Generation and characterization of a cold-adapted influenza A H9N2 reassortant as a live pandemic influenza virus vaccine candidate

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Abstract

H9N2 subtype influenza A viruses have been identified in avian species worldwide and were isolated from humans in 1999, raising concerns about their pandemic potential and prompting the development of candidate vaccines to protect humans against this subtype of influenza A virus. Reassortant H1N1 and H3N2 human influenza A viruses with the internal genes of the influenza A/Ann Arbor/60 (H2N2) (AA) cold-adapted (ca) virus have proven to be attenuated and safe as live virus vaccines in humans. Using classical genetic reassortment, we generated a reassortant virus (G9/AA ca) that contains the hemagglutinin and neuraminidase genes from influenza A/chicken/Hong Kong/G9/97 (H9N2) (G9) and six internal gene segments from the AA ca virus. When administered intranasally, the reassortant virus was immunogenic and protected mice from subsequent challenge with wild-type H9N2 viruses, although it was restricted in replication in the respiratory tract of mice. The G9/AA ca virus bears properties that are desirable in a vaccine for humans and is available for clinical evaluation and use, should the need arise.

Keywords: Live attenuated vaccines; Influenza H9N2; Pandemic influenza vaccines

1. Introduction

Influenza A viruses are divided into subtypes on the basis of the antigenicity of their surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA); influenza viruses bearing 15 HA and 9 NA subtypes have been isolated from birds, but only H1N1, H2N2, and H3N2 subtype viruses have circulated widely and caused epidemic disease in humans in the last century. Aquatic birds serve as a reservoir from which new subtypes of influenza A viruses enter the human population. In the last 10 years, human infections with avian influenza viruses (AIV) of three subtypes, H7, H5 and H9, have been detected on four occasions [1]. In 1996, an H7N7 virus was isolated from the eye of a woman with conjunctivitis who kept ducks in UK [2]. In 1997, H5N1 AIV transmitted from birds to humans resulted in 18 infections and 6 deaths in Hong Kong [3–5]. H9N2 subtype influenza viruses were isolated from pigs in Hong Kong in 1998 and subsequently from two sick children in 1999; six additional human infections were reported from China [6–9].

Unlike H7N7 and H5N1 viruses, whose circulation is often geographically restricted, H9N2 subtype AIVs have been isolated worldwide from wild and domestic birds [10–13]. Detailed antigenic and molecular analysis of the H9N2 influenza viruses isolated from avian species in southeastern China revealed the presence of three groups, represented by A/quail/Hong Kong/G1/97 (G1: G1 group), A/chicken/Hong Kong/G9/97 (G9: G9 group) and A/duck/Hong Kong/Y439/97 (Korea group) [6]. The two human H9N2 isolates from Hong Kong belong to the G1 group, while at least one human isolate from China and the two isolates from pigs in Hong Kong belong to the G9 group [7,14]. G1 and G9-like H9N2 viruses are still widespread in domestic poultry in China and thus remain a potential source for further human infections [15]. However, vaccines to protect humans against H9N2 viruses are not currently available.
2. Materials and methods

2.1. Viruses and antisera

Influenza viruses G9 and AA ca were propagated in the allantoic cavity of 10-day-old embryonated specific-pathogen free (SPF) eggs; the AA ca virus Clone 7-P1 was grown, aliquoted as Lot E-172 and tested for adventitious contamination. The AA ca virus was biologically cloned three times by limiting dilution and attenuation (att) phenotypes which are specified by mutations in the internal genes. Reassortant H1N1 and H3N2 human influenza A viruses with the six internal gene segments of the AA ca virus have been repeatedly demonstrated to bear these phenotypes and extensive evaluation in humans has proven them to be attenuated and safe as live virus vaccines (reviewed in [16-18]). An H3N1/AA ca reassortant virus that bears a modified avian influenza virus HA and avian NA gene in the AA ca background expressed the ca, ts, and att phenotypes that were displayed by the AA ca donor strain and by reassortant human ca influenza viruses [19]. Although several issues must be considered before a live attenuated influenza vaccine bearing HA, NA and HA genes is used widely, all options could be considered in the event of a pandemic only if appropriate candidate vaccines were available. Therefore, we generated a 6:2 reassortant virus, G9/AA ca, that contains the HA and NA genes from the avian influenza A/chicken/Hong Kong/G9/97 (H9N2) virus and six internal protein genes from the AA ca virus. The reassortant virus expressed ca and ts phenotypes, was restricted in replication in mice, and did not exhibit a high pathogenicity phenotype in chickens. The protective efficacy of the reassortant virus as a live attenuated vaccine was established in a mouse model.

2.2. Laboratory facility

All experiments, including animal studies with infectious wild-type (wt) avian H9 viruses and the reassortant virus, were conducted using biosafety level (BSL) 3 + containment procedures. Investigators wore appropriate respirator equipment (RACAL Health and Safety Inc., Frederick, MD).

2.3. Generation of the reassortant virus

To generate the reassortant virus by co-infection, 100 μl of ultraviolet light-treated G9 virus and 50 μl of the AA ca virus were mixed with 850 μl phosphate-buffered saline (PBS), and the mixture was injected into the allantoic cavity of 10-day-old embryonated eggs. The allantoic fluid from the eggs was harvested after incubation at 35 °C for 24 h. To select for viruses that lack the HA and NA of the AA ca donor virus, 50 μl fluid was mixed with 600 μl of serially (four-fold) diluted anti-AA ca post-infection SPF mouse antisera and incubated at room temperature for 30 min prior to being injected into eggs. Allantoic fluid was harvested after incubation at 35 °C for 48 h and tested for evidence of hemagglutination. The sample with hemagglutinating activity at the lowest dilution of antisera was used for a second round of antibody selection and the resulting virus was biologically cloned three times by limiting dilution in eggs. At each passage, the genotype of the virus was determined by reverse transcription-polymerase chain reaction (RT-PCR), using strain- and segment-specific primers (primer sequences available upon request). RNA was extracted from allantoic fluid by using an RNeasy Mini Kit (Qiagen, Valencia, CA), and RT-PCR was performed by using QIAGEN OneStep RT-PCR Kit (Qiagen, Chatsworth, CA). Sequencing reactions were performed with a Big Dye-Terminator Cycle Sequencing Ready Reaction Kit (PE/Applied Biosystems, Foster City, CA). Samples were electrophoresed and analyzed on a model 3100 DNA sequencer (PE/Applied Biosystems).

2.4. Immunization of ferrets and analysis of the antigenicity of the reassortant G9/AA ca virus

Two 8-month-old female ferrets (Jackson Laboratories) were anesthetized with ketamine-xylazine and immunized i.n. with 10⁶ EID₅₀ of the G9/AA ca virus at 14 days post-infection (p.i.), blood samples were collected. Sera were treated with Vibrio cholerae (Denka-Seiken, Tokyo, Japan) receptor-destroying enzyme before being tested for the presence of hemagglutination-inhibiting (HI) antibody.

2.5. Phenotypic analysis of the reassortant G9/AA ca virus

The ca phenotype of the parent and reassortant viruses was determined by comparing the infectivity at 25 and 35 °C of
virus stocks that were propagated at 35 °C. This was done by inoculating serial 10-fold dilutions of virus stock into eggs (four eggs per dilution) and incubating them at 25 and 35 °C. After 48 h, allantoic fluid from each egg was harvested and tested for the ability to agglutinate turkey red blood cells, and the infectivity of the stock virus (expressed as EID50 titer) at the two temperatures was calculated. Cold-adapted viruses have adapted to replicate efficiently at low temperatures; the ca phenotype is defined as a less than 100-fold reduction of virus titer at 25 °C compared with that at 33–36 °C. Viruses whose ability to form plaques at high temperatures (≥39 °C) is reduced more than 100-fold compared with that seen at permissive temperatures of 33–36 °C are considered ts. The ts phenotype of the parent and reassortant viruses was determined by evaluating the efficiency of plaque formation in MDCK cells at 32, 37, 38, 39 and 40 °C [20].

Replication of the reassortant virus in the upper and lower respiratory tract of mice was evaluated as a measure of the at phenotype. A dose of 103 50% tissue culture infectious doses (TCID50) of the virus was administered i.n. to eighteen 6–8-week-old female BALB/c mice; nasal turbinates and lungs of six mice per day were collected on days 2, 3 and 4, respectively, for virus titration in MDCK cells [21].

2.6. Infectivity of the reassortant G9/AA ca virus in chickens

Ten chickens were inoculated with 106 EID50 of each virus in 0.1 ml i.n. to determine the level of replication of the viruses in different tissues. In a separate experiment, eight chickens per group received the standard 0.2 ml of a 10-fold dilution of each stock virus by the intravenous (i.v.) route for pathotyping [22]; oropharyngeal and cloacal swabs were collected from each chicken on day 3 p.i. or on the day of death if chickens died prior to day 3, and all samples were inoculated into embryonated eggs for virus isolation. Two chickens per group were euthanized on day 3 p.i. and tissues were stained for routine histologic examination or for demonstration of influenza nucleoprotein by immunohistochemistry [23]. The remaining chickens were observed for clinical signs of illness and death for 14 days, at which time serum samples were harvested and tested for presence of antibodies by agar gel precipitation (AGP). In order to assess the infectivity of the parent and reassortant viruses for chickens, 50% chicken infectious doses (CID50) titer were determined by inoculating 10 chickens (4-week-old White Leghorn or White Rock) i.n. with 0.1 ml of serial 10-fold dilutions of each virus. At 14 days p.i., sera were collected and tested by AGP for serologic evidence of infection; CID50 titers were calculated by the method of Spearman and Karber [24].

For statistical analysis, virus titers between the three groups (G9, AA ca and G9/AA ca) for each route of administration and type of swab were subjected to a test for normality followed by a Kruskal–Wallis test to compare. Data from each route of administration and swab type were compared in a Student–Newman–Keuls all pairwise multiple comparison procedure.

2.7. Efficacy of protection against homologous and antigenically distinct wt H9N2 viruses

Groups of twelve 6–8-week-old BALB/c mice were immunized i.n. with 50 μl of 105 TCID50 G9, AA ca or G9/AA ca viruses or 50 μl of PBS. Sera were collected 4 weeks after infection for HI antibody analysis. Mice were then challenged i.n. with 103 TCID50 of either G9 or HK/1073 (G1-like) virus. Nasal turbinates and lungs were collected on day 3 after challenge and the titer of virus present in the tissue homogenates was determined in MDCK cells. Virus titers were compared in a two-tailed t-test.

3. Results and discussion

3.1. Generation and genetic characterization of an H9N2 cold-adapted reassortant virus, G9/AA ca

A reassortant virus, G9/AA ca, which derives the HA and NA genes from the avian H9N2 G9 virus and six internal protein genes from the AA ca vaccine donor virus, was generated by co-infection of SPF eggs with the two parent viruses and selection of the appropriate progeny viruses in the presence of mouse anti-H2N2 antibody. Sequencing the full-length HA and NA genes and partial sequence analysis of each internal protein gene confirmed the genotype of the reassortant G9/AA ca virus.

3.2. The ca, ts, and att phenotypes of the reassortant G9/AA ca virus

As shown in Fig. 1A, the AA ca and G9/AA ca viruses replicated equally well in eggs at 25 and 35 °C, while the G9 virus was clearly restricted in replication at 25 °C. These results confirmed that the reassortant G9/AA ca virus exhibits the ca phenotype specified by the internal protein genes of the AA ca virus.

As shown in Fig. 1B, the G9/AA ca and AA ca viruses replicated equally well at 32 and 37 °C, but failed to replicate at higher temperatures. In contrast, as is typical of AIVs, the G9 virus plaqued efficiently at 32–40 °C. These results indicate that the G9/AA ca reassortant and the AA ca parent viruses exhibited the ts phenotype with a shut-off temperature of 38 °C, while the G9 virus did not display the ts phenotype.

The level of replication of the G9/AA ca reassortant virus in the respiratory tract of mice, which have a body temperature of 37 °C like that of humans, is shown in Table 1. Virus was isolated from the nasal turbinates and lungs of all G9 virus-inoculated mice; mean titers ranged from 2.0 to 4.0 log10 TCID50 in the nasal turbinates and 3.0 to 3.7 log10 TCID50 in the lungs on days 2, 3, or 4 p.i. In the AA ca
Fig. 1. The reassortant G9/AA ca virus exhibits ca and ts phenotypes. (A) The ca phenotype was verified by titrating the viruses in eggs at 25 and 35 °C; the G9/AA ca and AA ca viruses replicate equally well in eggs at 25 and 35 °C. (B) The ts phenotype was tested by plaque assay at five different temperatures; the G9 virus replicates equally well at temperatures between 32 and 40 °C, while the G9/AA ca and AA ca viruses do not plaque at temperatures of 38 °C or higher.

Table 1

<table>
<thead>
<tr>
<th>Viruses</th>
<th>Virus replication in nasal turbinates on day 2</th>
<th>Virus replication on lungs on day 3</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Virus detected/total</td>
<td>Titer</td>
</tr>
<tr>
<td>G9</td>
<td>6/6</td>
<td>2.0 ± 0.4</td>
</tr>
<tr>
<td>AA ca</td>
<td>6/6</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>G9/AA ca</td>
<td>4/6</td>
<td>1.4 ± 0.4</td>
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</tbody>
</table>

*Groups of 6–8-week-old BALB/c mice received 10^5 TCID50 of virus i.n. Six mice from each group were euthanized on the indicated day; nasal turbinate and lung tissues were removed and homogenized in 1 ml PBS and cultured in MDCK cells; virus titers were expressed as mean ± S.E. log10 TCID50/ml. Samples from which the virus was not detected in 0.1 ml of organ homogenate were assigned a numeric value of 0.5 for calculation.

The reassortant and the parent viruses were administered to SPF chickens to determine the level of tissue-specific replication and their ability to cause severe morbidity and mortality following i.v. injection (pathotyping). On i.v. administration, although no clinical disease signs or deaths were observed, the wt G9 virus replicated to high titer in the oropharynx and cloaca. All chickens developed antibodies not isolated from the lungs of any mice on any of the 3 days. Thus, the replication of the G9/AA ca virus was significantly restricted in the upper respiratory tract and completely restricted in the lower respiratory tract, reflecting the presence of the att phenotype in the G9/AA ca virus.

3.3. Antigenicity of the reassortant G9/AA ca virus in ferrets

Post-infection ferret antisera were prepared in order to determine whether the antigenicity of the HA of the G9 virus was preserved in the reassortant virus. HI antibody titters induced by the G9/AA ca virus against the homologous G9 virus and antigenically heterogeneous H9N2 viruses, HK/1073 (G1-like), were 1280 and 40, respectively. The HI titers induced by the G9 virus against the same two viruses (G9 and HK/1073) were 5120 and 160, respectively. The Korean H9N2 virus failed to react in the HI assay with either of the antisera. Although the titer of antibodies induced in ferrets by the reassortant virus was lower than that induced by the G9 parent virus, the pattern of HI reactivity elicited by the G9 /AA ca virus was similar to that elicited by the G9 parent virus, indicating that the antigenicity of the G9 HA was conserved in the reassortant virus. The reduced immunogenicity of the G9 /AA ca reassortant virus in ferrets is likely a result of reduced level of replication of a ts virus in an animal with a high body temperature; AA ca reassortant viruses have consistently exhibited restricted replication in ferrets [25].

3.4. Pathotyping and replication in chickens

The reassortant and the parent viruses were administered to SPF chickens to determine the level of tissue-specific replication and their ability to cause severe morbidity and mortality following i.v. injection (pathotyping). On i.v. administration, although no clinical disease signs or deaths were observed, the wt G9 virus replicated to high titer in the oropharynx and cloaca. All chickens developed antibodies...
to influenza (Table 2). The chickens that were euthanized on day 3 showed mild tracheitis and severe nephrosis, with abundant avian influenza (AI) viral antigen observed in the necrotic tubules. The AA ca virus did not cause illness or deaths in chickens; although virus was not recovered from the oropharynges or cloaca of chickens that received the AA ca virus, five of eight chickens developed an antibody response. When the reassortant G9/AA ca virus was administered to chickens, one chicken died on day 4 and one on day 5. However, there were no signs of illness or death or evidence of viral replication in the oropharynges or cloaca of the surviving chickens and only 33% developed an antibody response. Because it was difficult to attribute the two deaths to influenza when the remaining chickens did not shed virus or exhibit signs of illness, the pathotyping experiment was repeated twice with reassortant G9/AA ca virus administered i.v. to 16 more chickens; there were no deaths in either experiment and it was concluded that the cause of death in the first experiment was unrelated to the replication of the G9/AA ca virus. Chickens that received the G9/AA ca reassortant virus and were euthanized on day 3 failed to demonstrate viral antigen in their respiratory tract. The combined data from the three experiments indicate that the G9/AA ca reassortant virus is not highly pathogenic for chickens.

When the viruses were administered i.n., seven (of eight) chickens that received the G9 virus shed high titers of virus in the oropharynx and low titers from the cloaca, and all eight chickens developed an antibody response. The chickens that were euthanized on day 3 had moderately severe heterophilic to necrotizing tracheitis and mild heterophilic to lymphocytic bronchitis. Moderate amounts of AI viral antigen were present in the respiratory epithelium of the trachea and bronchi. In contrast, none of the chickens that received the AA ca and G9/AA ca viruses shed detectable virus and none developed an antibody response (Table 2). The antibody response that was detected in 30–60% of chickens that received AA ca and G9/AA ca i.v. may have been induced by direct delivery of antigen to the immune system; similar results were found with the HSN1 ca reassortant virus [19].

The CID50 of the wt G9 virus was $\leq 2.9 \log_{10}$ EID50, while an endpoint was not reached in determining the CID50 of the G9/AA ca virus (greater than $5.97 \log_{10}$ EID50), indicating that the genotype of the G9/AA ca virus made the virus poorly infectious for chickens by the i.n. route. The lack of replication of the G9/AA ca virus in chickens is likely a result of poor replication of a ts virus in a host with a high body temperature and is not an unexpected finding for a ts virus with a 38 °C shut-off temperature in an animal host with a body temperature $>40$ °C. These observations suggest that the manufacture and use of the G9/AA ca virus as a live virus vaccine for humans will not pose a threat to the poultry industry.

### Table 2

<table>
<thead>
<tr>
<th>Route of administration</th>
<th>Virus</th>
<th>Morbidity (dead/total)</th>
<th>Mortality (dead/total)</th>
<th>Virus isolation from swabs1</th>
<th>Antibody detected/total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Oropharyngeal</td>
<td>Cloacal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.v.</td>
<td>G9</td>
<td>0/8</td>
<td>0/8</td>
<td>$4.3 \pm 0.9$ A</td>
<td>0/8</td>
</tr>
<tr>
<td></td>
<td>AA ca</td>
<td>0/8</td>
<td>0/8</td>
<td>$\geq 0.9B$</td>
<td>0/8</td>
</tr>
<tr>
<td></td>
<td>G9/AA ca</td>
<td>0/24</td>
<td>0/24</td>
<td>$\geq 0.9B$</td>
<td>0/8</td>
</tr>
<tr>
<td>i.n.</td>
<td>G9</td>
<td>0/8</td>
<td>0/8</td>
<td>$4.7 \pm 1.3A$</td>
<td>0/8</td>
</tr>
<tr>
<td></td>
<td>AA ca</td>
<td>0/8</td>
<td>0/8</td>
<td>$\geq 0.9B$</td>
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<tr>
<td></td>
<td>G9/AA ca</td>
<td>0/8</td>
<td>0/8</td>
<td>$\geq 0.9B$</td>
<td>0/8</td>
</tr>
</tbody>
</table>

1. Groups of chickens were infected i.v. with 0.2 ml 1:10 dilution of stock viruses or i.n. with 0.1 ml $10^6$ EID50 of each virus.
2. Oropharyngeal and cloacal swabs were collected for virus titration on day 3 after inoculation. Virus titers are expressed as log_{10} mean ± S.D.
3. EID50/ml from eight chickens. All oropharyngeal and cloacal swabs from which virus was not isolated were assigned a numeric value of 0.9 for calculations.
4. In the first experiment, two of eight chickens died on days 4 and 5 p.i., respectively; however, no viral antigens were detected.
5. Chickens were euthanized 2 weeks after infection and sera were collected; presence of antibody was determined by agar gel precipitin test.
6. The results were found with the H5N1 wt virus vaccine as a live virus vaccine was evaluated in mice.
Fig. 2. G9/AA virus live virus vaccine provides complete protection from replication of homologous and heterologous H9N2 challenge viruses in the A (upper) and B (lower) respiratory tract. Mice (12 per group) were immunized i.n. with 10^7 TCID_{50} of G9, G9/AA ca, AA ca or PBS, and challenged 4 weeks later with 10^7 TCID_{50} of G9 or HK/1073 H9N2 wt viruses. Nasal turbinates (A: upper respiratory tract) and lungs (B: lower respiratory tract) were collected on day 3 after challenge and titrated in MDCK cells. The lower limit of detection, indicated with a dashed line, was 0.5 TCID_{50}. Titer are expressed as mean virus titer ± S.E.; *P < 0.05 compared with the titer in the PBS immunized group.

AA ca-inoculated group were lower than those of the PBS inoculated mice, presumably as a result of heterosubtypic immunity [26] or because the viruses share an N2 NA [27]. Thus, a reassortant virus bearing unmodified avian H9 HA and N2 NA glycoproteins was restricted in replication in mice but could infect and elicit HI antibodies and protect mice against subsequent challenge with homologous and heterologous wt H9N2 viruses. The G9/AA ca virus induced complete protection of the respiratory tract in mice against challenge with wt H9N2 viruses, even though the titer of HI antibodies induced by G9/AA ca against the G9 virus was four-fold lower than that induced by the G9 wt virus; the lower HI antibody titers induced by the reassortant virus are likely a result of the restricted replication of the virus.

In summary, the G9/AA ca reassortant virus exhibits the ca, ts, and ut phenotypes of the AA ca parent virus and is antigenically similar to the G9 parent virus. The data from this study provide further evidence that the internal protein genes of the AA ca virus specify these phenotypes irrespective of the subtype of the accompanying HA and NA genes, in contrast to the avian human reassortant viruses that were discarded as potential vaccine candidates because their reag- totenicity was clearly influenced by the accompanying surface glycoprotein genes [28]. A single dose of G9/AA ca administered i.n. as a live virus vaccine was immunogenic and protected mice against subsequent challenge with homologous and antigenically heterologous H9N2 wt viruses. The restricted replication of the G9/AA ca virus in chickens suggests that large-scale manufacturing will not pose a threat to the poultry industry. The in vitro and in vivo phenotypes and the safety profile in chickens exhibited by the G9/AA ca reassortant virus make this virus a suitable candidate pandemic influenza vaccine for evaluation in humans. Clinical trials are planned to establish the safety, infectivity, and immunogenicity of this vaccine in healthy adults. In order to decrease the risk of reassortment of the vaccine virus with circulating human influenza viruses, the clinical trials will be carried out at a time of year when human influenza vaccines are not circulating in the community and the contacts of the vaccine recipients will receive the human influenza vaccine within 4 weeks of the study. The vaccine recipients will be housed in an isolation unit until they cease to shed the vaccine virus from their respiratory tract.

The need for such candidate vaccines is based on the fact that H9N2 subtype influenza viruses have been isolated from domestic poultry and wild birds worldwide and that the receptor specificity of G1- and G9-like H9N2 viruses is similar to that of human influenza A H3N2 viruses [29]. H9N2 viruses have the ability to infect mammals without prior adaptation in an intermediate host, suggesting that this AIV subtype currently presents a substantial pandemic threat. An optimal public health response in the event of a potential pandemic requires that vaccines be available to prevent infection with minimum delay. An important approach to pandemic preparedness is to generate and evaluate candidate vaccines against influenza A subtypes that are recognized to have pandemic potential, prior to their actual spread. The generation and evaluation of an H9N2 vaccine is a model of this approach. A significant concern associated with the use of a live attenuated influenza vaccine bearing genes derived from an avian influenza virus is the risk of reassortment of the vaccine virus with a circulating influenza virus, resulting in a novel subtype of influenza that could spread in the human population. Although such a reassortment event may not be of great significance in the face of widespread disease due to a pandemic strain of influenza, it would clearly be an unfavorable outcome if the threatened pandemic did not materialize. For example, it would not be advisable to implement a live attenuated vaccine in an abortive epidemic like the H5N1 outbreak in Hong Kong in 1997, but it may be a valuable control measure when an epidemic is widespread and involves several continents. This risk would be carefully considered by public health authorities before a decision is made to introduce a live attenuated vaccine in a threatened pandemic.

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References