

Generation of candidate human influenza vaccine strains in cell culture – rehearsing the European response to an H7N1 pandemic threat

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Background Although H5N1 avian influenza viruses pose the most obvious imminent pandemic threat, there have been several recent zoonotic incidents involving transmission of H7 viruses to humans. Vaccines are the primary public health defense against pandemics, but reliance on embryonated chickens eggs to propagate vaccine and logistic problems posed by the use of new technology may slow our ability to respond rapidly in a pandemic situation.

Objectives We sought to generate an H7 candidate vaccine virus suitable for administration to humans whose generation and amplification avoided the use of eggs.

Methods We generated a suitable H7 vaccine virus by reverse genetics. This virus, known as RD3, comprises the internal genes of A/Puerto Rico/8/34 with surface antigens of the highly pathogenic avian strain A/Chicken/Italy/13474/99 (H7N1). The

multi-basic amino acid site in the HA gene, associated with high pathogenicity in chickens, was removed.

Results The HA modification did not alter the antigenicity of the virus and the resultant single basic motif was stably retained following several passages in Vero and PER.C6 cells. RD3 was attenuated for growth in embryonated eggs, chickens, and ferrets. RD3 induced an antibody response in infected animals reactive against both the homologous virus and other H7 influenza viruses associated with recent infection by H7 viruses in humans.

Conclusions This is the first report of a candidate H7 vaccine virus for use in humans generated by reverse genetics and propagated entirely in mammalian tissue culture. The vaccine has potential use against a wide range of H7 strains.

Keywords Influenza, pandemic, reverse genetics, vaccine.

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Introduction

Influenza A virus is a major human pathogen which causes annual epidemics in the young and the old. Periodically a new influenza variant emerges in the human population causing a pandemic. The 1918 Spanish pandemic was the most severe to date, causing up to 40 million deaths worldwide.¹ Two well-documented influenza pandemics of the 20th Century, Asian influenza in 1957 and Hong Kong influenza in 1968, were caused by novel viruses generated by the process of antigenic shift; these viruses contained mixtures of genes derived from an avian influenza and a human influenza virus. However, more recently, there have

been several incidents of avian influenza viruses transmitting directly to humans.^{2–11} The avian viruses particularly implicated in these direct transmissions have hemagglutinin genes of two subtypes, H5 and H7, and usually have been highly pathogenic in chickens. This feature occurs mainly as a result of the presence of a stretch of several basic amino acids at the HA cleavage site, enabling the HA to be cleaved by ubiquitous proteases and facilitating the systemic spread of virus within chickens.^{12–14} It is not known whether the multi-basic cleavage site also plays a role in the transmission to or virulence of avian influenza virus in humans.

In none of the recent outbreaks have avian influenza viruses become adapted for human to human spread and

the numbers of people infected have been small, yet there is the possibility that if a virus acquires this ability, a pandemic virus with propensity to cause high mortality would emerge.

Since the first outbreak of H5N1 influenza in Hong Kong in 1997^{2,3} several candidate pandemic vaccine H5 viruses have been developed by reverse genetics,^{15–23} although the difficulties of translating this research into a product that can be tested in humans should not be underestimated.²⁴ In each case, the multi-basic cleavage site has been removed from the hemagglutinin (HA) gene of the candidate vaccine strain to attenuate the virus.

There have been several instances of highly pathogenic H7 viruses causing human disease.^{7,25,26} In the largest outbreak, in the Netherlands in 2003, most of the 89 people affected had symptoms of conjunctivitis, yet there were reports of respiratory disease and one fatality.²⁷ There was also evidence of human to human spread of this virus. It is therefore possible that the source of the next influenza pandemic could be an H7 virus.

In 1999, an outbreak of low pathogenicity avian influenza (LPAI) of the H7N1 subtype occurred in northern Italy in domestic poultry.^{28,29} This virus circulated for several months in the region until a highly pathogenic virus of the same subtype emerged, which caused the deaths of over 13 million birds in 3 months. The highly pathogenic avian influenza (HPAI) arose from the LPAI viruses circulating at the time by acquiring a multi-basic HA cleavage site, PEIPKGSRRVRRGLF.³⁰ During this outbreak, viruses emerged with a deletion of 22 amino acids in the stalk region of the neuraminidase, a mutation which may be an adaptation to growth in domestic poultry.³⁰ Thus, the HPAI viruses isolated in Italy in 1999 have shown a propensity for mutation within poultry. The HPAI H7N1 virus A/Chicken/Italy/13474/99 (Ck/It) was chosen as the candidate for a reverse genetics vaccine. This particular isolate did not contain a glycosylation site at position 149 in HA that many of the other viruses isolated during the same outbreak had acquired.³⁰

Using now standard reverse genetics techniques,^{31,32} we have generated an H7 N1 vaccine virus containing the surface antigens from Ck/It and the internal protein genes from the vaccine donor strain A/Puerto Rico/8/34 (PR8) (H1N1). The recovered virus was not passaged in eggs at all, but underwent passage through two different mammalian cell substrates suitable for vaccine production. The cleavage site of HA which had been engineered to contain only a single basic residue was stable following passage through the mammalian cells. The virus was neutralized by anti-H7 sera raised to a range of H7 strains and was itself immunogenic, inducing antibodies that could cross-neutralize other H7 viruses. Vaccine material produced from this virus is currently undergoing clinical trials. This is the

first report of a candidate vaccine virus produced in a cell culture system that can be used to protect humans against a potential H7 pandemic virus.

Materials and methods

Viruses

Virus strains A/Chicken/Italy/13474/99 (H7 N1), A/Turkey/Italy/3889/99 (H7N1), A/Turkey/Italy/214845/02 (H7N3) and A/Mallard/Italy/33/01 (H7N3) were propagated at the Istituto Superiore di Sanita, Rome, Italy. A/PR/8/34 virus, (PR8) was obtained from NIBSC and is the strain used annually to generate standard vaccine reassortants.

A/England/481/06 (H7N3) was isolated from a human case of conjunctivitis in a farm worker exposed to infected turkeys. A/Mallard/Netherlands/12/2000 and A/Mallard/Sweden/105/02 (H7N7) are LPAI related to viruses that caused an outbreak in the Netherlands in 2003. These viruses were propagated in eggs at the Health Protection Agency Laboratories, UK.

Plasmid DNA

The six segments encoding the internal genes of PR8 were cloned into plasmids supplied by T. Zurcher, Glaxo-SmithKline, UK that are suitable for virus recovery by reverse genetics as previously described.³³ The PR8 vRNA used was obtained from NIBSC and is the strain used annually to produce vaccine seeds. The HA segment from Ck/It was amplified by PCR, sequenced (Accession number: AJ491720) and cloned into the TOPO vector (Invitrogen, Paisley, UK). Site-directed mutagenesis (Stratagene, Amsterdam, the Netherlands) was carried out on the HA gene to remove the multi-basic cleavage site using the primers AH7ΔMBS5' (CCC GAG ATT CCG AAG GGC CGC GGC CTA TTT GGT GC) and AH7ΔMBS3' (GCA CCA AAT AGG CCG CGG CCC TTC GGA ATC TCG GG). A *SacII* restriction site was engineered into the HA cleavage site for diagnostic purposes. The HA segment was subsequently cloned into a plasmid suitable for recovery by reverse genetics.³³ The NA segment from Ck/It was cloned unmodified into a rescue plasmid. Four helper plasmids, expressing the PB1, PB2, PA, and NP genes from A/Victoria/3/75 (H3N2) were supplied by T. Zurcher, at Glaxo-SmithKline, UK. In our hands, these helper plasmids enabled more efficient recovery of recombinant virus than those derived from the PR8 strain (data not shown). cDNAs from the helper plasmids do not become incorporated into the rescued virus. All plasmids were isolated from bacteria propagated in Luria broth manufactured using soya tryptone to avoid contamination with bovine products. Plasmids were sequenced by Lark Technologies Ltd (Takeley, UK).

Cells

Vero cells that are validated for use in vaccine manufacture were provided by Sanofi Pasteur, France. PER.C6 cells were provided by Crucell, Netherlands, BV. Both cell lines were propagated within the Cell Biology & Imaging (CBI) section of NIBSC under Class A clean area conditions using MEM supplemented with 10% fetal bovine serum. A chick embryo cell (CEC) suspension was prepared from 10-day-old SPF embryos using 1% trypsin (Sigma, Gillingham, UK) and irradiated fetal calf serum (JRH Biosciences, Andover, UK).

Transfection

The reverse genetics process took place in a Class III Microbiological Safety Cabinet (MSC) at NIBSC at Biosafety Level 4 (BSL4) following guidelines set out in accordance with WHO [http://www.who.int/csr/resources/publications/influenza/WHO_CDS_CSR_RMD_2003_5/en/]. On day 0, Vero cells for transfection were sub-cultured to provide 10^6 cells in suspension in each well of a 6-well plate, and were transferred from the CBI unit to the BSL4 facility. The cells were transfected with 12 plasmids using Lipofectamine 2000TM (Invitrogen) reagent using a 1:3 ratio of volume of reagent to microgram DNA in serum free MEM. Cells were transfected with 0.3 µg of the rescue plasmids and 0.5 µg of the helper plasmids. Six hours post-transfection, 10^6 CEF cells were added to each well and 1 h later 1 µg/ml porcine trypsin (Sigma) was added. On days 3 and 6, the supernatant was sampled for virus and a fresh aliquot of medium containing trypsin was added. On day 6, the supernatant was harvested and passaged once in Vero cells and three times in PER.C6 cells. In each passage, the medium was supplemented with 0.5 µg/ml of porcine trypsin, with the exception of the first PER.C6 passage when 0.25 µg/ml was used. The material recovered from the third passage in PER.C6 cells was used for pathogenicity studies as described below.

Hemagglutination and hemagglutination inhibition assays

Hemagglutination assays were performed by standard methods.³⁴ HI assays were performed using horse erythrocytes.³⁵

Plaque assays

Plaque assays were performed on confluent monolayers of MDCK cells in 6-well plates. Ck/It and RD3 were assayed in parallel; duplicate \log_{10} series titrations of each virus were inoculated into separate plates. The plates were overlaid with 1% agarose medium which for one plate for each strain was supplemented with 5 µg/ml TPCK trypsin (Worthington, Lakewood, NJ, USA). Assays were incubated

at 35°C for 6 days. On day 3, the plates were overlaid with medium containing neutral red.

Egg embryo test

Ten-fold dilution series from 10^{-4} to 10^{-9} of Ck/It and RD3 were prepared and inoculated into 11-day embryonated chicken eggs using five eggs per dilution. Eggs were incubated at 35°C for 3 days and were candled to assess embryo survival and allantoic fluids sampled for hemagglutination assay.

Chicken pathogenicity test

The chicken pathogenicity test was performed according to OIE procedures at NIBSC and BSL4 by staff from VLA, Weybridge, UK. Ten 6-week-old specific pathogen-free (SPF) chickens were inoculated i.v. with 0.1 ml of RD3 virus containing 10^5 EID₅₀ and observed over a 10-day period. The intravenous pathogenicity index (IVPI) was determined as described.³⁶

Ferret pathogenicity test

The ferret pathogenicity test was performed according to WHO guidelines at BSL4. The pathogenicity of the RD3 virus was assessed in comparison to that of the two parent viruses Ck/It and PR8. For each strain 0.2 ml of virus containing 10^7 EID₅₀ was administered intranasally to four young adult male ferrets anesthetized with ketamine/xylazine. On day 3, two ferrets from each group were killed, nasal washings taken and the lungs, spleens, and brains were collected for virus recovery. The brain was divided longitudinally and half was fixed in formaldehyde for histological examination. The remaining two animals from each group were assessed for clinical signs at 24 h intervals for 14 days. On day 14, brain tissue was collected for histological examination. Virus titrations of tissue homogenates and nasal washes were performed in eggs.

Mouse pathogenicity test

Twenty microliter volumes of a 10-fold dilution series of virus were administered intranasally to groups of Balb/c mice anesthetized with ketamine/xylazine. On days 4 and 7, the virus present in lungs, brain, or spleen was determined by inoculation onto MDCK cells.

Microneutralization test

Ferrets were inoculated intranasally with approximately 10^5 plaque forming units (pfu) of egg grown virus, and serum samples were collected 2 weeks post-inoculation. Each serum sample was assayed in microneutralization assays in duplicate in accordance with the methods described elsewhere.³⁷ All microneutralization assays were performed with MDCK cells derived from the European Cell Culture

collections. Antibody titres greater than 20 were considered positive.

Immunogenicity in rabbits

Male and female rabbits (eight male rabbits and eight female rabbits per group) received three IM injections (days 0, 21, and 42) of an A/H7N1 monovalent β -propionolactone-inactivated split virion vaccine (31 μ g) produced from RD3 virus in PER.C6 cells with or without aluminum hydroxide (AIOH) adjuvant (600 μ g AIOH per dose). A control group received only PBS. Blood samples were collected on days 0, 21, 44, and 56 post-immunization. The immune response was evaluated by measuring H7-specific functional antibodies by HI assay using turkey erythrocytes.

Results

Construction and generation of the recombinant virus RD3

The highly pathogenic virus A/Chicken/Italy/13474/99 (Ck/It) isolated during an outbreak in Italy in 1999 was chosen to be the focus of this study. Genetic characterization of the surface antigens suggested that this virus was typical of viruses circulating in Italy at that time and also that it might be broadly cross-reactive because of the predicted glycosylation sites on HA.^{30,31} However, in an HI test of a post-infection ferret serum to Ck/It, titres were somewhat reduced with more recent (2001–2006) H7 viruses, which is discussed further in relation to antigenic properties of RD3 virus (Table 1). The Ck/It virus is highly pathogenic in chickens (IVPI = 3.0) and is also lethal to mice without prior adaptation. The LD₅₀ of Ck/It in mice was 10^{5.1}EID₅₀ and the ID₅₀ was 10⁴EID₅₀. Virus was detected in the nasal washings of infected mice from days 1–6 and also in the lungs of 2/3 mice killed on day 4 and 3/3 mice killed on day 6. The mean titre of the positive

samples was 10^{5.5}TCID₅₀/g (Table 4). Virus was not detected in the brains or spleens of any of the killed animals on either day 4 or 6. Ck/It virus replicated in eggs but also killed them. Thus, the Ck/It virus chosen as the basis for a vaccine candidate has the characteristics of a lethal virus in at least some mammalian species, and in killed eggs, which is a 'worst case' scenario for pandemic vaccine production.

The HA of Ck/It was cloned and the multi-basic cleavage site was removed by site-directed mutagenesis to convert the HA to the low-pathogenic form seen in LPAI isolates, such as A/Tk/It/3889/99 from the same outbreak (Fig. 1). In addition, the arginine codon at the cleavage site was changed from AGA to CGC, creating a unique *Sac*II restriction enzyme site for diagnostic purposes. Silent third base changes to remove adenine nucleotides in codons upstream of the cleavage site were also introduced, in an attempt to reduce the likelihood of viral RNA polymerase slippage that might result in the regeneration of a multi-basic site.^{14,38} The NA of Ck/It was cloned without modification. RNA segments for the six internal genes of PR8 virus obtained from NIBSC and used annually to generate

A/Ck/It/13474/99 WT:

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P E I P K G S R V R R ↓ G L F
ccc gaa att cca aaa gga tcg cgt gtg agg aga ggc cta ttt

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RD3 single basic cleavage site:

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P E I P K G           R   G L F
ccc gag att cag aag ggc   cgc ggc cta ttt

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Figure 1. Removal of the multi-basic site of H7 HA by site-directed mutagenesis. A diagnostic *Sac*II site was introduced during the process (underlined). Third base changes were also made in codons preceding the cleavage site (bold). The arrow indicates the HA cleavage site.

Table 1. HI reactions of H7 viruses with a panel of post-infection or post-immunization sera

Virus strain	Hemagglutination inhibition titre							
	α Ck/It, Ferret	α Ck/It, Sheep*	α Tk/3889, Ferret*	α Tk/214845, Ferret	α Mal/33, Ferret	α Eng/481, Ferret,	α NL/12, Ferret	α RD3, Ferret
A/Chicken/Italy/13474/99 (Ck/It) (H7 N1)	320	2560	320	40	40	40	40	160
A/Turkey/Italy/3889/99 (Tk/3889) (H7 N1)	80	2560	160	40	40	40	160	20
A/Turkey/Italy/214845/02 (Tk/214845) (H7 N3)	80	640	160	320	640	160	1280	20
A/Mallard/Italy/33/01 (Mal/33) (H7 N3)	320	1024	320	320	320	1280	640	20
A/England/481/06 (Eng/481) (H7 N3)	80	5120	160	320	320	640	640	20
A/Mallard/Netherlands/12/2000 (Mal/12) (H7 N7)	160	2560	640	320	640	320	1280	40
RD3 PER.C6	320	5120	320	40	40	40	40	160

*Post-immunization serum.

standard vaccine reassortants were cloned into plasmids suitable for virus recovery by reverse genetics. The modified H7 HA and the N1 NA were rescued into the background of NIBSC PR8 using a reverse genetics method involving the transfection of WHO-approved Vero cells and co-culture in SPF chick embryo cells (CECs).²³ The resulting recombinant virus was termed RD3.

RD3 requires trypsin for growth *in vitro*

Following the generation of RD3, the virus was subsequently passaged once through WHO-approved Vero cells and three times in PER.C6 cells. PER.C6 cells are derived from primary human retina cells, immortalized upon transfection with an E1 minigene of adenovirus type 5.³⁹ PER.C6 cell banks were prepared according to FDA and European Medicines Agency (EMA) guidelines and the cell substrate is being used for the manufacture of diverse biopharmaceutical products to be tested in clinical trials in Europe and the USA. PER.C6 cells have been shown to support efficiently the influenza virus propagation,⁴⁰ thus providing a suitable cell line for the manufacture of influenza vaccines. The entire HA gene of RD3 was sequenced following passage in PER.C6 cells to confirm the absence of the multi-basic cleavage site and to monitor any changes that might be selected for upon passage. The HA gene was stable and identical to the sequence of the cDNA from which the virus was generated. RD3 grew to an HA titre of 960 in PER.C6 cells, and the same preparation had an infectivity titre of $10^{7.7}$ TCID₅₀/ml measured in MDCK cells. The multi-basic cleavage site in an HA molecule enables viruses to plaque in tissue culture in the absence of trypsin.⁴¹ In MDCK cells Ck/It virus with a titre of $10^{8.4}$ pfu/ml in presence of trypsin also gave a titre of $10^{7.8}$ pfu/ml without trypsin, whereas RD3 with a titre of $10^{9.2}$ pfu/ml was unable to plaque without the addition of exogenous trypsin.

The antigenic properties of RD3 are identical to those of A/Ck/It/13474/99

The antigenicity of RD3 was compared with that of Ck/It in HI assays using sera raised against several different H7N1 or H7N3 viruses. The LPAI virus A/Turkey/Italy/3889/99 is antigenically related to Ck/It. The H7N3 viruses A/Turkey/Italy/214845/02 and A/Mallard/Italy/33/01 are LPAI-related antigenically to the group of viruses isolated during the outbreaks of H7N3 infection in Italy during 2001 and 2002. They differ antigenically from the 1999 H7 N1 Italian viruses and in relation to Ck/It/99 display two additional potential glycosylation sites at residues 123 and 149. Also included in the virus panel were A/England/481/06, an H7N3 virus isolated from a poultry worker with conjunctivitis in the UK,¹¹ and A/Mallard/Netherlands/12/2000, antigenically representa-

tive of the H7N7 outbreak in poultry in the Netherlands in 2003.²⁷ HI assays were performed using either turkey (data not shown) or horse erythrocytes (Table 1). The titres were higher using horse erythrocytes, but the overall pattern of reactivity was unaltered. Although there was some cross-reactivity between the Italian H7N1 viruses from 1999 with the later H7 strains, in general, the later strains behaved as a separate antigenic group. RD3 was antigenically like the original Ck/It virus and its antigenicity was not altered whether it was grown in Vero cells or in PER.C6 cells (data not shown). In particular, RD3 virus reacted with ferret or sheep sera raised against Ck/It or ferret sera raised against A/Tk/Italy/3889/99. This indicates that the removal of the multi-basic site from RD3 had not affected the antigenic properties of the virus. However, like Ck/It itself, RD3 was poorly recognized by sera raised against the more recent H7N3 isolates, A/Tk/It/214845/02, A/Mallard/It/33/01, or A/Eng/481/06 or against the H7 N7 virus A/Mallard/Netherlands/12/2000 (Table 1). In contrast to the specificity of post-infection ferret sera to Ck/It and RD3 viruses, post-immunization ferret serum to A/Turkey/Italy/3889/99 was more cross-reactive with later H7 viruses. This is probably a feature of the immunization schedule (priming infection with A/Port Chalmers/1/73 (H3N2) and two 50 µg intramuscular injections of inactivated A/Tk/It/3889/99 virus), compared with the single infection used for the Ck/It and RD3 ferret sera.

Removal of the multi-basic site attenuates RD3 pathogenicity in embryonated eggs

The parental virus Ck/It is highly pathogenic in avian species and is lethal when injected into embryonated chicken eggs, rendering it unsuitable for use in vaccine production. However, RD3 virus was shown to be non-pathogenic for chick embryos when five embryonated eggs were inoculated at a high MOI ($10^{4.3}$ EID₅₀/egg) with RD3 and all chick embryos survived the infection.

RD3 is attenuated in chickens and ferrets

The IVPI of Ck/It is 3.0, indicating that this virus is highly virulent in chickens. The IVPI of RD3 was determined by inoculating 6-week-old SPF chicks intravenously with a dose of $10^{6.3}$ EID₅₀ and monitoring the chicks over a 10-day-period. All the 10 inoculated chicks survived over this period. Thus, with an IVPI of 0, RD3 is completely apathogenic in chickens.

In order to determine the pathogenicity of RD3 in ferrets, four ferrets were inoculated intranasally with 10^7 EID₅₀ of either PR8, Ck/It or RD3 virus. The animals were weighed and monitored daily for clinical symptoms and on day 3 post-inoculation, two of the animals inoculated with each virus were killed and the presence of virus in organs was determined. The remaining ferrets were

Table 2. Pathogenicity testing of RD3 in ferrets

	No. animals infected (EID ₅₀ /ml or /g*)				Lesions in brain on 14 d p.i.	Clinical signs
	Nasal wash	Lung	Spleen	Brain		
PR8	2/2 (10 ^{4.8})	1/2 (10 ^{0.2})	0/2 (0)	2/2 (10 ^{1.1})	None observed	Mild respiratory
Ck/It	2/2 (10 ^{4.5})	2/2 (10 ^{3.3})	0/2 (0)	0/2 (0)	None observed	None observed
RD3	2/2 (10 ^{3.75})	1/2 (10 ^{0.65})	0/2 (0)	0/2 (0)	None observed	None observed

Groups of ferrets were inoculated i.n. with 10⁷ EID₅₀ of PR8, A/Ck/It/13474/99 or RD3. *Mean of two animals.

monitored daily for a further 10 days. Although all animals had been infected as shown by the presence of virus in nasal turbinates, only ferrets inoculated with PR8 showed any clinical symptoms or spread of virus outside the respiratory tract (Table 2). None of the animals showed evidence of lesions in the brain on 14 days post-infection. The only evidence of a difference between RD3 virus and either parental virus was the reduced virus titres in nasal tissues (10^{3.75}) compared with PR8 (10^{4.8}) and Ck/It (10^{4.5}) and in lung tissues (10^{0.65}) compared with Ck/It (10^{3.3}). These data show that neither the parental Ck/It nor the recombinant virus, RD3 are pathogenic in ferrets.

In addition, sera were collected from the ferrets infected intranasally with RD3 as part of the pathogenicity trials. These sera were obtained at 14 days post-infection. Although homologous HI antibodies of the sera were generally low, one of the sera reacted with two of the H7 viruses, A/Mallard/Italy/33/01 and A/Mallard/Netherlands/12/2000 (data not shown). This is further proof that the ferrets had successfully been infected with RD3 virus despite the complete absence of symptoms.

A further ferret was infected with RD3 virus by the intranasal route and immune sera collected at 21 days after infection. This serum was used in a neutralization assay to establish the immunogenicity of RD3 (Table 3). Antiserum to RD3 neutralized the homologous RD3 virus with a titre of 92–104. The same antiserum also showed a neutralization titre of 87 against the H7N3 virus A/England/481/06. Antiserum raised to A/England/481/06 virus had a homologous neutralization titre of 551 and also neutralized RD3 virus with a titre of 167, indicating in this case a two-way antigenic relationship between these viruses. These two viruses show 97.3% amino acid homology in the HA1 region of the HA protein.

Both the anti-RD3 ferret serum and the anti-Eng/481/06 H7N3 ferret serum also neutralized infectivity of an H7N7 virus, A/Mallard/Sweden/105/02 which is antigenically related to the viruses that caused an outbreak of HPAI

Table 3. Geometric means of 50% neutralization titres of sera from ferrets infected with H7N1 RD3 virus or H7N3 A/England/481/06 virus

Virus	Ferret antiserum	
	Ferret 709 (Anti-RD3 H7N1)	Ferret 707 (Anti-A/England/481/06 H7N3)
RD3 H7N1 (egg)	92 (2)	167 (2)
RD3 H7N1 (Per.C6)	104 (4)	Not done
A/England/481/06-H7N3	87 (4)	551 (3)
A/Mallard/Sweden/105/02-H7N7	144 (1)	2054 (1)

Numbers in parentheses indicate number of duplicates used to calculate mean.

associated with conjunctivitis and influenza-like illness in exposed individuals in the Netherlands in 2003.

RD3 infection of mice

RD3 virus was infectious and pathogenic in mice (Table 4). The MID₅₀ for RD3 was intermediate between that for each of the parental viruses, Ck/It and PR8. RD3 replicated to high titres in the lungs of the infected mice, but no virus was recovered from brain or spleen. RD3 was less pathogenic in mice than either parent. Nonetheless, although these experiments are preliminary and were not designed to compare formally the pathogenicity, it is clear that at moderate doses, RD3 retains lethality for mice.

RD3-derived monovalent vaccine is immunogenic in rabbits

A monovalent β -propiolactone-inactivated split virion vaccine preparation of RD3 was prepared by Sanofi Pasteur in PER.C6 cells and used to immunize rabbits by an intramuscular route with or without alum adjuvant. Rabbits received

Table 4. Infectivity and pathogenicity of RD-3 and parents in Balb/c mice

Strain	MID ₅₀	LD ₅₀	Lung titres (TCID ₅₀ /g)		Virus recovery in	
			Day 4 post-infection	Day 7 post-infection	Brain	Spleen
Ck/It	10 ⁴ EID ₅₀ 10 ^{2.5} TCID ₅₀	10 ^{5.1} EID ₅₀ 10 ^{3.6} TCID ₅₀	10 ^{5.5} /g (2 mice) (SD 10 ^{0.283})	10 ^{5.36} /g (3 mice) (SD 10 ^{0.416})	Negative	Negative
PR8	10 ² EID ₅₀ 10 ¹ TCID ₅₀	10 ^{1.1} MID ₅₀ 10 ^{4.2} EID ₅₀ 10 ^{3.2} TCID ₅₀ 10 ^{2.2} MID ₅₀	not tested	not tested	Not tested	Not tested
RD3	10 ^{2.7} EID ₅₀ 10 ^{2.2} TCID ₅₀	10 ^{6.1} EID ₅₀ 10 ^{5.6} TCID ₅₀ 10 ^{3.4} MID ₅₀	10 ⁶ /g (3 mice) (SD 10 ^{0.723})	Not tested	Negative	Negative

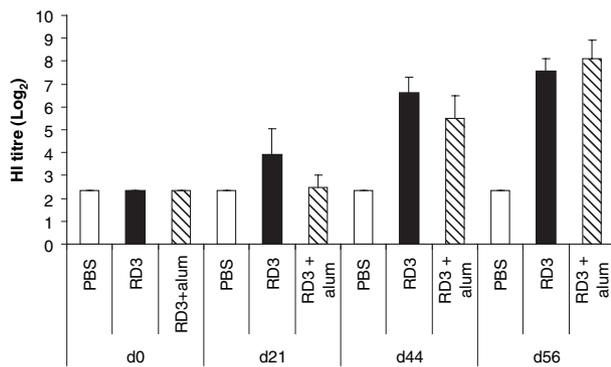


Figure 2. Functional antibodies against H7N1 virus measured by HI in the sera of rabbits immunized with PERC6-produced RD3 H7N1 vaccine. Groups of 16 (d0, d21, d44) or 8 (d56) rabbits were inoculated with PBS (clear bars), 31 µg H7N1 vaccine preparation (black bars) or 31 µg H7N1 vaccine preparation with alum adjuvant. Sera collected from the rabbits on days 0, 21, 44, and 56 after immunization were tested for HI activity against RD3 virus. Geometric mean titres for each group of sera with standard errors are shown.

3 IM injections on day 0, 21, and 42 of 31 µg of HA. Sera were collected from the rabbits on day 0, 21, 44, and 56 post-immunization and tested for reactivity to RD3 virus by HI assay (Fig. 2). The results showed that the monovalent vaccine preparation derived from RD3 virus was immunogenic in rabbits and antibodies were detected even after just one injection (day 21). Alum adjuvant did not show any statistically significant alteration of the immune response, although it should be noted that a relatively high dose of H7 N1 vaccine (31 µg) was given.

Discussion

It has been 39 years since the last influenza pandemic and it is inevitable that a pandemic strain will emerge in the near future. The recent episodes of H5 and H7 avian influ-

enza infections in humans have strongly suggested that one of these subtypes may be the source of the next influenza pandemic. It is vital that during this interpandemic period we prepare by generating potential vaccine seed stocks and acquire knowledge about the pitfalls involved in the generation of vaccines so that in the event of a pandemic a vaccine can be generated as efficiently as possible. We report the generation by reverse genetics of an H7 subtype vaccine reference virus, termed RD3, that will be available for use against a human H7 pandemic. Although an H7 subtype reverse genetics vaccine for use in chickens has previously been reported,²² and a recombinant low pathogenicity H7 N7 virus vaccine was generated and tested in mice,⁴² the H7 N1 vaccine virus produced in our cell culture system is suitable for use in the human population. Recently, another H7 N1 vaccine virus has been described that was produced by reverse genetics in substrates suitable for human use.²³ However, safety testing and further characterization beyond initial recovery of this virus was not described. Moreover, the HA chosen for this vaccine virus was from a virus of the North American lineage of H7 viruses, and may show low cross-reactivity with Eurasian H7 isolates.⁴³ Bearing in mind the international drive towards production of influenza vaccine in cell cultures, the generation of the RD3 virus entirely in cell culture is a significant achievement. The RD3 virus was recovered following transfection of WHO approved Vero cells, co-cultured with CECs, and then passaged in the PER.C6 cell line. It is possible that since PER.C6 cells are appropriate for biopharmaceutical production, and are highly transfectable (L.C.S. Hartgroves and W.S. Barclay, unpublished), recombinant influenza viruses could be recovered directly following PER.C6 transfection in the future.

The annual influenza vaccine is generated by virus passage in embryonated chickens' eggs, yet in a pandemic situation it is possible that the availability of eggs suitable for vaccine production will be limited, due to either eggs not

being ordered in advance or to an avian outbreak reducing the supply of eggs. In addition, it has been shown in animal studies that vaccines produced in mammalian tissue culture can be more protective than those grown in eggs,^{44–46} an advantage in a pandemic situation. Further studies are required to determine whether this is the case for the RD3 vaccine.

The removal of the multi-basic site from the RD3 HA has been shown to attenuate the virus for chickens and ferrets without altering the antigenicity. The single basic cleavage site present in RD3 is stably retained following four passages in tissue culture (one in Vero, three in PER.C6). The demonstration of non-pathogenicity enables RD3 to be re-categorized as a BSL2 enhanced (pandemic influenza vaccine) pathogen according to guidelines prepared to advise vaccine manufacturers about the handling of such recombinant influenza viruses [http://www.who.int/vaccine_research/diseases/influenza/ECBS_2005_Annex_5_Influenza.pdf]. The reclassification is important to facilitate bulk production of virus for a vaccine under lower level containment. It has been shown that multiple passage of an H7 subtype virus in chicken embryo cells has led to the generation of a virus which is highly pathogenic for chickens, a phenotype which is linked to the acquisition of an HA cleavable by highly abundant proteases.^{47,48} However, in the same study, the H7 virus was passaged through mammalian cells and although the HA acquired the ability to be cleaved in the absence of trypsin in MDCK cells, this did not render the virus pathogenic for chickens.⁴⁸ This indicates that the use of mammalian cells for vaccine development in place of embryonated chicken eggs may reduce the propensity of the virus to acquire chicken virulence.

In contrast, the RD3 virus was both infectious and pathogenic in mice. Although the LD₅₀ was higher for RD3 than for either of the pathogenic parental strains Ck/It or PR8, RD3 was more infectious in mice and higher viral loads accumulated in the lungs than for infection with Ck/It. Although some H5 recombinant viruses that lack the HA multi-basic cleavage site are attenuated in mice^{17,49} the basis of mouse virulence is not understood. PR8 virus, the genetic backbone of all influenza A vaccine viruses, is virulent in mice, and a recent study has also found that other H7 avian influenza viruses that lack the multi-basic cleavage motif are lethal in mice.⁴³ Therefore, interpretation of mouse virulence data is complicated and might not be useful as a predictor of vaccine safety. This important point should be considered during safety testing of other recombinant influenza virus vaccine strains to avoid unnecessary use of animals.

In 2002/2003, there was a further outbreak of LPAI in turkeys in Italy.⁵⁰ The virus causing the outbreak was of the H7N3 subtype and was closely related to an H7N3

virus isolated from wild ducks in Italy in 2001. Although the virus did not mutate to a highly pathogenic form, it did contain a deletion of 23 amino acids in the NA stalk which was not present in the virus isolated from wild ducks,^{50,51} indicating that the virus had the propensity to mutate. Unlike the H7N1 viruses, these and other H7N2 and H7N3 viruses have been transmitted to exposed humans.^{10,11,52} This indicates that the H7 viruses currently circulating may have the ability to infect humans and underlines the importance of generating a vaccine against H7 subtype viruses. The candidate vaccine strain we have generated stimulates antibody which cross-reacts with many H7 isolates in HI tests and neutralizes H7 viruses from Italy, from England, and also from the Netherlands. RD3 is therefore considered suitable for use in phase I clinical trials. We did not test the RD3 antisera against any H7 viruses of the North American lineage. We note that the HA amino acid sequence of Ck/It differs from that of the British Columbian H7N3 virus that infected two people in 2004 by 27.6%, whereas the divergence between Ck/It and the H7N7 Netherlands virus A/Neth/219/03 is only 3.1%. Therefore, it is prudent to generate vaccine seeds for both divergent lineages of H7 avian influenza viruses as either may pose a pandemic threat. The recent publication from²³ describes a vaccine virus bearing a North American lineage H7 antigen, A/Rhea/NC/39482/93. However, data from⁴³ suggest that antisera against H7 viruses of the Eurasian lineages may confer some protection against North American H7 viruses, although the relationship was not reciprocal. It would be important in future studies to test the level of cross protection conferred by RD3 vaccine against a number of different H7 viruses.

We chose to generate an H7 vaccine virus for use in a human population in an attempt to prepare for the possibility of an influenza pandemic caused by an H7 subtype virus. Recently, an H7N7 low pathogenicity recombinant virus was generated by reverse genetics and shown to protect mice against lethal infection with H7N7 HPAI. Importantly, protection was only achieved when an adjuvant was employed,⁴² a finding reminiscent of reports from earlier clinical trials with an H5N3 surrogate vaccine in humans.⁵³ Although in this study, rabbits immunized with RD3 vaccine produced a strong antibody response even in the absence of adjuvant, it is expected that in clinical trials of RD3 vaccine an adjuvant will be needed.

The generation of RD3 was part of an EC-funded project, 'FLUPAN' (Quality of Life and Management of Living Resources). The FLUPAN project began before the escalation of human H5N1 cases in 2003 and 2004 and before the use of reverse genetics to develop H5N1 vaccine viruses. This rehearsal of the European pandemic response has illustrated that the generation of recombinant influenza strains using reverse genetics could be a fast and robust

approach in the European setting. The RD3 virus has in fact taken more than 3 years to reach clinical trials since the conception of the FLUPAN project, but during that time many regulatory hurdles had to be overcome and efficient procedures put in place in the European laboratories involved. Therefore, in addition to producing and testing a candidate H7 vaccine, the FLUPAN rehearsal has facilitated increasingly rapid responses to contemporary H5N1 pandemic threats.

The next stage in this process is to use a β -propiolactone-inactivated split virion cell culture vaccine to evaluate the safety and immunogenicity of this vaccine in humans. Clinical trials are now ongoing.

Author contribution

Alison Whitely generated the H7N1 recombinant virus, RD3. Diane Major supervised virus rescue and performed HA and pathogenicity tests. Isabelle Legastelois developed the methodology for virus rescue. Laura Campitelli and Isabella Donatelli provided the Ck/Italy/99 virus and the sequence thereof. Catherine Thompson generated cDNAs for virus rescue. Maria Zambon generated ferret antisera to RD3 and supervised neutralization tests. John Wood coordinated this EU project, FLUPAN. Wendy Barclay was the principal investigator for this workpackage of FLUPAN.

In addition, Rachel Brierley, NIBSC, Potters Bar, UK propagated RD3 virus. Mirjam Kuhne, HPA, Colindale, UK performed neutralization tests.

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Conflicts of interest

JW and DM have received funding from Sanofi Pasteur for contract research unconnected with this work. WB has received funds for research and consultancy relating to the production of influenza vaccines from Sanofi Pasteur and Crucell. The Health Protection Agency receives funding from several vaccine companies, including Sanofi Pasteur,

for work unrelated to the present study carried out in MZ's Laboratory at HPA Colindale.

References

- Potter CW. Chronicle of influenza pandemics; in Nicholson KG, Webster RG, Hay AJ (eds): *The Textbook of Influenza*. Oxford: Blackwell, 1998; 3–18.
- de Jong JC, Class ECJ, Osterhaus ADME, Webster RG, Lim WL. A pandemic warning? *Nature* 1997;389:554.
- Subbarao K, Klimov A, Katz J *et al.* Characterization of an avian influenza A (H5 N1) virus isolated from a child with a fatal respiratory illness. *Science* 1998;279:393–396.
- Peiris M, Yuen KY, Leung CW *et al.* Human infection with influenza H9 N2. *Lancet*. 1999;354:916–917.
- Lin YP, Shaw M, Gregory V *et al.* Avian-to-human transmission of H9 N2 subtype influenza A viruses: relationship between H9 N2 and H5 N1 human isolates. *Proc Natl Acad Sci USA* 2000;97:9654–9658.
- Peiris JS, Yu WC, Leung CW *et al.* Re-emergence of fatal human influenza A subtype H5 N1 disease. *Lancet* 2004;363:617–619.
- Koopmans M, Wilbrink B, Conyn M *et al.* Transmission of H7 N7 avian influenza A virus to human beings during a large outbreak in commercial poultry farms in the Netherlands. *Lancet* 2004;363:587–593.
- Tran TH, Nguyen TL, Nguyen TD *et al.* World Health Organization International Avian Influenza Investigative Team. Avian influenza A (H5 N1) in 10 patients in Vietnam. *N Engl J Med* 2004;350:1179–1188.
- Chotpitayasunondh T, Ungchusak K, Hanshaoworakul W *et al.* Human disease from influenza A (H5 N1), Thailand, 2004. *Emerg Infect Dis*. 2005;11:201–209.
- Skowronski DM, Tweed SA, Petric M, Booth T, Li Y, Tam T. Human illness and isolation of low-pathogenicity avian influenza virus of the H7 N3 subtype in British Columbia, Canada. *J Infect Dis* 2006;193:899–900.
- Nguyen-Van-Tam JS, Nair P, Acheson P and the Incident Response Team. Outbreak of low pathogenicity H7 N3 avian influenza in UK, including associated case of human conjunctivitis. *Eurosurveillance* 2006;11:60504.
- Bosch FX, Garten W, Klenk HD, Rott R. Proteolytic cleavage of influenza virus hemagglutinin: primary structure of the connecting peptide between HA1 and HA2 determines proteolytic cleavability and pathogenicity of avian influenza virus. *Virology* 1981;113:725–735.
- Webster RG, Rott R. Influenza A virus pathogenicity: the pivotal role of hemagglutinin. *Cell* 1987;50:665–666.
- Perdue M, Garcia M, Senne D, Fraire M. Virulence-associated sequence duplication at the hemagglutinin cleavage site of avian influenza viruses. *Virus Res* 1997;49:173–186.
- Li S, Liu C, Klimov A *et al.* Recombinant influenza A virus vaccines for the pathogenic human A/Hong Kong/97 (H5 N1) viruses. *J Inf Dis* 1999;179:1132–1138.
- Takada A, Kuboki N, Okazaki K *et al.* Avirulent avian influenza virus as a vaccine strain against a potential human pandemic. *J Virol* 1999;73:8303–8307.
- Subbarao K, Chen H, Swayne D *et al.* Evaluation of a genetically modified reassortant H5 N1 influenza A virus vaccine candidate generated by plasmid-based reverse genetics. *Virology* 2003;305:192–200.
- Webby RJ, Perez DR, Coleman JS *et al.* Responsiveness to a pandemic alert: use of reverse genetics for rapid development of influenza vaccines. *Lancet* 2004;363:1099–1103.

- 19 Nicolson C, Major D, Wood JM, Robertson JS. Generation of influenza vaccine viruses on Vero cells by reverse genetics: an H5 N1 candidate vaccine strain produced under a quality system. *Vaccine* 2005;23:2943–2952.
- 20 Suguitan AL Jr, McAuliffe J, Mills KL *et al.* Live, attenuated influenza A H5 N1 candidate vaccines provide broad cross-protection in mice and ferrets. *PLoS Med* 2006; 1541–1555.
- 21 Liu M, Wood JM, Ellis T *et al.* Preparation of a standardized, efficacious agricultural H5 N3 vaccine by reverse genetics. *Virology* 2003;314:580–590.
- 22 Lee CW, Senne DA, Suarez DL. Generation of reassortant influenza vaccines by reverse genetics that allows utilization of a DIVA (differentiating infected from vaccinated animals) strategy for the control of avian influenza. *Vaccine* 2004;22:3175–3181.
- 23 Legastelois I, Garcia-Sastre A, Palese P *et al.* Preparation of genetically engineered A/H5 N1 and A/H7 N1 pandemic vaccine viruses by reverse genetics in a mixture of Vero and chicken embryo cells. *Influenza and Other Respiratory Viruses*. 2007;1:95–104.
- 24 Wood JM, Robertson JS. Reference viruses for seasonal and pandemic influenza vaccine preparation. *Influenza and Other Respiratory Viruses*. 2007;1:5–9.
- 25 Banks J, Speidel E, Alexander DJ. Characterisation of an avian influenza virus isolated from a human – is an intermediate host necessary for the emergence of pandemic influenza viruses? *Arch Virol* 1998;143:781–787.
- 26 Tweed SA, Skowroski DM, David ST *et al.* Human illness from avian influenza H7 N3, British Columbia. *Emerg Infect Dis* 2004;10:2196–2199.
- 27 Fouchier RAM, Schneeberger PM, Rozendaal FW *et al.* Avian influenza A virus (H7 N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. *Proc Natl Acad Sci USA* 2004;101:1356–1361.
- 28 Capua I, Marangon S. The avian influenza epidemic in Italy, 1999–2000: a review. *Avian Path* 2000;29:289–294.
- 29 Capua I, Mutinelli F, Pozza MD, Donatelli I, Puzelli S, Cancellotti FM. The 1999–2000 avian influenza (H7 N1) epidemic in Italy: veterinary and human health implications. *Acta Trop* 2002;83:7–11.
- 30 Banks J, Speidel ES, Moore E *et al.* Changes in the haemagglutinin and the neuraminidase genes prior to the emergence of highly pathogenic H7 N1 avian influenza viruses in Italy. *Arch Virol* 2001;146:963–973.
- 31 Fodor E, Devenish L, Engelhardt OG, Palese P, Brownlee GG, Garcia-Sastre A. Rescue of influenza A virus from recombinant DNA. *J Virol* 1999;73:9679–9682.
- 32 Neumann G, Watanabe T, Ito H *et al.* Generation of influenza A viruses entirely from cloned cDNAs. *Proc Natl Acad Sci USA* 1999;96:9345–9350.
- 33 Thompson C, Barclay W, Zambon M. Changes in *in vitro* susceptibility of influenza H3 N2 viruses to a neuraminidase inhibitor drug during evolution in the human host. *J Antimicrob Chem* 2004;53:759–765.
- 34 Kendal AP, Pereira MS, Skehel JJ. Concepts and Procedures for Laboratory-Based Influenza Surveillance. U.S. Department of Health and Human Services (Centers Dis. Control, Atlanta). 1982.
- 35 Stephenson I, Wood JM, Nicholson KG, Zambon MC. Sialic acid receptor specificity on erythrocytes affects detection of antibody to avian influenza haemagglutinin. *J Med Virol* 2003;70:391–398.
- 36 OIE. Manual of Standards for Diagnostic Tests and Vaccines, 4th Edn. Office Internationale des Epizooties, 12 rue de Prony, 75017, Paris, France; 2001.
- 37 Rowe T, Abernathy RA, Hu-Primmer J *et al.* Detection of antibody to avian influenza A (H5 N1) virus in human serum by using a combination of serologic assays. *J Clin Microbiol* 1999;37(4):937–943.
- 38 Garcia M, Crawford JM, Latimer JW, Rivera-Cruz E, Perdue ML. Heterogeneity in the haemagglutinin gene and emergence of the highly pathogenic phenotype among recent H5 N2 avian influenza viruses from Mexico. *J Gen Virol* 1996;77:1493–1504.
- 39 Fallaux FJ, Bout A, van der Velde I *et al.* New helper cells and matched early region 1-deleted adenovirus vectors prevent generation of replication-competent adenoviruses. *Hum Gene Ther* 1998;9:1909–1917.
- 40 Pau MG, Ophorst C, Koldijk MH, Schouten G, Mehtali M, Uytendaele F. The human cell line PER.C6 provides a new manufacturing system for the production of influenza vaccines. *Vaccine* 2001;19:2716–2721.
- 41 Klenk HD, Rott R, Orlich M, Blodorn B. Activation of influenza A viruses by trypsin treatment. *Virology* 1975;68:426–439.
- 42 de Wit E, Munster VJ, Spronken MI *et al.* Protection of mice against lethal infection with highly pathogenic H7 N7 influenza A virus by using a recombinant low-pathogenicity vaccine strain. *J Virol* 2005;79:12401–12407.
- 43 Joseph T, McAuliffe J, Lu B, Jin H, Kemble G, Subbarao K. Evaluation of replication and pathogenicity of avian influenza A H7 subtype viruses in a mouse model. *J Virol* 2007; DOI:10.1128/JVI.00970-07.
- 44 Katz JM, Webster RG. Efficacy of inactivated influenza A virus (H3 N2) vaccines grown in mammalian cells or embryonated eggs. *J Inf Dis* 1989;160:191–198.
- 45 Wood JM, Oxford JS, Dunleavy U, Newman RW, Major D, Robertson JS. Influenza A (H1 N1) vaccine efficacy in animal models is influenced by two amino acid substitutions in the haemagglutinin molecule. *Virology* 1989;171:214–221.
- 46 Alymova IV, Koodihalli S, Govorkova EA, Fanget E, Gerdil C, Webster RG. Immunogenicity and protective efficacy in mice of influenza B virus vaccines grown in mammalian cells or embryonated chicken eggs. *J Virol* 1998;72:4472–4477.
- 47 Khatchikian D, Orlich M, Rott R. Increased viral pathogenicity after insertion of a 28S ribosomal RNA sequence into the haemagglutinin gene of an influenza virus. *Nature* 1989;340:156–157.
- 48 Orlich M, Khatchikian D, Teigler A, Rott R. Structural variation occurring in the haemagglutinin of influenza virus A/turkey/Oregon/71 during adaptation to different cell types. *Virology* 1990;176:531–538.
- 49 Hatta M, Gao P, Halfmann P, Kawaoka Y. Molecular basis for high virulence of HongKong H5N1 influenza A viruses. *Science* 2001;293:1840–1842.
- 50 Campitelli L, Mogavero E, De Marco MA *et al.* Interspecies transmission of an H7 N3 influenza virus from wild birds to intensively reared domestic poultry in Italy. *Virology* 2004;323:24–36.
- 51 Di Trani L, Bedini B, Cordioli P *et al.* Molecular characterization of low pathogenicity H7 N3 avian influenza viruses isolated in Italy. *Avian Dis* 2004;48:376–383.
- 52 Puzelli S, Di Trani L, Fabiani C *et al.* Serological analysis of humans exposed to avian H7 influenza viruses in Italy between 1999 and 2003. *J Inf Dis* 2005;192:1318–1322.
- 53 Nicholson KG, Colegate AE, Podda A *et al.* Safety and antigenicity of non-adjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5 N3) vaccine: a randomised trial of two potential vaccines against H5 N1 influenza. *Lancet* 2001;357(9272):1937–1943.