed in the egg reassortant virus. This highlights one of the significant problems that may occur with novel viruses using this method. Although for future synthetic viruses the mutations identified here can be corrected prior to DNA synthesis, it is possible that similar, unidentified mutations may be present which would prevent replication of synthetic virus, but may adapt in wild-type isolates upon egg passage during the traditional reassorting process. The time period shown here for generation of a reverse genetics virus derived from a wild-type isolate supports the 9–12 days recently shown by Zhou *et al.*¹⁴; however, this does not allow for the time potentially required for further manipulation of viral genes, such as removal of the polybasic region from the HA gene of an H5N1 isolate.

We demonstrate that the use of synthetic DNA, or site-directed mutagenesis of existing template DNA, provides alternative approaches to the rapid generation of influenza virus vaccine candidate strains. This is particularly relevant to cases where transport of wild-type virus is delayed or for high-pathogenicity isolates requiring high-level containment facilities for the wild-type virus. This study indicates that the time from receipt of DNA to rescue of reverse genetics virus in eggs may be expedited to approximately 7 days, significantly shorter than the 21 days required to generate a reassortant virus in eggs. These methods have the capacity to reduce the risks associated with the development of appropriate vaccine candidate strains, and the preparation time required to manufacture a vaccine in the event of an influenza pandemic.

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Conflict of interest statement

Authors Erin Verity, Chi Ong, Catherine Agius, Sarina Camuglia, and Steve Rockman are employees of CSL Ltd. CO, CA, SC, and SR also hold shares in CSL Ltd.

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