Responsiveness to a pandemic alert: use of reverse genetics for rapid development of influenza vaccines

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Summary

Background In response to the emergence of severe infection capable of rapid global spread, WHO will issue a pandemic alert. Such alerts are rare; however, on Feb 19, 2003, a pandemic alert was issued in response to human infections caused by an avian H5N1 influenza virus, A/Hong Kong/213/03. H5N1 had been noted once before in human beings in 1997 and killed a third (6/18) of infected people.[1,2] The 2003 variant seemed to have been transmitted directly from birds to human beings and caused fatal pneumonia in one of two infected individuals. Candidate vaccines were sought, but no avirulent viruses antigenically similar to the pathogen were available, and the isolate killed embryonated chicken eggs. Since traditional strategies of vaccine production were not viable, we sought to produce a candidate reference virus using reverse genetics.

Methods We removed the polybasic aminoacids that are associated with high virulence from the haemagglutinin cleavage site of A/Hong Kong/213/03 using influenza reverse genetics techniques. A reference vaccine virus was then produced on an A/Puerto Rico/8/34 (PR8) backbone on WHO-approved Vero cells. We assessed this reference virus for pathogenicity in in-vivo and in-vitro assays.

Findings A reference vaccine virus was produced in Good Manufacturing Practice (GMP)-grade facilities in less than 4 weeks from the time of virus isolation. This virus proved to be non-pathogenic in chickens and ferrets and was shown to be stable after multiple passages in embryonated chicken eggs.

Interpretation The ability to produce a candidate reference virus in such a short period of time sets a new standard for rapid response to emerging infectious disease threats and clearly shows the usefulness of reverse genetics for influenza vaccine development. The same technologies and procedures are currently being used to create reference vaccine viruses against the 2004 H5N1 viruses circulating in Asia.

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Introduction

In February, 2003, two family members were admitted to intensive care wards in Hong Kong Special Administrative Region with influenza-like respiratory illness. Avian-like H5N1 influenza viruses were isolated from both patients, one of whom succumbed to infection. This was the first time since 1997 that H5N1 viruses had been identified in human beings, and WHO responded by issuing a pandemic alert. Candidate vaccines were immediately sought. The recent outbreak of severe acute respiratory syndrome (SARS) had been a striking example of the rapid and global spread of an emerging infectious disease. However, even the effects of SARS could be dwarfed by those that could arise with the emergence of an influenza pandemic.

Infection caused by the influenza A virus is a zoonosis, and the animal reservoir of this virus is the aquatic bird populations of the world. The compelling epidemiological link between the presence of the virus in poultry in live-bird markets and the appearance of H5N1 in human beings in 1997 suggested that influenza A viruses can be transmitted directly from avian species to man and can cause severe respiratory disease.[1–3] Although control of the 1997 outbreak was achieved by culling millions of birds in the Hong Kong markets,[4] this episode demonstrated that the capability for an effective global response to emerging influenza threats was poor because of technical, legislative, and infrastructural limitations. A disturbing finding that emerged from this event was that the scientific community was unable to produce an effective vaccine even after several years.

The inactivated human influenza vaccines in use today are derived from essentially modified viruses. By exploiting the segmented nature of the influenza A genome, vaccine manufacturers and the laboratories of the WHO influenza network have produced a reassortant virus carrying the circulating virus's gene segments that encode haemagglutinin and neuraminidase, the major targets of neutralising antibodies. The remaining six-gene segments are supplied from PR8, a laboratory-adapted avirulent H1N1 strain.[5] The resulting reassortant virus has the antigenic properties of the circulating strain and the safety and high-yield properties of PR8.

The first batch of inactivated material against the 1997 H5N1 virus was not ready for clinical trial until 7 months after the second case of human infection arose, and even today the effectiveness of vaccine against this virus has not been proven.[6] A key reason for this delay in the production of an H5N1-specific vaccine was the nature of the virus itself. The H5N1 virus is highly pathogenic in human beings and poultry. The agent must be handled only under conditions of at least biosafety level 3 (BSL3), and it can kill fertilised chicken eggs, the standard medium for the reassortment and

propagation of influenza virus before its inactivation and formulation for use in vaccines. These same traits are present in the 2003 H5N1 virus.

The pathogenic nature of these H5N1 viruses is linked to the presence of additional basic residues in haemagglutinin at the site of cleavage, a step required for haemagglutinin activation and, thus, for virus entry into cells.[7–9] To overcome the high pathogenicity of the virus, polybasic aminoacids have to be eliminated. A rapid, reproducible system to achieve these modifications—ie, plasmid-based reverse genetics—has been developed only in the past 4–5 years[10–12] The potential benefits of reverse genetics for the generation and attenuation of vaccine candidates against highly pathogenic and low pathogenic influenza viruses are enormous.[13–15] However, the host specificity of the RNA polymerase I promoter used in the influenza reverse-genetics systems and the required use of an approved cell line limits the practical options for the system's use in the manufacture of human vaccines. The vaccine-candidate reference virus stock described in this report has been produced entirely on a cell substrate licensed for the manufacture of human vaccine, and as such, is—to our knowledge—the first reverse genetically derived influenza vaccine suitable for testing in clinical trials. We describe the construction of a vaccine reference virus in Good Manufacturing Practice (GMP)-grade facilities in less than 4 weeks from the time of virus isolation. Our findings highlight the speed with which new technologies can be implemented in response to influenza pandemic alerts.

Methods

Cells and A/Puerto Rico/8/34 plasmids

We obtained WHO-approved Vero cells (WHO-Vero, X38, p134) from the American Type Culture Collection (Manassas, Virginia, USA). Passage-142 cells (five passages since their removal from a working cell bank) were used for the rescue of the vaccine-candidate virus. The plasmids containing the genes from PR8 have been described elsewhere.[13]

Virus propagation, RNA extraction, PCR amplification, and haemagglutinin and neuraminidase gene cloning

We obtained A/Hong Kong/213/03 (H5N1) that had been passaged in eggs from the WHO influenza network. The virus was isolated and propagated in 10-day-old embryonated chicken eggs. Total RNA was extracted from infected allantoic fluid with use of the RNeasy kit (Qiagen, Valencia, CA, USA) in accordance with manufacturer's instructions. Reverse transcription was carried out with the uni12 primer (5′-AGCA AAAGCAGG-3′) and AMV reverse transcriptase (Roche, Indiana Biochemicals Indianapolis, USA). The removal of the connecting peptide of the haemagglutinin was done with use of PCR with the following primer sets: (1) Bm-HA-1 (5′-TATTCGTCTCAGGGAGCAA AAGCAGGGG-3′) and 739ΔR (5′-TAATCGTC TCGTTTCAATTTGAGGGCTATTTCTGAGCC-3′); and (2) 739ΔF (5′-TAATCGTCTCTGAAA CTAGAGGATTATTTGGAGCTATAGC-3′) and Bm-NS-890r (5′-ATATCGTCTCGTATTAGTAG AAACAAGGGTGTTTT-3′). We amplified the neuraminidase gene of A/Hong Kong/213/03 using the primer pair Ba-NA-1 (5′-TATTGGTCTC AGGGAGCAAAAGCAGGAGT-3′) and Ba-NA-1413r (5′-ATATGGTCTCGTATTAGTAGAAACAAG GAGTTTTTT-3′). PCR products were purified and cloned into the vector pHW2000 as described previously.[11]

Rescue of virus from Vero cells

The rescue of infectious virus from cloned cDNA was done under GMP conditions. Vero cells were grown to 70% confluency in a 75 cm^2 flask, trypsinised (with trypsin-versene), and resuspended in 10 mL of Opti-MEM I (Invitrogen, Carlsbad CA, USA). To 2 mL of cell suspension we added 20 mL of fresh Opti-MEM I; then, we added 3 mL of this diluted suspension to each well of a six-well tissue culture plate (about 1×10^6 cells per well). The plates were incubated at 37°C overnight. The next day, 1 μg of each plasmid and 16 μL of TransIT LT-1 transfection reagent (Panvera, Madison, WI, USA) were added to Opti-MEM I to a final volume of 200 μL and the mixture incubated at room temperature for 45 min. After incubation, the medium was removed from one well of the six-well plate, 800 μL of Opti-MEM I added to the transfection mix, and this mixture added dropwise to the cells. 6 h later, the DNA-transfection mixture was replaced by Opti-MEM I. 24 h after transfection, 1 mL of Opti-MEM I that contained 1 μg/mL L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin (Worthington Biochemicals, Lakewood, NJ, USA) was added to the cells. About 72 h after the addition of TPCK-trypsin, the culture supernatants were harvested and clarified by low-speed centrifugation; we then injected 100 μL of the clarified supernatant into the allantoic cavity of individual 10-day-old pathogen–free embryonated research grade eggs (Charles River SPAFAS, North Franklin, CT, USA).

Pathogenicity testing in chickens

Ten 4-week-old chickens received intravenous injections of 0·1 mL diluted virus (dilution ratio, 1/10). We monitored chickens for signs of disease for 10 days using the Intravenous Pathogenicity Index, approved by the Office of International Epizooites (OIE). Additionally, we took tracheal and cloacal swabs (in 1 mL of media) 3 days and 5 days after infection, and we did assays for the presence of virus by injection of 0·1 mL into all of three 10-day-old embryonated chicken eggs. Haemagglutination activity in the allantoic fluid of these eggs was assessed after incubation at 35°C for 2 days.

Pathogenicity testing in ferrets

We tested pathogenicity of the vaccine in five young adult male ferrets (Marshall's Farms, North Rose, NY, USA) aged 4–8 months (weight about 1·5 kg) that were shown by haemagglutination inhibition assays to be seronegative for currently circulating human influenza A viruses (H3N2, H1N1) and H5N1 viruses. We anaesthetised the ferrets with inhaled isoflurane, and they were then infected intranasally with 10^6 50% egg infectious dose (EID_{50})/mL of vaccine reassortant virus or wildtype virus. We monitored the ferrets once per day for signs of sneezing, inappetence, and inactivity, and we recorded rectal temperatures and bodyweights. 3, 5, and 7 days after infection, the ferrets were anaesthetised with ketamine (25 mg/kg), and we collected nasal washes using 1 mL of sterile phosphate-buffered saline (PBS) containing antibiotics. We measured titres of virus in these washes with EID_{50} assays.

To further assess the pathogenicity of the viruses, we collected tissue samples from lungs, brain, olfactory bulb, spleen, and intestine for virus isolation and histopathological analysis at the time of death or in the case of three ferrets, after euthanasia at day 3 after infection. The tissues were fixed in 10% neutral buffer formalin, processed and embedded in paraffin, sectioned at 5 μg, stained with haematoxylin and eosin and examined by light microscopy in a blinded fashion.

Stability testing in eggs

To test the stability of the vaccine virus on propagation, we made 16 consecutive passages of the virus in embryonated chicken eggs. A 10^{-4} dilution of the virus was made in PBS, and 0·1 mL of the solution was injected into the allantoic cavities of all of four 10-day-old embryonated chicken eggs. Eggs were incubated at 35°C for 1·5–2 days. After incubation, each egg was candled to determine embryo viability before chilling at 4°C. We harvested 2 mL of allantoic fluid from each egg harvested, and samples were pooled together, tested for haemagglutination activity, and then reinjected into another four eggs.

Role of the funding source

The sponsor had no role in study design, in the collection, analysis, and interpretation of data, in the writing of the report or decision to submit this manuscript for publication.

Results

Alteration of haemagglutinin cleavage site and virus rescue

The first challenge we faced in producing a vaccine against A/Hong Kong/213/03 (H5N1) was to attenuate the virus in preparation for mass production. Previous experiences have shown that removal of the basic aminoacids at the haemagglutinin cleavage site substantially attenuates pathogenic influenza viruses.[15–17] Using a PCR-based mutagenesis approach, we replaced the cleavage site encoded by the haemagglutinin gene of A/Hong Kong/213/03 (H5N1) with that of the avirulent A/teal/Hong Kong/W312/97 (H6N1) (figure 1); this modified haemagglutinin gene and the neuraminidase gene of A/Hong Kong/213/03 (H5N1) were cloned individually into the vector pHW2000.[11] The two resulting plasmids and the six plasmids encoding the remaining proteins of PR8[13] were transfected into WHO-approved Vero cells under GMP conditions to rescue the vaccine seed virus, Δ213/PR8. 36–48 h after transfection, isolated areas of cytopathic effect could be seen on the Vero monolayers. Although addition of further 1 μg aliquots of TPCK-treated trypsin every 24 h led to a proportional increase in the cytopathic effect, it was not required for successful virus rescue. The candidate vaccine strain grew to high titres on subsequent amplification in eggs (haemagglutination titres of 1024–2048) and did not cause embryo death. The vaccine seed virus was unable to form plaques on Madin-Darby canine kidney (MDCK) cells in the absence of trypsin, a trait consistent with that of influenza viruses that lack the polybasic cleavage site, and was antigenically indistinguishable from the parental H5N1 virus in haemagglutination inhibition assays. The rescued virus was fully sequenced and was identical to the plasmids used in its creation.

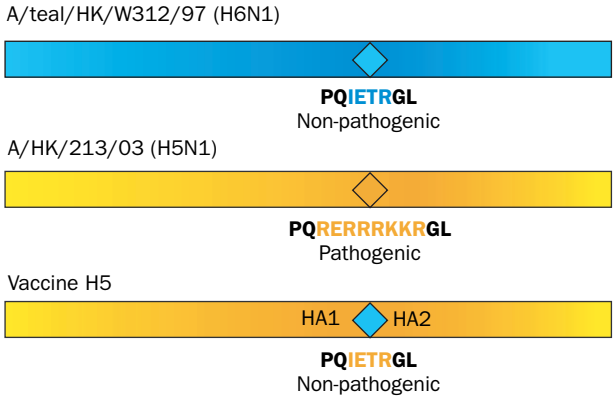

Figure 1: **Creation of haemagglutinin protein of candidate vaccine seed**

Haemagglutinin protein of the candidate vaccine seed (Δ213/PR8) was produced by replacing the connecting peptide of the A/Hong Kong/213/03 haemagglutinin gene with that of the A/Teal/Hong Kong/W312/97 gene.

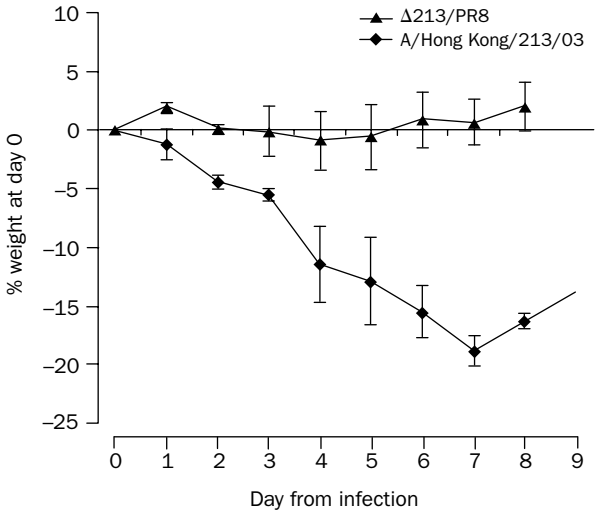

Figure 2: **Weight changes of ferrets infected with wildtype A/Hong Kong/213/03 or Δ213/PR8**

Vertical bars show SD.

Pathogenicity testing of the candidate reference virus

To assess the pathogenicity of the H5N1 vaccine seed virus, we compared the properties of this virus with those of the wildtype A/Hong Kong/213/03 (H5N1) in ferrets and in chickens. By stark contrast with the wildtype virus, which was lethal to all chickens within 48 h of infection, intravenous administration of a 1/10 dilution of Δ213/PR8 did not result in any signs of infection in chickens, and we were unable to detect any virus in swabs of cloacae or tracheae from inoculated birds. Compared with A/Hong Kong/213/03 (H5N1), Δ213/PR8 was attenuated in ferrets that had been inoculated intranasally with 10^6 EID_{50} of virus. Ferrets infected with A/Hong Kong/213/03 had inappetence and weight loss (figure 2), with one infected animal dying 6 days after infection and a second killed 10 days after infection because of hind-limb paralysis. Infection in these animals was characterised by viral shedding until 7 days after infection and replication of virus in the lower respiratory tract and olfactory bulb (as determined by virus isolation). In the A/Hong Kong/213/03 infected animals, there was a mild mononuclear cell infiltrate in the meninges and tracheal submucosal mucous glands and an extensive bronchopneumonia. The pneumatic infiltrate progressed in severity from the bronchi to the pleura. The bronchi and bronchioles contained sloughed necrotic epithelial cells, numerous mononuclear cells, and a few neutrophils. The alveoli were consolidated with inflammatory cells and fibrin (figure 3). By contrast, those ferrets infected with Δ213/PR8 did not lose weight (figure 2) and seemed to remain healthy during the study (14 days) (figure 3). Virus was detected in the nasal washes of these animals at 5 days but not 7 days after infection, and virus was recovered from the upper respiratory tract only. By light microscopy, the meninges and trachea of the Δ213/PR8 infected ferrets did not have an inflammatory infiltrate and only a few neutrophils were noted occasionally in pulmonary bronchi. Our results clearly show that

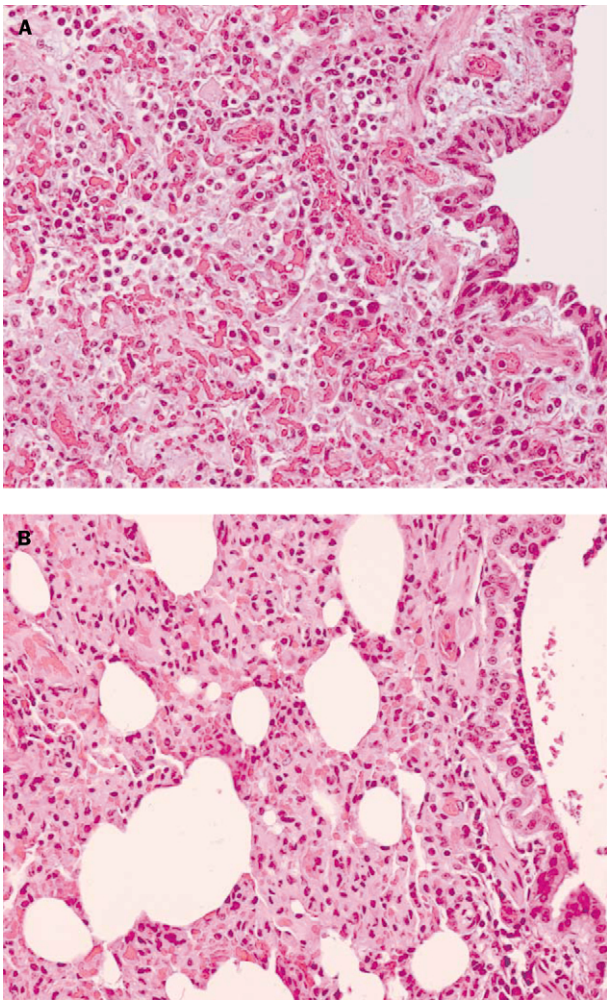

Figure 3: **Ferret lung 3 days after infection with wildtype virus (A) and the reverse genetic virus Δ213/PR8 (B)**
(A) Alveoli are filled with inflammatory cells and the bronchiolar submucosa is oedematous. (B) Alveoli are free of inflammatory cells and there are a few neutrophils on the surface of the bronchiolar epithelium. Magnification ×20.

Δ213/PR8 was attenuated. In view of our findings, this virus can be safely handled with standard precautions in BSL2 containment facilities.

Stability of non-pathogenic phenotype

Because the mechanisms and requirements for the accumulation of basic aminoacids at the haemagglutinin cleavage site are not entirely understood, we wanted to confirm that the altered cleavage site remained stable on multiple passages in embryonated chicken eggs. Such passaging in eggs would occur in transition and amplification of the reference virus to vaccine stock. The rescued virus was stable on continued serial passage in embryonated eggs, and we did not detect any change in nucleotide sequence of the haemagglutinin cleavage site after 16 passages. There was no evidence of changing pathogenicity of the virus and we noted only one dead embryo at passage 15. No haemagglutination activity was evident in this egg and no embryo death was seen in passage 16, which strongly suggests that the death was not related to virus replication. Haemagglutination titres at each passage ranged from 512 to 2048 with no apparent trend of increasing or decreasing titres in subsequent passages.

Discussion

The rapid response in terms of potential vaccine reference virus production to the 2003 H5N1 outbreak differs strikingly from the response to the 1997 episode. This difference is attributable to the new scientific technology available in 2003 and, just as importantly, to the infrastructure for virus surveillance in Hong Kong developed since 1997. The first case of H5N1 influenza in Hong Kong was in May, 1997; yet several months elapsed before this virus was finally characterised as an H5N1 virus. In 2003, the causative agent was identified only hours after admission of the patients to the hospital. The increased awareness, surveillance, and availability of reagents to identify influenza viruses of all subtypes bode well for the rapid identification of viruses that arise from future interspecies transfer events and for the coordination of international vaccine development by WHO. The timely distribution of candidate viruses is a very important step in the development of vaccines for pandemic emergencies. Despite the heightened security and documentation requirements for shipping and receiving potential bioterrorism agents, the H5N1 and SARS outbreaks have shown that in true emergencies, global distribution is feasible.

Although it is pertinent to prepare for future pandemics by stockpiling potential vaccine strains, the H5N1 situation in 2003—and the ongoing H5N1 outbreaks throughout Asia in 2004 (http://www.who.int)—have highlighted the fact that some of the focus of pandemic planning must go into the implementation of technology to rapidly produce vaccines from field isolates. Although viruses similar to A/Hong Kong/213/03 (H5N1) had been circulating in bird populations, these viruses were antigenically distinct, despite high genetic similarities (Guan Y and Peiris JS, unpublished data). That the aminoacid differences are on the globular head of haemagglutinin and seem to be responsible for much of the antigenic difference means that even a vaccine previously prepared from genetically similar precursor viruses might not provide adequate protection. We may well be faced with potential pandemic situations in the future and the rapid production of a matched vaccine will be needed—a point again highlighted by H5N1 outbreaks in 2004. Although the reference virus described in this report was prepared from a virus isolated in a similar geographic region and only a year earlier, it shares only limited antigenic cross-reactivity to the 2004 H5N1 viruses. Hyperimmune sheep serum samples produced against the purified haemagglutinin of Δ213/PR8 has at least a six-fold reduced haemagglutination inhibitory activity against A/Vietnam/1203/04 as compared with A/Hong Kong/213/03. As our findings show, we have the technical capabilities to respond rapidly to outbreaks with a safe and stable reference virus, but there is still much to be accomplished before such viruses can be fully used in pandemic and interpandemic influenza vaccine production.

The use of reverse genetics introduces a number of new processes into influenza vaccine manufacture that are not encountered with standard reassortment methods. One of the most obvious is the need for cultured cells. Although both Vero[18] and MDCK[19,20] cells are in development as substrates for the growth of influenza vaccine, there are additional requirements for the use of cells in reverse genetics. Unfortunately, the number of suitable cell lines is very small. In addition to the regulatory requirements, the choice of cell is also limited by the technology. The plasmid based reverse-genetics systems[10–12] use the species-specific human RNA polymerase I promoter, which

necessitates the use of cells from primate origin. The Vero cell line is probably the only option currently able to meet both regulatory and technical demands. We have shown that Vero cells can be used to successfully rescue H1N1, H3N2, H6N1, and H9N2 viruses on the PR8 backbone using the 8-plasmid system.[21] Others have demonstrated the suitability of Vero cells for alternative influenza virus reverse-genetics systems.[10] Although cultures of Vero cells are easily obtained, only cells from fully tested and licensed cell banks are likely to be acceptable for vaccine manufacture. This issue must be acknowledged and access to such cells must be incorporated as part of future pandemic plans.

That future threats of influenza pandemics will be addressed by the use of the technology described in this report seems inevitable. Despite the presence of low pathogenic surrogate strains, the recent human death from influenza-like illness caused by highly pathogenic H7N7 virus in the Netherlands[22] reinforces the fact that future outbreaks will probably occur in which this reverse-genetics technology provides the logical—and, possibly, the only—way to respond rapidly and effectively. Although our response to the outbreak of H5N1 influenza in 2003 has shown that current scientific capabilities are sufficient to respond to the threat, there are still legal and infrastructural barriers to be overcome.[23] These barriers include licensing and intellectual property issues surrounding what is, essentially, a genetically modified organism. Yet, these difficulties are not insurmountable and pandemic scares such as the 2003 and ongoing 2004 H5N1 outbreaks are forcing commercial and regulatory parties to address these issues with some urgency. With the development of the 2003 H5N1 vaccine reference virus, and ongoing attempts to create the same for the 2004 virus, the challenge in responding to a threat of an influenza pandemic must now be supported by the large-scale manufacture of the vaccine and by clinical trials of a new vaccine manipulated by reverse genetics.

Contributors

R J Webby, D R Perez, J S Coleman, J H Knight, E I Tuomanen, R G Webster designed the study; R J Webby did much of the construction of the vaccine seed virus; D R Perez developed and constructed plasmid templates; Y Guan and J S Peiris characterised and isolated the initial H5N1 virus; J E Rehg participated in the design and analysis of animal safety testing of the candidate H5N1 vaccine seed virus; E A Govorkova participated in the safety testing of the candidate H5N1 vaccine seed virus; L R McClain-Moss participated in the preparation of GMP documentation of the process and was involved in the reconstitution of the vaccine seed virus.

Conflict of interest statement

None declared. The corresponding author has had full access to all the data in the study and has had the final responsibility for the decision to submit this manuscript for publication.

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