shi, Yamagata 992-0025, Japan. [9]Department of Virology III, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-shi, Tokyo 208-0011, Japan. [10]Influenza Virus Research Center, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-shi, Tokyo 208-0011, Japan. [11]Infectious Disease Surveillance Center, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-shi, Tokyo 208-0011, Japan.

Authors' contributions

AF, HK, HT, MT, SY, and NO designed research; MA, KGS, HT, KM, MN, MY, and AN performed research; HT, AF, and MA contributed analytic tools, HK, AR, YO, YK, and MM analyzed data; HK, HT, and YO wrote the paper. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests. The authors alone are responsible for the content and writing of the paper.

Received: 9 February 2011 Accepted: 10 June 2011
Published: 10 June 2011

References

1. Tregoning JS, Schwarze J: **Respiratory viral infections in infants: causes, clinical symptoms, virology, and immunology.** *Clin Microbiol Rev* 2010, **23(1)**:74-98.
2. Monto AS: **Occurrence of respiratory virus: time, place and person.** *Pediatr Infect Dis J* 2004, **23(1 Suppl)**:S58-64.
3. Fabbiani M, Terrosi C, Martorelli B, Valentini M, Bernini L, Cellesi C, Cusi MG: **Epidemiological and clinical study of viral respiratory tract infections in children from Italy.** *J Med Virol* 2009, **81(4)**:750-6.
4. Busse WW, Lemanske RF Jr, Gern JE: **Role of viral respiratory infections in asthma and asthma exacerbations.** *Lancet* 2010, **376(9743)**:826-34.
5. Braman SS: **The global burden of asthma.** *Chest* 2006, **130(1 Suppl)**:4S-12S.
6. Pierangeli A, Gentile M, Di Marco P, Pagnotti P, Scagnolari C, Trombetti S, Lo Russo L, Tromba V, Moretti C, Midulla F, Antonelli G: **Detection and typing by molecular techniques of respiratory viruses in children hospitalized for acute respiratory infection in Rome, Italy.** *J Med Virol* 2007, **79(4)**:463-8.
7. Kusel MM, de Klerk NH, Kebadze T, Vohma V, Holt PG, Johnston SL, Sly PD: **Early-life respiratory viral infections, atopic sensitization, and risk of subsequent development of persistent asthma.** *J Allergy Clin Immunol* 2007, **119(5)**:1105-10.
8. Kato M, Tsukagoshi H, Yoshizumi M, Saitoh M, Kozawa K, Yamada Y, Maruyama K, Hayashi Y, Kimura H: **Different cytokine profile and eosinophil activation are involved in rhinovirus- and RS virus-induced acute exacerbation of childhood wheezing.** *Pediatr Allergy Immunol* 2011, **22(1 Pt 2)**:e87-94.
9. Kuehni CE, Spycher BD, Silverman M: **Causal links between RSV infection and asthma: no clear answers to an old question.** *Am J Respir Crit Care Med* 2009, **179(12)**:1079-80.
10. Cane PA: **Molecular epidemiology of respiratory syncytial virus.** *Rev Med Virol* 2001, **11(2)**:103-16.
11. Mackay IM: **Human rhinoviruses: the cold wars resume.** *J Clin Virol* 2008, **42(4)**:297-320.
12. Turner RB, Couch RB: **Rhinoviruses.** In *Fields Virology*.. 5 edition. Edited by: Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Strausl SE. Philadelphia: Lippincott Williams 2007:895-909.
13. Henrickson KJ: **Parainfluenza viruses.** *Clin Microbiol Rev* 2003, **16(2)**:242-64.
14. Kahn JS: **Newly discovered respiratory viruses: significance and implications.** *Curr Opin Pharmacol* 2007, **7(5)**:478-83.
15. Piralla A, Rovida F, Campanini G, Rognoni V, Marchi A, Locatelli F, Gerna G: **Clinical severity and molecular typing of human rhinovirus C strains during a fall outbreak affecting hospitalized patients.** *J Clin Virol* 2009, **45(4)**:311-7.
16. Hishinuma-Igarashi I, Mizuta K, Saito Y, Ohuchi Y, Noda M, Akiyama M, Sato H, Tsukagoshi H, Okabe N, Tashiro M, Kimura H: **Phylogenetic analysis of human bocavirus (HBoV) detected from children with acute respiratory infection in Japan.** *J Infect* 2009, **58(4)**:311-3.
17. Mizuta K, Hirata A, Suto A, Aoki Y, Ahiko T, Itagaki T, Tsukagoshi H, Morita Y, Obuchi M, Akiyama M, Okabe N, Noda M, Tashiro M, Kimura H: **Phylogenetic and cluster analysis of human rhinovirus species A (HRV-A) isolated from children with acute respiratory infections in Yamagata, Japan.** *Virus Res* 2010, **147(2)**:265-74.
18. Parveen S, Sullender WM, Fowler K, Lefkowitz EJ, Kapoor SK, Broor S: **Genetic variability in the G protein gene of group A and B respiratory syncytial viruses from India.** *J Clin Microbiol* 2006, **44(9)**:3055-64.
19. Ishiko H, Shimada Y, Yonaha M, Hashimoto O, Hayashi A, Sakae K, Takeda N: **Molecular diagnosis of human enteroviruses by phylogeny-based classification by use of the VP4 sequence.** *J Infect Dis* 2002, **185(6)**:744-54.
20. Olive DM, Al-Mufti S, Al-Mulla W, Khan MA, Pasca A, Stanway G, Al-Nakib W: **Detection and differentiation of picornaviruses in clinical samples following genomic amplification.** *J Gen Virol* 1990, **71(Pt 9)**:2141-7.
21. Takao S, Shimozono H, Kashiwa H, Matsubara K, Sakano T, Ikeda M, Okamoto N, Yoshida H, Shimazu Y, Fukuda S: **The first report of an epidemic of human metapneumovirus infection in Japan: clinical and epidemiological study.** *Kansenshogaku Zasshi* 2004, **78(2)**:129-37.
22. Echevarría JE, Erdman DD, Swierkosz EM, Holloway BP, Anderson LJ: **Simultaneous detection and identification of human parainfluenza viruses 1, 2, and 3 from clinical samples by multiplex PCR.** *J Clin Microbiol* 1998, **36(5)**:1388-91.
23. WHO information for laboratory diagnosis of pandemic (H1N1) 2009 virus in humans - revised:[http://www.who.int/csr/resources/publications/swineflu/diagnostic_recommendations/en/index.html].
24. Miura-Ochiai R, Shimada Y, Konno T, Yamazaki S, Aoki K, Ohno S, Suzuki E, Ishiko H: **Quantitative detection and rapid identification of human adenoviruses.** *J Clin Microbiol* 2007, **45(3)**:958-67.
25. Allander T, Tammi MT, Eriksson M, Bjerkner A, Tiveljung-Lindell A, Andersson B: **Cloning of a human parvovirus by molecular screening of respiratory tract samples.** *Proc Natl Acad Sci USA* 2005, **102(36)**:12891-6.
26. Saitou N, Nei M: **The neighbor-joining method: a new method for reconstructing phylogenetic trees.** *Mol Biol Evol* 1987, **4(4)**:406-25.
27. Goto-Sugai K, Tsukagoshi H, Mizuta K, Matsuda S, Noda M, Sugai T, Saito Y, Okabe N, Tashiro M, Kozawa K, Tanaka R, Morita Y, Nishina A, Kimura H: **Genotyping and phylogenetic analysis of major genes in respiratory syncytial virus isolated from infants with bronchiolitis in Japan.** *Jpn J Infect Dis* 2010, **63(6)**:393-400.
28. Rosenthal LA, Avila PC, Heymann PW, Martin RJ, Miller EK, Papadopoulos NG, Peebles RS Jr, Gern JE, Infections and Asthma Committee, Environmental and Occupational Respiratory Diseases Interest Section, American Academy of Allergy, Asthma & Immunology: **Viral respiratory tract infections and asthma: the course ahead.** *J Allergy Clin Immunol* 2010, **125(6)**:1212-7.
29. Gern JE: **Mechanisms of virus-induced asthma.** *J Pediatr* 2003, **142(2 Suppl)**:S9-13, discussion S13-4.
30. Nakamura M, Itokazu K, Taira K, Kawaki T, Kudaka J, Nidaira M, Okano S, Koja Y, Tamanaha K, Kimura H, Noda M: **Genotypic and phylogenetic analysis of the G gene of respiratory syncytial virus isolates in Okinawa, Japan, 2008.** *Jpn J Infect Dis* 2009, **62(4)**:326-7.
31. Martinello RA, Chen MD, Weibel C, Kahn JS: **Correlation between respiratory syncytial virus genotype and severity of illness.** *J Infect Dis* 2002, **186(6)**:839-42.
32. Riccetto AG, Ribeiro JD, Silva MT, Almeida RS, Arns CW, Baracat EC: **Respiratory syncytial virus (RSV) in infants hospitalized for acute lower respiratory tract disease: incidence and associated risks.** *Braz J Infect Dis* 2006, **10(5)**:357-61.
33. Awomoyi AA, Rallabhandi P, Pollin TI, Lorenz E, Sztein MB, Boukhvalova MS, Hemming VG, Blanco JC, Vogel SN: **Association of TLR4 polymorphisms with symptomatic respiratory syncytial virus infection in high-risk infants and young children.** *J Immunol* 2007, **179(5)**:3171-7.
34. Collins PL, Graham BS: **Viral and host factors in human respiratory syncytial virus pathogenesis.** *J Virol* 2008, **82(5)**:2040-55.
35. Rawlinson WD, Waliuzzaman Z, Carter IW, Belessis YC, Gilbert KM, Morton JR: **Asthma exacerbations in children associated with rhinovirus but not human metapneumovirus infection.** *J Infect Dis* 2003, **187(8)**:1314-8.
36. Miller EK, Edwards KM, Weinberg GA, Iwane MK, Griffin MR, Hall CB, Zhu Y, Szilagyi PG, Morin LL, Heil LH, Lu X, Williams JV, New Vaccine Surveillance Network: **New Vaccine Surveillance Network. A novel group of rhinoviruses is associated with asthma hospitalizations.** *J Allergy Clin Immunol* 2009, **123(1)**:98-104.

37. Chung JY, Han TH, Kim SW, Kim CK, Hwang ES: **Detection of viruses identified recently in children with acute wheezing.** *J Med Virol* 2007, **79(8)**:1238-43.
38. Calvo C, Casas I, García-García ML, Pozo F, Reyes N, Cruz N, García-Cuenllas L, Pérez-Breña P: **Role of rhinovirus C respiratory infections in sick and healthy children in Spain.** *Pediatr Infect Dis J* 2010, **29(8)**:717-20.
39. Winther B, Hayden FG, Hendley JO: **Picornavirus infections in children diagnosed by RT-PCR during longitudinal surveillance with weekly sampling: Association with symptomatic illness and effect of season.** *J Med Virol* 2006, **78(5)**:644-50.
40. Fairchok MP, Martin ET, Chambers S, Kuypers J, Behrens M, Braun LE, Englund JA: **Epidemiology of viral respiratory tract infections in a prospective cohort of infants and toddlers attending daycare.** *J Clin Virol* 2010, **49(1)**:16-20.
41. Williams JV, Edwards KM, Weinberg GA, Griffin MR, Hall CB, Zhu Y, Szilagyi PG, Wang CK, Yang CF, Silva D, Ye D, Spaete RR, Crowe JE Jr: **Population-based incidence of human metapneumovirus infection among hospitalized children.** *J Infect Dis* 2010, **201(12)**:1890-8.
42. Mallia P, Johnston SL: **How viral infections cause exacerbation of airway diseases.** *Chest* 2006, **130(4)**:1203-10.
43. Nagayama Y, Tsubaki T, Toba T, Nakayama S, Kiyofumi O: **Analysis of sputum taken from wheezy and asthmatic infants and children, with special reference to respiratory infections.** *Pediatr Allergy Immunol* 2001, **12(6)**:318-26.

Pre-publication history

The pre-publication history for this paper can be accessed here:
http://www.biomedcentral.com/1471-2334/11/168/prepub

doi:10.1186/1471-2334-11-168
Cite this article as: Fujitsuka *et al.*: A molecular epidemiological study of respiratory viruses detected in Japanese children with acute wheezing illness. *BMC Infectious Diseases* 2011 11:168.

Vaccine 29 (2011) 8330–8337

Contents lists available at SciVerse ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Inactivated and adjuvanted whole-virion clade 2.3.4 H5N1 pre-pandemic influenza vaccine possesses broad protective efficacy against infection by heterologous clades of highly pathogenic H5N1 avian influenza virus in mice

Yuichi Harada[a], Ai Ninomiya-Mori[b], Yoshimasa Takahashi[c], Masayuki Shirakura[a], Noriko Kishida[a], Tsutomu Kageyama[a], Yoshikazu Tada[d], Masato Tashiro[a], Takato Odagiri[a,*]

[a] Influenza Virus Research Center, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashi-Murayama, Tokyo 208-0011, Japan
[b] Kobe Institute of Health, Kobe City 650-0046, Hyogo, Japan
[c] Department of Immunology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan
[d] The Research Foundation for Microbial Diseases of Osaka University, Kagawa 768-0061, Japan

ARTICLE INFO

Article history:
Received 21 June 2011
Received in revised form 8 August 2011
Accepted 18 August 2011
Available online 10 September 2011

Keywords:
H5N1 avian influenza
Adjuvanted H5N1 whole virion
pre-pandemic influenza vaccine
Cross-neutralizing antibodies
Cross-protective immunity

ABSTRACT

In this study, we evaluated the immunogenicity and protective efficacy of a candidate attenuated H5N1 pre-pandemic influenza vaccine of clade 2.3.4, rgAnhui, which was reverse genetically generated from highly virulent A/Anhui/01/2005 (H5N1) wild-type virus. When a low-dose antigen (0.3 μg HA) vaccine was combined with aluminum hydroxide adjuvant, virus neutralization and anti-HA IgG antibodies induced in the sera of vaccinated mice showed similar levels as those in mice vaccinated with non-adjuvanted high-dose antigen (3 μg HA) vaccine. Serum antibodies had broad reactivity against highly pathogenic H5N1 viruses of both homologous and heterologous clades. All mice vaccinated with adjuvanted and non-adjuvanted rgAnhui vaccines at low and high antigen doses survived, without any significant weight loss, lethal challenge infection with homologous clade 2.3.4 viruses, including antigenic variant virus and heterologous clade 2.1.3. Mice vaccinated with low-dose antigen without adjuvant, however, exhibited 20% and 60% survival rates against clade 1 and clade 2.2 viruses, respectively; but, addition of adjuvant improved these rates to 80% and 100%, respectively. The data strongly suggest that aluminum hydroxide-adjuvanted rgAnhui vaccine can elicit broad cross-reactive and protective immunities against homologous and heterologous clades, and that the rgAnhui vaccine is a useful pre-pandemic H5N1 vaccine.

1. Introduction

H5N1 highly pathogenic avian influenza virus (H5N1 HPAIV) has become enzootic in some countries and has the potential to cause an influenza pandemic. The direct avian-to-human transmission of H5N1 HPAIV, with a high mortality rate, was first reported in Hong Kong in 1997 [1,2]. Subsequently, the number of reported human infections has increased in various countries around the world, particularly in Southeast Asia, the Middle East and Africa. As of August 2, 2011, the World Health Organization (WHO) has confirmed 563 cases of human infections and 330 deaths in 15 countries [3]. Most cases of H5N1 HPAIV infection in humans appear to be caused by direct avian transmission. However, several suspected cases of human-to-human transmission have also been reported [4–6], and these viruses have the potential to become human-adapted viruses due to the accumulation of mutations in their genome. Although there have been no reports of human infections in Japan, the isolation of H5N1 HPAIV from wild birds and outbreaks at poultry farms have been intermittently reported since 2003. In particular, 30 cases of virus detection in wild birds and 24 outbreaks in poultry farms were confirmed to be caused by clade 2.3.2 viruses in 2010 and 2011 [7]. Furthermore, anti-H5N1 HPAIV antibodies were detected in workers exposed to H5N1 HPAIV-positive poultry, although they showed no symptoms suggestive of viral infection [8]. Thus, sustained measures against H5N1 HPAIV by more rigorous monitoring of both wild birds and poultry, and by the national stockpiling of pre-pandemic H5N1 vaccines remain a high priority for pandemic preparedness.

The development of attenuated H5N1 vaccines from H5N1 HPAIVs isolated from wild birds, poultry and humans is performed by reverse-genetics (rg) to modify the virulent H5 hemagglutinin (HA) gene and to reassort the six backbone genes of A/Puerto Rico/8/1934 (H1N1) virus [9–11]. These rg H5N1 virus vaccines generally have low immunogenicity without adjuvant [12]. Conse-

* Corresponding author. Tel.: +81 42 848 7162; fax: +81 42 561 6149.
E-mail address: todagiri@nih.go.jp (T. Odagiri).

0264-410X/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2011.08.091

quently, we [13] and other groups [14,15] have assessed the effects of aluminum hydroxide adjuvant (alum), which is the most widely used licensed adjuvant for human use [16], and have confirmed positive results with respect to strengthened immunogenicity in mouse models and human clinical studies. However, other oil-in-water adjuvants, such as MF-59 [17] and AS03 [18], have recently been licensed and are now used in human H5N1 vaccines [19–22].

The H5N1 HPAIVs isolated worldwide have diversified both genetically and antigenically, and the current major isolates are clade 2.3.2 viruses detected in Hong Kong (SAR), China, Korea and Japan and clade 2.2.1 viruses detected in Egypt, although clade 1 viruses, which were the major isolates in 2004 [23], are still sporadically detected in Cambodia [24]. Therefore, multiple H5N1 vaccine candidate viruses from these clades, or a vaccine virus that can elicit broad reactive and protective immunities against various clades of H5N1 HPAIVs, must be prepared for pandemic preparedness.

In the present study, we assessed the immunogenicity and protective efficacy of an inactivated H5N1 whole virus vaccine, rgAnhui, with or without alum adjuvant against homologous and heterologous clades of H5N1 HPAIVs using a mouse model.

2. Materials and methods

2.1. Vaccine virus and adjuvant

Recombinant avirulent A/Anhui/01/05-PR8-IBCDC-RG5 (rgAnhui01/05) virus generated from the virulent A/Anhui/01/05 strain by reverse genetics (rg) was obtained from the United States Centers for Disease Control and Prevention (USCDC, Atlanta, USA). Virus was grown in the allantoic cavity of 10-day-old embryonated chicken eggs and purified by velocity density gradient centrifugation through a 10–50% linear sucrose gradient. The purified virus pellet obtained by ultracentrifugation was re-suspended in phosphate buffered saline (PBS) and inactivated by 0.05% formalin, as described previously [13].

A portion of the purified virus was separated by SDS-PAGE using a 12.5% polyacrylamide gel. The gel was stained with Coomassie brilliant blue, and the gel image was captured using a CS cool saver (ATTO, Tokyo, Japan). The digitized gel image was then analyzed with a CS analyzer (ATTO), and the percentage HA content (%HA) was calculated by (HA1 + HA2)/(HA1 + NP + M1 + HA2) × 100. Protein concentration of the purified virus was determined using a DC protein assay kit (Bio-Rad Laboratories K.K., Tokyo, Japan) based on the modified Lowry method. HA content (μg/mL) was calculated by (%HA) × (protein concentration). Purified virus was appropriately diluted and mixed with aluminum hydroxide gel or PBS in order to obtain a final concentration of 0.3 mg/mL.

2.2. Animals, immunization and H5N1 HPAIV challenge

Eight-week-old female BALB/c mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). Procedures involving mice were performed in accordance with the institutional guidelines for animal care.

Five mice per group were subcutaneously immunized twice at a 3-week interval with 100 μl of formalin-inactivated virus vaccine containing 0.3 or 3 μg HA with or without alum adjuvant. Mice in the control group were immunized according to the same schedule with alum adjuvant only. Three weeks after the last immunization, mice were anesthetized and then intranasally inoculated with 20 μl of virus solution containing 20× 50% mouse lethal dose (MLD_{50}) of H5N1 HPAIV. The H5N1 HPAIVs used in this study were: A/Viet Nam/JP1203/2004 (VN) [clade 1], A/Indonesia/5/2005 (Indo) [clade 2.1.3], A/Turkey/12/2006 (Tk) [clade 2.2], A/Japanese White Eye/HK/1038/2005 (JWE) [clade 2.3.4], A/Laos/JP127/2007 (Laos) [clade 2.3.4] and A/Myanmar JPA007-07/2007 (Myan) [clade 2.3.4]. For 2–3 weeks after lethal H5N1 HPAIV challenge, mice were monitored daily for their survival and weight. During monitoring, mice losing 30% of their body weight were euthanized.

2.3. Hemagglutination inhibition (HI) assay

Post-infection ferret anti-sera raised against JWE, rgAnhui and Laos viruses were provided by the USCDC. Sera were treated with RDE (Denka Seiken, Niigata, Japan) for 18–20 h at 37 °C and were then inactivated by incubation for 30 min at 56 °C. Sera were treated with packed turkey red blood cells for 60 min at room temperature in order to remove non-specific hemagglutinating factors in the sera. Using U-bottom 96-well microtiter plates (AGC Techno Glass Co., Ltd., Chiba, Japan), 25 μl of sera were serially diluted 2-fold with PBS and mixed with an equivalent volume of test antigens containing 8 hemagglutinating units (HAU) of virus. The mixture of diluted sera and virus was incubated for 30 min at room temperature. Fifty microliters of 0.5% turkey red blood cells was added to the antigen/serum mixture and incubated for 45 min at room temperature. After determination of HI (positive or negative), endpoint antibody titers were expressed as the reciprocal value of the last dilution at which complete inhibition of hemagglutination was observed.

2.4. Titration of antigen-specific antibodies in mouse sera

Virus-neutralizing antibody titers were determined as follows. Sera were collected from mice at 21 days after the last vaccination, and were treated with RDE for 18–20 h at 37 °C, followed by inactivation via incubation for 30 min at 56 °C. Sera were serially diluted and mixed with an equivalent volume of H5N1 HPAIV containing 200× 50%-tissue-culture infectious dose ($TCID_{50}$) of virus. The mixture of diluted sera and virus was incubated for 30 min at 37 °C. Confluent monolayers of MDCK cells in 96-well microtiter plates were washed with PBS, and serum/virus mixtures were transferred into the wells. After incubation for 4 days at 37 °C under a humidified atmosphere containing 5% CO_2, cells were fixed with 10% formalin and then stained with NB staining solution (0.1% naphthol blue black, 0.1% sodium acetate and 9% acetic acid). Stained cells were eluted with 100 mM NaOH, and optical densities at 655 nm were measured using a microplate reader (Model 550; Bio-Rad Laboratories, Tokyo, Japan). Endpoint antibody titers were expressed as the reciprocal value of the last dilution with an optical density that was at least a half unit greater than the optical density of the uninfected control.

Viral HA-specific antibody titers were determined according to the method described by Takahashi et al. [25]. Briefly, baculovirus-produced recombinant HA proteins were used as the coating antigen, and viral HA-specific IgG1 and IgG2a antibodies in the sera of mice were measured by ELISA. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG1 and IgG2a antibodies (Southern Biotechnology, Birmingham, AL, USA) were used as detection antibodies.

2.5. Statistical analysis

For comparison among virus-neutralizing antibody titers against various HPAIVs, we performed Steel's method for multiple comparison. The virus-neutralizing antibody titers against 3–4 virus strains were compared with anti-Myan or anti-VN virus-neutralizing antibody titers. The p-value of each comparison under 0.05 was regarded as statistically significant difference.

Table 1
Antigenic properties of rgAnhui-H5N1 vaccine virus and HPAIVs.

Viruses	Genetic clade	Post-infection ferret sera		
		JWE	rgAnhui	Laos
VN	1	<10	20	40
Indo	2.1.3	80	1280	160
Tk	2.2	160	80	40
JWE	2.3.4	**320**	320	320
rgAnhui	2.3.4	160	**1280**	1280
Laos	2.3.4	160	1280	**1280**
Myan	2.3.4	40	160	320

Bold type refers to homologous HI titers for indicated viruses.

2.6. The survival curve was compared using Log-rank test

Student's *t*-test or Mann–Whitney U test was performed to compare viral HA-specific IgG1 and IgG2a antibodies against various virus strains.

All statistical analyses, except for Steel's method, were performed with PASW statistical software (IBM Japan Ltd., Tokyo, Japan) and the *p*-value of each comparison under 0.05 was regarded as statistically significant difference.

3. Results

3.1. Antigenic properties of rgAnhui vaccine virus and H5N1 HPAIVs

First, we analyzed the antigenic properties of all viruses used in this study by performing HI assay with post-infection ferret antisera. As shown in Table 1, rgAnhui, Laos and JWE viruses of clade 2.3.4 exhibited similar antigenicity, as indicated by HI titers within 4-fold of the HI titers for each homologous antiserum. Myan virus, however, showed 8-fold lower HI titers against anti-rgAnhui and anti-JWE sera, and 4-fold lower HI titer against anti-Laos serum, indicating that the virus was antigenically different from rgAnhui and the two clade 2.3.4 viruses, although the Myan virus also belongs to clade 2.3.4. With respect to the heterologous clade viruses, the HI titer of Indo (clade 2.1.3) virus against anti-rgAnhui serum was the same as the homologous HI titer, whereas VN (clade 1) and Tk (clade 2.2) viruses exhibited 64-fold and 16-fold lower HI titers against anti-rgAnhui serum, respectively. Thus, HI assay clearly indicated that VN and Tk viruses were antigenically different from rgAnhui vaccine virus, while Indo virus was closely related to the vaccine virus, despite the heterologous genetic clade.

3.2. Induction of virus-neutralizing antibodies in sera from vaccinated mice

In order to investigate the immunogenicity of the rgAnhui vaccine, mice were subcutaneously vaccinated with a high (3 μg HA) or low (0.3 μg HA) doses of vaccine antigen with or without alum. On day 42 (21 days after the 2nd vaccination), sera were collected from vaccinated mice and virus-neutralizing antibody titers against various clades of wild-type H5N1 HPAIVs were measured (Fig. 1). JWE was used as a homologue of the wild-type virus in rgAnhui vaccine, as wild-type A/Anhui/01/2005 was not available and JWE virus is antigenically similar (Table 1). The virus-neutralizing antibody titer against homologous JWE virus was correlated with both the antigen dose and the presence of alum. Mice vaccinated with 0.3 μg HA antigen with adjuvant produced almost the same level of serum virus-neutralizing antibody as that induced by vaccination with 3 μg HA antigen without adjuvant, indicating that one-tenth the amount of vaccine antigen could be used with addition of alum. Similar reactivity occurred against the Laos strain, which is from

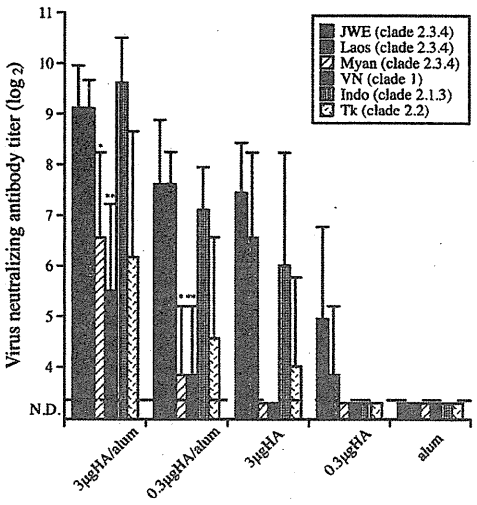

Fig. 1. Virus-neutralizing antibody titers in sera from vaccinated mice. Mice were vaccinated twice with H5N1 rgAnhui vaccine (clade 2.3.4) at a 3-week interval and with or without alum. On day 21 after the second vaccination, sera were collected, and the virus-neutralizing antibody titers against various clades (2.3.4, 1, 2.1.3 and 2.2) of H5N1 viruses were measured. Each bar represents the mean titer of virus-neutralizing antibodies, and error bars represent standard deviations. The dashed line represents the detection limit. *$p < 0.05$ for comparison with JWE or Laos strain and $p > 0.05$ for comparison with VN strain using Steel's method. **$p < 0.05$ for comparison with JWE, Laos or Indo strain and $p > 0.05$ for comparison with Tk strain using Steel's method.

the same clade (2.3.4) and is antigenically similar to the rgAnhui vaccine virus (Table 1). In contrast, the antibody reactivity to an antigenic variant virus of clade 2.3.4, the Myan strain (Table 1), was significantly weaker than that of JWE and Laos strain ($p < 0.05$, Steel's method for multiple comparison), and the titer was similar to that against heterologous clade 1 virus, the VN strain. Furthermore, virus-neutralizing antibodies against the Myan strain were not detectable at any antigen dose with non-adjuvanted vaccine.

In order to assess cross-reactivity against heterologous clade viruses, virus-neutralizing antibody titers against the VN strain (clade 1), Indo strain (clade 2.1.3) and Tk strain (clade 2.2) were also measured (Fig. 1). Mice vaccinated with adjuvanted vaccine showed a high level of cross-reactive virus-neutralizing antibodies against Indo virus and an intermediate level of virus-neutralizing antibody against Tk virus. These cross-reactive virus-neutralizing antibodies were also detected by vaccination with high-dose non-adjuvanted vaccine. Virus-neutralizing antibody against VN strain was induced only in mice receiving vaccine antigen with adjuvant. Anti-VN virus-neutralizing antibody titers were similar to those of anti-Tk, but were significantly lower than anti-JWE, -Laos or -Indo antibody titers ($p < 0.05$, Steel's method for multiple comparison).

3.3. Protective efficacy of inactivated rgAnhui vaccine against lethal-dose infections with homologous clade H5N1 HPAIVs

The protective efficacy of rgAnhui vaccine was evaluated in mice that were subcutaneously vaccinated twice, as outlined in Fig. 1. On day 42, mice were anesthetized and intranasally infected with a lethal dose ($20 \times MLD_{50}$) of homologous clade viruses (JWE, Laos or Myan). All mice vaccinated with either dose of antigen with or without adjuvant survived the lethal-dose infection (Fig. 2). Moreover, none of the infected mice lost a significant amount of weight during the 14-day observation period (Fig. 3). In contrast, the weight of control mice (inoculated with alum adjuvant alone) began to decrease at 4–6 days after HPAIV infection, and all control mice were dead by day 10 after infection (Figs. 2 and 3).

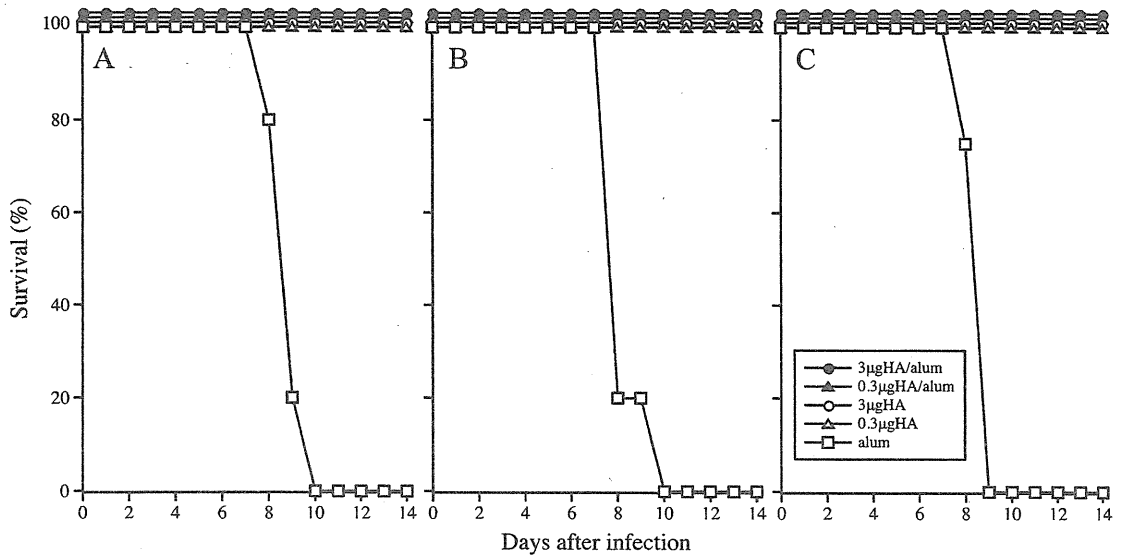

Fig. 2. Survival rate of vaccinated mice after lethal infection with homologous clade 2.3.4 viruses. Five mice in each group were vaccinated twice with H5N1 rgAnhui vaccine (with or without alum) at a 3-week interval (●, 3 μg HA with alum; ▲, 0.3 μg HA with alum; ○, 3 μg HA; △, 0.3 μg HA; □, alum). On day 21 after the second vaccination, mice were anesthetized and inoculated intranasally with a lethal dose of homologous clade JWE (A), Laos (B) or Myan (C) strain, and their survival was monitored for 2 weeks.

3.4. *Cross-protective efficacy of inactivated rgAnhui vaccine against lethal-dose infections with heterologous clade H5N1 HPAIVs*

Next, we examined the cross-protective efficacy of rgAnhui vaccine by challenge infection with 20 × MLD_{50} of various heterologous clades of H5N1 HPAIVs. After clade 1 VN virus infection, only 20% of mice vaccinated with a low dose (0.3 μg HA) of non-adjuvanted vaccine survived (Fig. 4A), and severe weight loss similar to that of the control mice occurred until day 9 after virus infection (Fig. 5A). However, the survival rate increased to 80% when mice were vaccinated with the same dose of adjuvanted vaccine (Fig. 4A). These mice lost some weight during days 3–7, after which they returned to a normal weight (Fig. 5A). At a high dose (3 μg HA) of adjuvanted vaccine, all mice survived and no significant weight loss was observed, whereas one mouse was dead at day 16 by vaccination with non-adjuvanted vaccine (Fig. 4A).

In a challenge infection with Indo virus (clade 2.1.3) (Figs. 4B and 5B), all vaccinated mice survived without significant weight loss, regardless of the dose and presence or absence of adjuvant, with the exception of mice vaccinated with 0.3 μg HA non-adjuvanted vaccine; these mice had a weight loss of approximately 10% during days 4–8, after which their weight recovered. In contrast, the control mice lost weight starting at 3 days after virus infection, and all control mice were dead by day 9 after infection (Fig. 5B).

On challenge infection with Tk (clade 2.2) virus (Fig. 4C), the survival rate of mice vaccinated with 0.3 μg HA non-adjuvanted

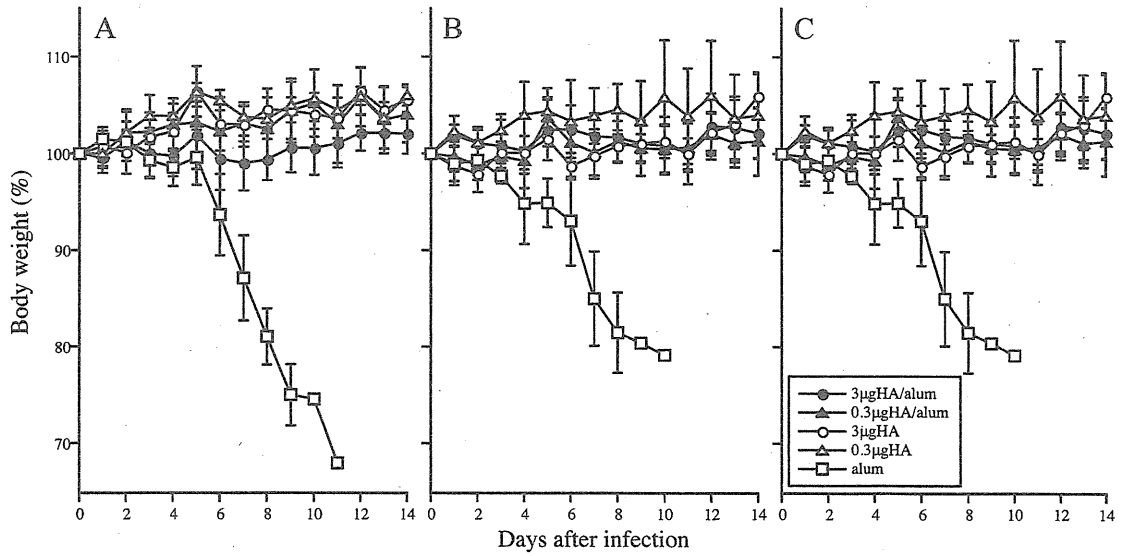

Fig. 3. Weight changes in vaccinated mice after lethal infection with homologous clade 2.3.4 viruses. Five mice in each group were vaccinated twice with H5N1 rgAnhui vaccine (with or without alum) at a 3-week interval (●, 3 μg HA with alum; ▲, 0.3 μg HA with alum; ○, 3 μg HA; △, 0.3 μg HA; □, alum). On day 21 after the second vaccination, mice were anesthetized and inoculated intranasally with a lethal dose of homologous clade JWE (A), Laos (B) or Myan (C) strain, and their weight was monitored for 2 weeks. Weight is expressed as a percentage of weight on day 0. Data represent the mean weight of mice, and error bars represent standard deviations.

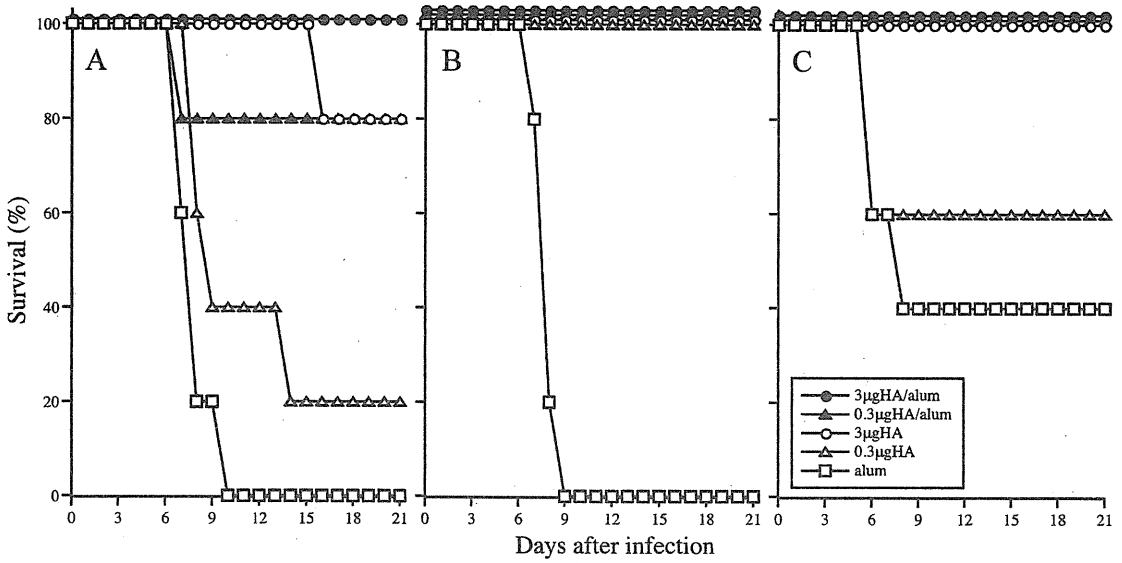

Fig. 4. Survival rate of vaccinated mice after lethal infection with heterologous clade viruses. Five mice in each group were vaccinated twice with H5N1 rgAnhui vaccine (clade 2.3.4) at a 3-week interval and with or without alum (●, 3 μg HA with alum; ▲, 0.3 μg HA with alum; ○, 3 μg HA; △, 0.3 μg HA; □, alum). On day 21 after the second vaccination, mice were anesthetized and inoculated intranasally with a lethal dose of heterologous clade 1 VN (A), clade 2.1.3 Indo (B) or clade 2.2 Tk (C) strain, and their survival was monitored for 3 weeks.

vaccine was 60%, which was not significantly higher than that of the control mice (Log-rank (Mantel-Cox) test, $p = 0.582$). However, the survival rate was 100% with the addition of adjuvant. At a high dose of vaccine antigen, all mice survived, regardless of the presence or absence of adjuvant. However, unlike other clades of challenge virus, Tk infection caused weight loss in all vaccinated mice starting at 1–2 days after infection (Fig. 5C). The severity of weight loss depended on the vaccine antigen dose (0.3 μg HA antigen was associated with more marked weight loss than 3 μg HA antigen), but all mice returned to a normal weight by day 14. Moreover, recovery from weight loss occurred earlier in the mice that received adjuvanted vaccine as compared to the non-adjuvanted vaccine at the same vaccine antigen dose (Fig. 5C).

3.5. Detection of viral HA-specific IgG1 and IgG2a antibodies in sera from vaccinated mice

HA protein from influenza virus is the major antigen for induction of protective antibodies. Alum is thought to enhance the induction of antigen-specific IgG1 antibody responses [26,27]. It has also been reported that virus-specific IgG2a antibodies are induced by live influenza virus infection and are important to

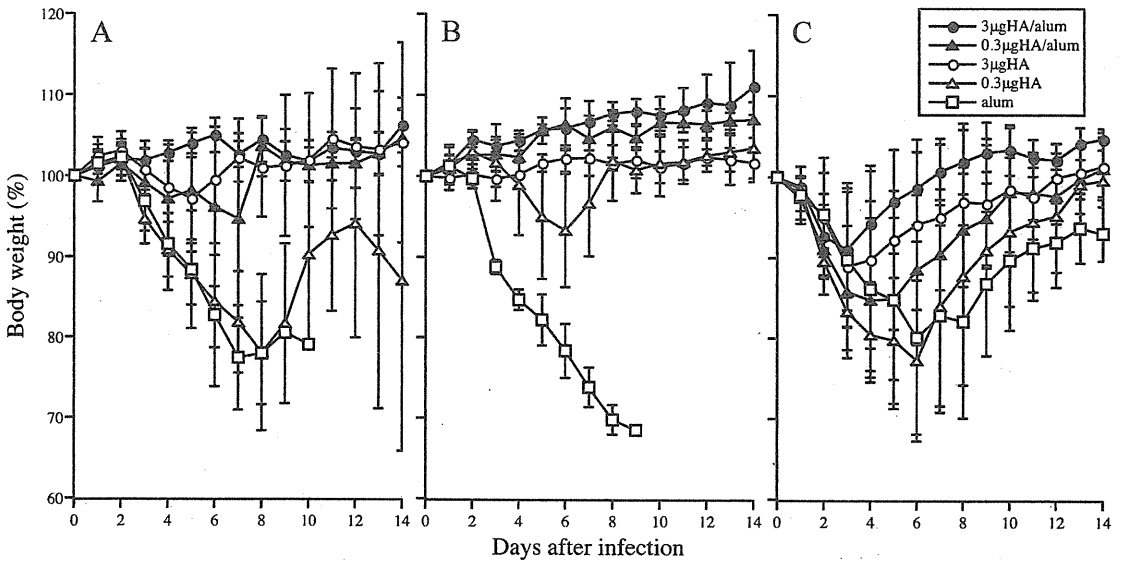

Fig. 5. Weight changes in vaccinated mice after lethal infection with heterologous clade viruses. Five mice in each group were vaccinated twice with H5N1 rgAnhui vaccine (clade 2.3.4) at a 3-week interval and with or without alum (●, 3 μg HA with alum; ▲, 0.3 μg HA with alum; ○, 3 μg HA; △, 0.3 μg HA; □, alum). On day 21 after the second vaccination, mice were anesthetized and inoculated intranasally with a lethal dose of heterologous clade 1 VN (A), clade 2.1.3 Indo (B) or clade 2.2 Tk (C) strain, and their weight was monitored for 2 weeks. Weight is expressed as a percentage of weight on day 0. Data represent the mean weight of mice, and error bars represent standard deviations.

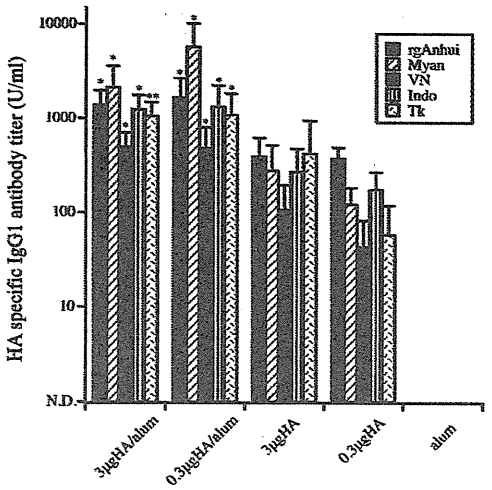

Fig. 6. Viral HA-specific IgG1 antibody titers in sera from vaccinated mice. Mice were vaccinated twice at a 3-week interval and with or without alum. On day 21 after the second vaccination, sera were collected, and viral HA-specific IgG1 antibody titers against various clades of H5N1 viruses were measured by ELISA. Each bar represents the mean value of viral HA-specific IgG1 antibody titers, and error bars represent standard deviations. *$p < 0.05$ for comparison against each vaccine antigen only using Student's t-test. **$p < 0.05$ for comparison against each vaccine antigen only using Mann–Whitney U test.

antibody-mediated protection against influenza A virus infection [28,29]. Thus, we used ELISA to measure HA-specific IgG1 and IgG2a antibody titers in the sera of vaccinated mice. Sera subjected to ELISA were the same as those used for the titration of virus-neutralizing antibodies.

All vaccinated mice developed HA-specific IgG1 and IgG2a antibodies (Figs. 6 and 7). These antibodies could not be detected in the sera of control mice. Alum significantly enhanced the induction of cross-reactive IgG1 ($p < 0.05$, Student's t-test or Mann–Whitney U test) and IgG2a ($p < 0.01$, Student's t-test or Mann–Whitney U test) antibodies against all test HAs from H5N1 viruses belonging to different clades. In particular, IgG1 antibody levels at a low antigen dose were efficiently enhanced by addition of adjuvant, and IgG1 antibody titers at the low antigen dose were as high against all test viral HAs as those at the high antigen dose (Fig. 6). It is noteworthy that IgG1 antibody titers against the HA from the antigenic variant Myan virus were higher, but not significantly so ($p = 0.055$, Student's t-test), than those against the HA from rgAnhui vaccine virus in the sera of mice vaccinated with adjuvanted vaccine (Fig. 6), although the virus-neutralizing antibody titer against Myan virus was significantly lower than that against JWE virus ($p < 0.05$, Steel's method for multiple comparison), and was as low as that against clade 1 virus VN (Fig. 1). Similarly, high levels of IgG2a antibodies against Myan and VN viruses were detected by ELISA.

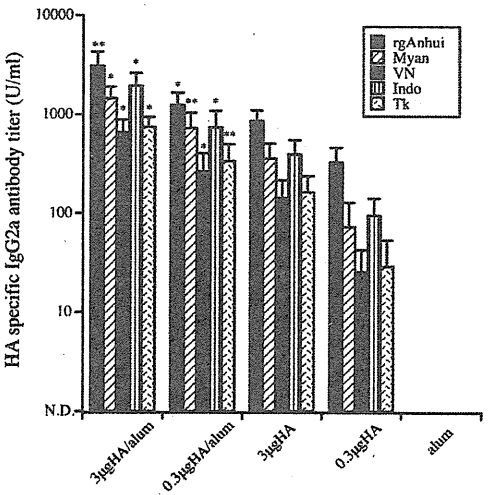

Fig. 7. Viral HA-specific IgG2a antibody titers in sera from vaccinated mice. Mice were vaccinated twice at a 3-week interval and with or without alum. On day 21 after the second vaccination, sera were collected, and viral HA-specific IgG2a antibody titers against various clades of H5N1 viruses were measured by ELISA. Each bar represents the mean value for viral HA-specific IgG2a antibody titers, and error bars represent standard deviation. *$p < 0.01$ for comparison against each vaccine antigen only using Student's t-test. **$p < 0.01$ for comparison against each vaccine antigen only using Mann–Whitney U test.

4. Discussion

In the present study, we evaluated the vaccine efficacy of rgAnhui virus (clade 2.3.4) with respect to immunogenicity and cross-protection against viruses of homologous and heterologous genetic clades, including antigenic variants. To assess immunogenicity, we detected virus-neutralizing antibody rather than HI antibody, as in our previous mouse model study of H5N1 vaccines, HI antibody was not detected after vaccination at the highest dose (2 μg HA) of adjuvanted vaccine, while sufficient levels of virus-neutralizing antibody were detected in many cases [13]. Furthermore, virus-neutralizing assay is a hallmark for assessing the immunogenicity of H5N1 vaccines due to its higher sensitivity. In the present mouse model experiments, vaccination with 0.3 μg HA enabled the production of virus-neutralizing antibodies against homologous clade 2.3.4 viruses, except for the antigenic variant Myan virus (Fig. 1).

As expected and as reported previously [13,15], the addition of alum to 0.3 μg HA markedly increased antibody levels to levels similar to those induced by vaccination with 3 μg HA antigen. Furthermore, these antibodies were also able to neutralize homologous Myan and heterologous VN strains, and were not observed in mice receiving 3 μg HA antigen without adjuvant. Thus, serum antibodies induced by 0.3 μg HA antigen with alum were able to neutralize all test viruses in clades 1, 2.1.3 and 2.2, as well as homologous clade 2.3.4, including Myan virus. These data clearly indicate that vaccine formulations with alum allow 10-times less vaccine antigen to be used in our mouse model.

Although it has been reported that addition of alum to H5N1 vaccine did not enhance immunogenicity in a clinical study [17], non-adjuvanted H5N1 vaccines required large amounts of vaccine antigen to induce serum antibodies that meet the evaluation criteria for vaccines by the Committee for Proprietary Medicinal Products (CPMP) [12]. The strategy of vaccine antigen sparing is crucial for the creation of a national stockpile of pre-pandemic H5N1 vaccines, as manufacturers have limited vaccine production capacity. Moreover, in our unpublished clinical trials of attenuated and inactivated H5N1 vaccine in 1997, 15 μg HA antigen from split and whole virion vaccines without adjuvant failed to elicit any detectable level of neutralizing antibodies in the sera of vaccine recipients, which led us to the conclusion that an adjuvanted formulation is necessary for H5N1 vaccines. Consequently, the use of alum or other recently approved oil-in-water adjuvants, such as MF59 [17] and AS03 [18], is the most promising and realistic H5N1 vaccine strategy for pandemic preparedness.

In homologous genetic clade 2.3.4, the neutralizing antibody titers induced by rgAnhui vaccine were high against JWE and Laos viruses, but were significantly lower against Myan virus (Fig. 1). Based on antigenic analysis by HI test, JWE and Laos viruses were antigenically related to rgAnhui virus, but the Myan virus differed from the rgAnhui virus (Table 1) through substitution of two amino acids at antigenic site B of the HA protein (data not shown). However, such low cross-reactivity against the Myan virus did not

correlate with the survival rate and weight loss after lethal challenge with Myan virus; all vaccinated mice, regardless of antigen dose and presence or absence of adjuvant, survived without significant weight loss, similarly to mice exposed to other clade 2.3.4 viruses (Figs. 2 and 3).

The protection observed in vaccinated mice should be mediated by virus-specific antibodies, particularly virus-neutralizing and/or viral HA-specific antibodies, as formalin-inactivated viral antigens and alum itself cannot induce cellular immunity [16,30–32]. In fact, on ELISA, the HA-specific IgG1/IgG2a antibodies against Myan virus were markedly elicited with similar levels as homologous vaccine-like virus by non-adjuvanted rgAnhui vaccination (Figs. 6 and 7), while virus-neutralizing antibodies against Myan virus could not be detected (Fig. 1). Moreover, Lu et al. [33] reported that mice vaccinated and survived from following lethal virus challenge did not always possess detectable levels of virus-neutralizing antibodies against challenge virus. It is therefore possible that the undetectable levels of neutralizing antibody induced by vaccination were sufficient to provide protection from the homologous clade of the Myan virus.

Among heterologous clade viruses, virus-neutralizing titers, survival rate and antigenic differences were well correlated. Indo virus (clade 2.1.3) exhibited antigenic similarity to rgAnhui virus on HI test (Table 1) and was well neutralized by antibodies induced by rgAnhui vaccine, similarly to homologous clade 2.3.4 viruses (Fig. 1), and all vaccinated mice survived (Fig. 4B). The results suggest that rgAnhui vaccine sufficiently neutralized Indo-like viruses in clade 2.1.3. On the other hand, rgAnhui vaccination at a low (0.3 μg HA) dose without adjuvant was less effective against clade 1 VN virus, as only 20% of mice challenged with VN virus survived, although the survival rate increased to 80% with addition of adjuvant (Fig. 4A). Such decreased cross-protective efficacy when compared with Indo and Myan viruses, particularly the undetectable levels of virus-neutralizing antibody, may be attributable to the magnitude of antigenic differences between VN and rgAnhui viruses, as HI titers of VN virus were 64-fold lower when compared to the homologous titer of anti-rgAnhui ferret antiserum, and the VN virus apparently differed greatly in antigenicity when compared with the rgAnhui vaccine virus (Table 1). The results indicate that, for the rgAnhui vaccine, enhanced immunogenicity due to addition of adjuvant and/or increasing the antigen dose is necessary to neutralize clade 1 viruses.

The rgAnhui vaccine induced intermediate levels of cross-reactive neutralizing antibody against clade 2.2 Tk virus (Fig. 1), and only a low dose of non-adjuvanted vaccine showed reduced cross-protection (60%) to challenge infection with Tk virus (Fig. 4C). This result was also correlated with the magnitude of antigenic difference between Tk virus and rgAnhui virus, as the HI titer of Tk virus was 16-fold lower when compared with the homologous titer (Table 1). The survival rate of control mice challenged with Tk virus was 40%, in contrast to the 0% seen with other viruses, despite inoculation with the $20 \times MLD_{50}$ dose calculated by the Reed and Muench method. However, 1–2 of the 5 control mice challenged with $20 \times MLD_{50}$, or higher doses, of Tk virus survived in several independent experiments (data not shown). As all control mice challenged with Tk virus showed unrecoverable weight loss (Fig. 5C) and virus-neutralizing antibody titers in the sera of surviving control mice on day 14 after challenge were comparable to those of vaccinated mice (data not shown), the higher survival rate in control mice was not due to a technical failure, but was attributable to the features of the TK virus itself.

Addition of adjuvant improved the protection efficacy against TK virus, but the severity of weight loss in vaccinated mice was markedly higher than with clade 1 VN virus (Fig. 5A and C), despite intermediate levels of neutralizing antibodies being elicited by rgAnhui vaccine. The MLD_{50} values for VN and Tk viruses were 5.1×10^2 $TCID_{50}$ and 2.3×10^6 $TCID_{50}$, respectively. Consequently, the input amount of Tk virus for lethal infection was 4510-times greater when compared to VN virus. This difference may have been reflected in the severity of weight loss.

Despite undetectable levels of virus-neutralizing antibody after non-adjuvanted vaccine, all mice infected with Myan and Indo viruses and 20–80% mice infected with VN virus, respectively, survived (Figs. 1, 2 and 4). Nonetheless, high levels of HA-specific IgG1 and IgG2a antibodies against these viruses were elicited (Figs. 6 and 7), but it is unlikely that non-neutralizing HA-specific antibodies contributed to the survival of virus-infected mice, as HA-specific IgG1 and IgG2a antibodies were also induced in the sera of mice vaccinated but did not survived from following lethal virus challenge. Consequently, the method of virus-neutralizing assay may be less sensitive for evaluating mouse survival after lethal challenge infection.

In the present study, the efficacy of H5N1 vaccine derived from clade 2.3.4, rgAnhui, was assessed based on broad immunogenicity and cross-protection by challenge infection. The rgAnhui vaccine was shown in a mouse model to produce broad-spectrum immunity and to protect against not only homologous infections, but also heterologous clade H5N1 HPAIVs, when the vaccine was conjugated with alum to enhance immunogenicity. Human infections with H5N1 HPAIVs have been continuously reported since 2003, and the antigenic features of the isolates from humans, poultry and wild birds have became variable among the HA genetic clades [34]. Although several H5N1 vaccine candidate viruses chosen from 8 clades (1, 2.1, 2.2, 2,2.1, 2.3.2, 2.3.4, 4 and 7) have been developed and updated [24], it is impossible to prepare stockpile vaccines using all recently available vaccine candidates. The rgAnhui vaccine virus possesses broad cross-immunity, and is therefore a promising candidate for the production of stockpile H5N1 vaccine. In fact, rgAnhui virus was selected for the Japanese national stockpile vaccine in 2007 [35].

Acknowledgements

We would like to thank Dr. Phengta Vongprachanh, National Center for Laboratory and Epidemiology, Lao PDR, Dr. Khin Yi Oo, National Health Laboratory, Myanmar, and Dr. L.T.Q. Mai, National Institute of Health and Epidemiology, Viet Nam, for providing clinical specimens for isolation of A/Laos/JP127/2009, A/Myanmar/JPA007-07/2007 and A/VietNam/JP1203/2004, respectively. We are also grateful to Dr. Nancy Cox, US-CDC, Dr. Alan Hay, National Institute of Medical Research, and Dr. Malik Peiris, University of Hong Kong, for providing rgAnhui01/2005 vaccine virus and post-infection ferret sera, A/Turkey/12/2006, A/Indonesia/5/2005 and A/Japanese White Eye/HK/1038/2005, respectively. These studies were supported by a Health Labor Sciences Research Grant from the Ministry of Health, Labor and Welfare of Japan and by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO).

References

[1] Claas EC, Osterhaus AD, van Beek R, De Jong JC, Rimmelzwaan GF, Senne DA, et al. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet 1998;351(February (9101)):472–7.
[2] Subbarao K, Klimov A, Katz J, Regnery H, Lim W, Hall H, et al. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. Science 1998;279(January (5349)):393–6.
[3] World Health Organization. Cumulative number of confirmed human cases of avian influenza A/(H5N1) reported to WHO; 2011, cited, Available from: http://www.who.int/csr/disease/avian_influenza/country/cases_table_2011_08_02/en/index.html.
[4] Normile D. Avian influenza human transmission but no pandemic in Indonesia. Science 2006;312(June (5782)):1855.

[5] Tran TH, Nguyen TL, Nguyen TD, Luong TS, Pham PM, Nguyen VC, et al. Avian influenza A (H5N1) in 10 patients in Vietnam. N Engl J Med 2004;350(March (12)):1179–88.
[6] Ungchusak K, Auewarakul P, Dowell SF, Kitphati R, Auwanit W, Puthavathana P, et al. Probable person-to-person transmission of avian influenza A (H5N1). N Engl J Med 2005;352(January (4)):333–40.
[7] Ministry of Agriculture FaFoJ; 2011, cited; Available from: http://www.maff.go.jp/j/syouan/douei/tori/pdf/110405_ai_japan_map.pdf.
[8] Ministry of Health LaW; 2004, cited; Available from: http://www.mhlw.go.jp/topics/bukyoku/kenkou/tori/041222/1.html.
[9] Nicolson C, Major D, Wood JM, Robertson JS. Generation of influenza vaccine viruses on Vero cells by reverse genetics: an H5N1 candidate vaccine strain produced under a quality system. Vaccine 2005;23(April (22)):2943–52.
[10] Dong J, Matsuoka Y, Maines TR, Swayne DE, O'Neill E, Davis CT, et al. Development of a new candidate H5N1 avian influenza virus for pre-pandemic vaccine production. Influenza Other Respi Viruses 2009;3(November (6)):287–95.
[11] Webby RJ, Perez DR, Coleman JS, Guan Y, Knight JH, Govorkova EA, et al. Responsiveness to a pandemic alert: use of reverse genetics for rapid development of influenza vaccines. Lancet 2004;363(April (9415)):1099–103.
[12] Treanor JJ, Campbell JD, Zangwill KM, Rowe T, Wolff M. Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine. N Engl J Med 2006;354(March (13)):1343–51.
[13] Ninomiya A, Imai M, Tashiro M, Odagiri T. Inactivated influenza H5N1 whole-virus vaccine with aluminum adjuvant induces homologous and heterologous protective immunities against lethal challenge with highly pathogenic H5N1 avian influenza viruses in a mouse model. Vaccine 2007;25(May (18)):3554–60.
[14] Brady RC, Treanor JJ, Atmar RL, Keitel WA, Edelman R, Chen WH, et al. Safety and immunogenicity of a subvirion inactivated influenza A/H5N1 vaccine with or without aluminum hydroxide among healthy elderly adults. Vaccine 2009;27(August (37)):5091–5.
[15] Lin J, Zhang J, Dong X, Fang H, Chen J, Su N, et al. Safety and immunogenicity of an inactivated adjuvanted whole-virion influenza A (H5N1) vaccine: a phase I randomised controlled trial. Lancet 2006;368(September (9540)):991–7.
[16] Naim JO, van Oss CJ, Wu W, Giese RF, Nickerson PA. Mechanisms of adjuvancy: I—metal oxides as adjuvants. Vaccine 1997;15(August (11)):1183–93.
[17] Bernstein DI, Edwards KM, Dekker CL, Belshe R, Talbot HK, Graham IL, et al. Effects of adjuvants on the safety and immunogenicity of an avian influenza H5N1 vaccine in adults. J Infect Dis 2008;197(March (5)):667–75.
[18] Leroux-Roels G. Prepandemic H5N1 influenza vaccine adjuvanted with AS03: a review of the pre-clinical and clinical data. Expert Opin Biol Ther 2009;9(August (8)):1057–71.
[19] Europian Medicines Agency. Aflunov, cited; Available from: http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/002094/human_med_001396.jsp&murl=menus/medicines/medicines.jsp&mid=WC0b01ac058001d125.
[20] Europian Medicines Agency. Prepandemic influenza vaccine (H5N1) Novartis Vaccines and Diagnostic, cited; Available from: http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/002269/human_med_001397.jsp&murl=menus/medicines/medicines.jsp&mid=WC0b01ac058001d125.
[21] Europian Medicines Agency. Prepandrix, cited; Available from: http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/000822/human_med_000986.jsp&murl=menus/medicines/medicines.jsp&mid=WC0b01ac058001d125.
[22] Europian Medicines Agency. Pandemic influenza vaccine (H5N1) (split virion, inactivated, adjuvanted) GlaxoSmithKline Biologicals, cited; Available from: http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/001206/human_med_001214.jsp&murl=menus/medicines/medicines.jsp&mid=WC0b01ac058001d125.
[23] World Health Organization. Antigenic and genetic characteristics of H5N1 viruses and candidate H5N1 vaccine viruses developed for potential use as pre-pandemic vaccines; 2005, cited; Available from: http://www.who.int/csr/disease/avian_influenza/guidelines/recommendationvaccine.pdf.
[24] World Health Organization. Antigenic and genetic characteristics of influenza A (H5N1) and influenza A(H9N2) viruses and candidate vaccine viruses developed for potential use in human vaccines; 2011, cited; Available from: http://www.who.int/csr/disease/avian_influenza/guidelines/2011_02_h5_h9_vaccinevirusupdate.pdf.
[25] Takahashi Y, Hasegawa H, Hara Y, Ato M, Ninomiya A, Takagi H, et al. Protective immunity afforded by inactivated H5N1 (NIBRG-14) vaccine requires antibodies against both hemagglutinin and neuraminidase in mice. J Infect Dis 2009;199(June (11)):1629–37.
[26] Brewer JM, Conacher M, Hunter CA, Mohrs M, Brombacher F, Alexander J. Aluminium hydroxide adjuvant initiates strong antigen-specific Th2 responses in the absence of IL-4- or IL-13-mediated signaling. J Immunol 1999;163(December (12)):6448–54.
[27] Brewer JM, Conacher M, Satoskar A, Bluethmann H, Alexander J. In interleukin-4-deficient mice, alum not only generates T helper 1 responses equivalent to freund's complete adjuvant, but continues to induce T helper 2 cytokine production. Eur J Immunol 1996;26(September (9)):2062–6.
[28] Fazekas G, Rosenwirth B, Dukor P, Gergely J, Rajnavolgyi E. IgG isotype distribution of local and systemic immune responses induced by influenza virus infection. Eur J Immunol 1994;24(December (12)):3063–7.
[29] Palladino G, Mozdzanowska K, Washko G, Gerhard W. Virus-neutralizing antibodies of immunoglobulin G (IgG) but not of IgM or IgA isotypes can cure influenza virus pneumonia in SCID mice. J Virol 1995;69(April (4)):2075–81.
[30] Bomford R. The comparative selectivity of adjuvants for humoral and cell-mediated immunity. II. Effect on delayed-type hypersensitivity in the mouse and guinea pig, and cell-mediated immunity to tumour antigens in the mouse of Freund's incomplete and complete adjuvants, alhydrogel, Corynebacterium parvum, Bordetella pertussis, muramyl dipeptide and saponin. Clin Exp Immunol 1980;39(February (2)):435–41.
[31] Reiss CS, Schulman JL. Cellular immune responses of mice to influenza virus vaccines. J Immunol 1980;125(November (5)):2182–8.
[32] Wijburg OL, van den Dobbelsteen GP, Vadolas J, Sanders A, Strugnell RA, van Rooijen N. The role of macrophages in the induction and regulation of immunity elicited by exogenous antigens. Eur J Immunol 1998;28(February (2)):479–87.
[33] Lu X, Edwards LE, Desheva JA, Nguyen DC, Rekstin A, Stephenson I, et al. Cross-protective immunity in mice induced by live-attenuated or inactivated vaccines against highly pathogenic influenza A (H5N1) viruses. Vaccine 2006;24(November (44–46)):6588–93.
[34] World Health Organization. Weekly epidemiological record; 2011, cited; Available from: http://www.who.int/wer/2011/wer8617.pdf.
[35] Ministry of Health LaW. Annual health, labour and welfare report 2007–2008; 2008, cited; Available from: http://www.mhlw.go.jp/english/wp/wp-hw2//part2//p2c1s7a.pdf.

Antiviral resistance during the 2009 influenza A H1N1 pandemic: public health, laboratory, and clinical perspectives

Aeron C Hurt, Tawee Chotpitayasunondh, Nancy J Cox, Rod Daniels, Alicia M Fry, Larisa V Gubareva, Frederick G Hayden, David S Hui, Olav Hungnes, Angie Lackenby, Wilina Lim, Adam Meijer, Charles Penn, Masato Tashiro, Timothy M Uyeki, Maria Zambon; on behalf of the WHO Consultation on Pandemic Influenza A (H1N1) 2009 Virus Resistance to Antivirals

Influenza A H1N1 2009 virus caused the first pandemic in an era when neuraminidase inhibitor antiviral drugs were available in many countries. The experiences of detecting and responding to resistance during the pandemic provided important lessons for public health, laboratory testing, and clinical management. We propose recommendations for antiviral susceptibility testing, reporting results, and management of patients infected with 2009 pandemic influenza A H1N1. Sustained global monitoring for antiviral resistance among circulating influenza viruses is crucial to inform public health and clinical recommendations for antiviral use, especially since community spread of oseltamivir-resistant A H1N1 2009 virus remains a concern. Further studies are needed to better understand influenza management in specific patient groups, such as severely immunocompromised hosts, including optimisation of antiviral treatment, rapid sample testing, and timely reporting of susceptibility results.

Published Online December 19, 2011 DOI:10.1016/S1473-3099(11)70318-8

WHO Collaborating Centre for Reference and Research on Influenza, North Melbourne, VIC, Australia (A C Hurt PhD); **Queen Sirikit National Institute of Child Health, Ministry of Public Health, Bangkok, Thailand** (Prof T Chotpitayasunondh MD); **WHO Collaborating Centre for Surveillance, Epidemiology, and Control of Influenza, Centers for Disease Control and Prevention, Atlanta, GA, USA** (N J Cox PhD, A M Fry MD, L V Gubareva PhD, T M Uyeki MD); **WHO Collaborating Centre for Reference and Research on Influenza, MRC National Institute of Medical Research, Mill Hill, London, UK** (R Daniels PhD); **University of Virginia School of Medicine, Charlottesville, VA, USA, and Wellcome Trust, London, UK** (Prof F G Hayden MD); **Stanley Ho Center for Emerging Infectious Diseases, The Chinese University of Hong Kong, Hong Kong SAR, China** (D S Hui MD); **Division of Infectious Disease Control, Norwegian Institute of Public Health, Oslo, Norway** (O Hungnes PhD); **Health Protection Agency, Colindale, London, UK** (A Lackenby PhD, Prof M Zambon PhD); **Centre for Health Protection, Public Health Laboratory Centre, Hong Kong SAR, China** (W Lim MD); **National Institute for Public Health and the Environment, Bilthoven, Netherlands** (A Meijer PhD); **Global Influenza Programme, WHO, Geneva, Switzerland** (C Penn PhD); **and WHO Collaborating Centre for Reference and Research on Influenza, National Institute of Infectious Diseases, Tokyo, Japan** (M Tashiro MD)

Introduction

In early 2009, a novel swine-origin influenza virus entered the human population and spread rapidly around the globe, prompting WHO to declare a pandemic on June 11, 2009. The emergence of the novel virus caused the first pandemic in an era when the neuraminidase inhibitors oseltamivir and, to a lesser extent, zanamivir had been stockpiled by many high-resource countries and by WHO for use in a pandemic response.[1,2] Although the adamantane (M2 blocker) antivirals amantadine and rimantadine have been used intermittently over the past 30 years to treat influenza A infections, widespread resistance to adamantanes has developed in seasonal H3N2 and Eurasian-lineage swine influenza A viruses. Because the 2009 virus carried the Eurasian swine *M* gene and was resistant to this class of drugs,[3] adamantanes are not considered further in this Review.

In early clinical trials of oseltamivir therapy in seasonal influenza, emergence of resistance was detected in up to 4% of adults who received treatment[4–6] and 5–6% of children,[7] although higher frequencies of resistance (up to 27% in children infected with seasonal H1N1 viruses) have been reported in observational studies.[8–10] Resistance was most often associated with single neuraminidase aminoacid His275Tyr substitutions (N1 numbering) in H1N1 viruses, and Glu119Val and Arg292Lys substitutions (N2 numbering) in H3N2 viruses. N1 aminoacid numbering is used throughout this Review when referring to substitutions in the H1N1 neuraminidase. Resistance to zanamivir in clinical isolates has been rarely reported,[11] presumably due to either the high similarity between the chemical structure of zanamivir and the natural substrate sialic acid,[12] or the relative lack of zanamivir use compared with oseltamivir.[13] In 2007–08, in the apparent absence of drug pressure, oseltamivir-resistant seasonal H1N1 viruses carrying the neuraminidase His275Tyr substitution began circulating in Europe and USA,[14–16] before spreading into the southern hemisphere over the next 6 months.[17,18] The rapid global dissemination of oseltamivir-resistant viruses, leading to replacement of susceptible seasonal H1N1 viruses, showed the potential for a virus resistant to neuraminidase inhibitor to retain or even show enhanced transmission fitness, because of additional permissive or compensatory mutations in the neuraminidase.[19,20] This potential contrasted with most of the oseltamivir-resistant viruses detected before 2007, which had compromised viral fitness in laboratory studies.[21–23]

From the time the new virus was identified in April, 2009, up to April, 2011, WHO received global data for more than 27 000 2009 pandemic H1N1 viruses tested for neuraminidase-inhibitor resistance, of which 447 oseltamivir-resistant viruses were detected (table 1).[24] This is the first time in the history of the Global Influenza Surveillance and Response System (GISRS) that there has been capacity for influenza antiviral susceptibility testing on this scale within WHO Collaborating Centres for Influenza Reference and Research, National Influenza Centres, and some hospital-based laboratories. This large-scale testing was a result of efforts in pandemic planning and upgrading of the technical capability of the GISRS. Most of the resistant viruses were detected in individuals undergoing oseltamivir treatment, many of whom were immunocompromised, but a small number were detected in individuals that were neither receiving oseltamivir nor in known contact with others receiving treatment (figure). Most of the oseltamivir-resistant viruses were detected in three WHO regions (table 1). The low number of resistant viruses detected in other regions might be due to low oseltamivir use, or other factors such as differences in surveillance, sampling strategies (community-based *vs* patients receiving treatment), or laboratory testing capacity. However, despite high use of oseltamivir in some countries,[13,25] the frequency of resistance in community-based surveillance samples of pandemic H1N1 was very low during this period.[26–28]

In this Review, we discuss some of the issues presented by antiviral resistance during the 2009 pandemic, by

Correspondence to:
Dr Aeron C Hurt, WHO Collaborating Centre for Reference and Research on Influenza, North Melbourne, VIC 3051, Australia
aeron.hurt@influenzacentre.org

addressing it from public health, laboratory, and clinical perspectives, and make recommendations relating to current testing, reporting, and management of patients infected with neuraminidase inhibitor-resistant influenza.

Public health

The main public health concerns about antiviral resistance are whether a resistant virus is transmissible and retains the ability to cause disease, whether spread of resistant virus is sustained, and to what extent resistant viruses are prevalent among all circulating influenza viruses locally and worldwide. Of the 447 oseltamivir-resistant 2009 pandemic H1N1 viruses identified up to April, 2011 (table 1), 14% were from patients with no history of oseltamivir use.[29,30] This finding demonstrates either spontaneous His275Tyr substitution in the virus or limited transmission of resistant strains from patients who received treatment, such as several instances of nosocomial transmission[31,32] (discussed in more detail in the clinical management section) and reports of community transmission of oseltamivir-resistant 2009 H1N1 virus to close contacts. Community transmission of resistant viruses was reported in a child in Israel,[30] between cabin-mates undergoing chemoprophylaxis at a school camp in North Carolina, USA,[33] and among students after a 42 h train journey in Vietnam.[34] Patients within the Vietnam train cluster were all asymptomatic during the trip, but developed fever within 12 h of arrival and were shedding an His275Tyr variant virus before any treatment. Although ongoing transmission from the cluster was not detected, it could not be ruled out.[34] A more widespread cluster of oseltamivir resistance within the community was recently reported in Australia, involving more than 20 patients, most within 50 km of a large city.[35] Although the numbers were small, recent data from the third wave of virus circulation in the UK suggested a possible increase in the frequency of transmission of oseltamivir resistance in the community,[36] further highlighting the need for surveillance to detect more widespread dissemination of resistant viruses.

Laboratory-based experiments have been used to assess the viral replication, virulence, and transmissibility of His275Tyr variants of the 2009 pandemic H1N1 virus in various animal models (table 2). Results are somewhat difficult to interpret because the viruses and methods used differed across studies, and inconsistent data were obtained. Some His275Tyr variants have slower viral replication in cell culture[37–39] but equivalent in-vivo replication compared with the wild-type virus in mice, ferrets, and guineapigs[38–41] (table 2). One resistant variant was outgrown by the corresponding wild-type strain in a competitive-mixtures experiment in ferrets,[37] but two other His275Tyr viruses generated by reverse genetics were not.[40] Transmissibility has been assessed in ferret and guineapig models with contact and aerosol (respiratory droplet) routes. In contact models, His275Tyr variants seemed to transmit between animals and replicate to equivalent virus titres at rates similar to wild-type viruses.[38,40–42] However, results differed for the three studies that assessed transmission with an aerosol model (table 2). Siebert and colleagues[40] reported equivalent transmission, compared with the wild-type virus, of two His275Tyr variants in ferrets and guineapigs, whereas Kiso and colleagues[39] reported delayed transmission, compared with the wild-type, of another variant in ferrets. By contrast, Duan and colleagues[37] reported that one His275Tyr variant virus did not transmit at all in an aerosol or respiratory droplet ferret model; however, the wild-type virus only transmitted in one of the two ferrets in the study. The differences reported in aerosol transmission might be attributed to variation in experimental conditions, but these results show that the effect of the His275Tyr substitution in different 2009 H1N1 viruses can range from no change in viral fitness and virulence to reduced respiratory droplet transmissibility and replication. Studies are ongoing to better understand the role of key mutations in haemagglutinin and neuraminidase on the viral fitness and transmissibility of the virus.[43,44]

	WPRO	EURO	AMRO	AFRO	SEARO	EMRO
Viruses tested for antiviral susceptibility	>10 000	>8000	>9000	140	47	59
Oseltamivir-resistant viruses reported	159	191	95	1	0	1

WPRO=western Pacific region. EURO=European region. AMRO=region of the Americas. AFRO=African region. SEARO=southeast Asia region. EMRO=eastern Mediterranean region.

Table 1: **Oseltamivir resistance in 2009 pandemic influenza A H1N1 viruses, by WHO region up to April, 2011**

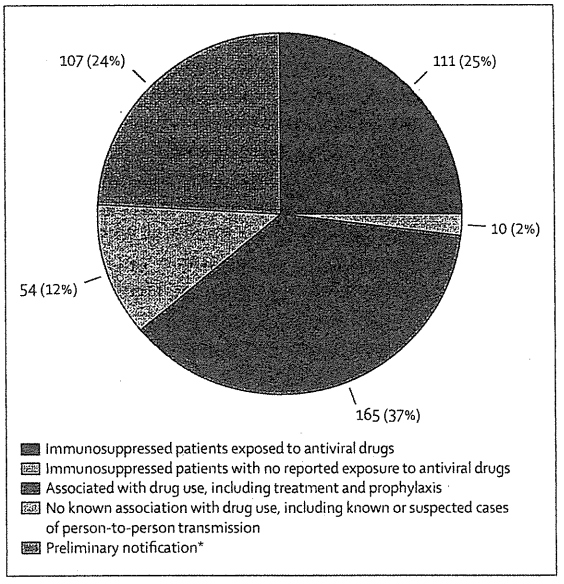

Figure: **Clinical background of reported cases of oseltamivir-resistant A H1N1 2009 viruses (n=447)**
*Insufficient clinical information available or investigations ongoing, as of April, 2011.

www.thelancet.com/infection Published online December 19, 2011 DOI:10.1016/S1473-3099(11)70318-8

	Viral replication (compared with wild-type*)		Virulence (compared with wild-type*)	Transmissibility (compared with wild-type*)	
	In vitro	In vivo		Contact model	Aerosol or respiratory droplet model
Duan et al (2010)[37]	Delayed initial growth	Equivalent, although variant was outgrown by wild-type in mixture experiment (ferrets)	Equivalent (ferrets)	Equivalent (ferrets)	Variant did not transmit, compared with transmission of wild-type in one of two ferrets
Hamelin et al (2010)[38]	Delayed initial growth	Equivalent (mice and ferrets)	Equivalent, although increased inflammation after infection with variant (mice and ferrets)	Transmitted, but equivalent studies with wild-type were not done (ferrets)	Not tested
Kiso et al (2010)[39]	Poorer growth for one His275Tyr variant, but equivalent for a different His275Tyr variant	Equivalent (mice and ferrets)	Equivalent (ferrets)	Not tested	Transmitted, but delayed compared with wild-type
Seibert et al (2010)[40]	Equivalent	Equivalent (ferrets and guineapigs)	Equivalent (ferrets)	Equivalent (ferrets and guineapigs)	Equivalent (ferrets and guineapigs)
Memoli et al (2011)[41]	Not tested	Equivalent (ferrets)	Equivalent (ferrets)	Equivalent (ferrets)	Not tested

*Wild-type viruses used were A/Denmark/524/2009 in Duan et al; A/Quebec/147023/2009 in Hamelin et al; A/Osaka/164/2009 and A/Vietnam/HCM9727/2009 in Kiso et al; A/California/04/2009 and A/Hansa Hamburg/01/2009 in Seibert et al; A/Bethesda/NIH107-D0/2009 and A/Bethesda/NIH106-D0/2009 in Memoli et al.

Table 2: **Summary of virus replication, virulence, and transmissibility of oseltamivir-resistant 2009 pandemic influenza A H1N1 2009 His275Tyr variants in vitro and in vivo**

Although influenza surveillance has improved in many regions of the world, there is an inevitable delay between taking specimens from patients, detecting the presence of influenza viruses by local laboratories, and shipping clinical specimens or virus isolates to reference centres for comprehensive antiviral resistance testing (genotypic and phenotypic analyses). Community-based studies have not reported any notable differences in clinical symptoms or disease severity between infections with oseltamivir-resistant and oseltamivir-susceptible viruses,[15,16] so community spread of viruses with resistance to neuraminidase inhibitors might go unnoticed. Representative and timely samples are needed so that neuraminidase inhibitor susceptibility data can be aligned with clinical (disease severity) and epidemiological data (age, underlying medical conditions, time from illness onset, dose and duration of antiviral exposure, and time from antiviral initiation), to monitor changes that might occur in the future.

Surveillance data on influenza antiviral resistance informs public health and clinical recommendations on the use of antiviral medications for prophylaxis and outbreak management; thus, a baseline level of susceptibility monitoring should be done routinely in countries in which antiviral drugs are used, so that changes in the detected prevalence of resistance can be aligned with changes in influenza activity and antiviral use. Similarly, increased testing of specimens from patients with influenza who receive treatment in hospital would provide much needed data to guide clinical management and public health recommendations, and should include collection of specimens before, during, and after antiviral therapy, particularly in specific groups of patients, such as severely immunocompromised hosts (eg, haemopoietic stem-cell transplantation [HSCT] or solid-organ transplant recipients, patients with haematological malignancy treated with chemotherapy, those with profound lymphocytopenia, advanced AIDS, or receiving higher doses of systemic glucocorticoids) and those with serious illness requiring mechanical ventilation or intensive care (panel 1).

Interpretation of test results should take into account the representativeness of specimens tested. The frequency of detection of a virus resistant to antiviral drugs should be considered for each specific type or influenza A

***Panel 1:* Key recommendations relating to the testing, reporting, and management of patients infected with influenza viruses resistant to neuraminidase inhibitors**

- Increase testing of specimens from patients who receive influenza treatment in hospital, particularly immunocompromised patients and severely ill patients with prolonged viral detection
- Where possible collect specimens before, during, and after antiviral therapy
- Develop a global database for sharing information on the detection of novel resistance mutations and the frequency of drug resistance in different locations
- Produce a panel of reference viruses for distribution to testing laboratories for comparison and standardisation of data and methods
- Where possible regularly test patients receiving treatment with methods that can detect minor proportions of resistant virus in mixtures, correlate virological findings with clinical outcomes
- Consider alternative antivirals (eg, zanamivir) if the virus population contains viruses resistant to oseltamivir, even if present at low proportions in a mixture
- Develop surveillance guidelines for the reporting of mixtures of resistant and sensitive viruses detected through surveillance networks
- Analyse the original clinical specimen, rather than a cell-culture-derived isolate, when reporting the presence of a resistant variant in a mixed population, because the proportions of viruses in mixtures can change in cell culture
- Develop clinical practices to prevent immunocompromised patients transmitting resistant viruses to vulnerable patients in nosocomial settings
- Rigorously study the use of new investigational antiviral drugs or combination therapy to reduce the selection of antiviral resistance

	Times increase in IC_{50} compared with wild-type*				Reference
	Zanamivir	Oseltamivir	Peramivir	Laninamivir	
His275Tyr	1–2	246–1268	84–404	1–2	56–59
His275Tyr + Ile223Arg	15–22	9053–12374	7530–13092	70–85	56,58, CDC unpublished data
Ile223Arg	9–46	5–10	6	NT	56,57
Ile223Lys	5–6	12–39	1–4	1–2	58, CDC unpublished data
Ile223Val	2	4	NT	NT	55
Ser247Asn	2–5	4–7	1–2	NT	59
Ser247Asn + His275Tyr	5	5880–7073	334–704	NT	59
Gln313Arg + Ile427Thr	20	43	4	11–16	3, CDC unpublished data
Ile117Val	3	4	1	NT	60

IC_{50}=concentration of drug required to inhibit neuraminidase activity by 50%. CDC=Centers for Disease Control and Prevention. NT=not tested. *Includes IC_{50} data from chemiluminescence-based and fluorescence-based assays.

Table 3: Neuraminidase-inhibitor susceptibility of 2009 pandemic influenza A H1N1 variants reported so far

subtype. Additionally, because influenza A subtyping analysis is commonly not available when clinicians are making decisions about therapy, the frequency of resistance as the total of all locally circulating influenza viruses identified should be taken into account. Up-to-date global information about resistance to neuraminidase inhibitors in 2009 pandemic H1N1 viruses is available from WHO.[45] Up-to-date regional information about resistance of all human influenza virus types and subtypes is available in regional and national biweekly reports on influenza activity—eg, from Europe,[46,47] China,[48] the USA,[49] Canada,[50] and Australia,[51] particularly during the periods of circulation of influenza in these regions. Development of consensus on thresholds for changing first-line drug recommendations should be considered (panel 1). A lower threshold (eg, 5%) could provide an alert that resistant viruses are circulating, with a higher threshold (eg, 15–25%) as the point where changes in first-line antiviral medication recommendations are considered. Such thresholds might need to be modified depending on the population of patients (eg, those with serious or progressive illness compared with outpatients) and the history of antiviral drug exposure. Regional differences in the frequency of circulating viruses and bias in the sampling of particular groups of patients need to be taken into account as part of an overall guidance for antiviral use.

Most viruses tested for antiviral susceptibility through the GISRS are isolated from untreated outpatients, providing information on antiviral resistance in isolates from patients seeking medical attention. Innovative surveillance schemes, such as the self-swabbing programme for patients receiving oseltamivir treatment in the UK, enable samples to be taken and tested for antiviral susceptibility before and during the course of antiviral therapy, increasing the opportunities for detecting infections by resistant variants due to community transmission and for assessing the emergence of resistance during treatment.[52] However, such schemes might not be possible in resource-limited settings and are highly dependent on health-care informatics infrastructure. Expanded surveillance to include testing of patients receiving antiviral treatment or chemoprophylaxis will help inform clinical recommendations on the use of antiviral agents and will be an important source of information regarding the emergence of novel mutations, especially in immunocompromised patients who show persistent shedding of influenza (panel 1).

Laboratory testing

Among the oseltamivir-resistant 2009 pandemic H1N1 viruses detected so far, nearly all have contained the neuraminidase His275Tyr substitution, which was also associated with the 2007–08 emergence of oseltamivir-resistant seasonal viruses.[53] In all N1-containing viruses studied, the His275Tyr substitution causes high-level oseltamivir resistance (generally >200-times loss of susceptibility), except in the setting of mixed virus populations. His275Tyr variants remain susceptible to zanamivir but have reduced susceptibility to peramivir.[54]

Laboratory identification of viruses resistant to neuraminidase inhibitors is typically done with a phenotypic neuraminidase enzyme-inhibition assay or a genotypic assay that detects mutations encoding aminoacid substitutions at key residues shown to confer resistance to one or more neuraminidase inhibitors. Apart from the His275Tyr substitution, a few other neuraminidase substitutions in A H1N1 2009 viruses isolated from patients receiving treatment have been shown to confer reduced susceptibility to neuraminidase inhibitors in phenotypic assays. These include substitutions of Ile to Arg, Lys, or Val at residue 223,[3,55–58] Ser to Asn at residue 247,[59] and Ile to Val at residue 117,[60] many of which have been detected in combination with His275Tyr, which causes even higher levels of resistance to oseltamivir and peramivir (table 3).[58,59] With the increasing number of newly identified neuraminidase substitutions that confer reduced susceptibility, a global database that facilitates rapid sharing of this information would greatly assist surveillance laboratories in the detection of such viruses, as has been pioneered in Europe (panel 1).[14]

How the IC_{50} of a virus (the concentration of drug required to inhibit neuraminidase activity by 50%) derived by a phenotypic assay in vitro relates to clinical effectiveness of the neuraminidase inhibitors is poorly understood. Although oseltamivir seems to be ineffective for the treatment of seasonal H1N1 His275Tyr variants,[61–63] the clinical effectiveness of oseltamivir against variants such as Ile223Arg or Ser247Asn that have much lower IC_{50} increases than that seen for oseltamivir in His275Tyr variants is currently unknown. Therefore, to define viruses with moderate increases in IC_{50} as being resistant might be misleading, particularly because different in-vitro assay systems have different dynamic ranges, and

susceptible influenza B viruses have higher IC_{50} values than their influenza A H1N1 and H3N2 counterparts. For example, with a fluorescence-based phenotypic assay, an increase of 20-times in oseltamivir IC_{50} for an influenza A virus might still represent a lower value than the mean oseltamivir IC_{50} value of so-called sensitive wild-type influenza B viruses.[18]

Attempts have been made to compare in-vitro IC_{50} values with pharmacokinetic data in plasma or respiratory secretions (eg, sputum, bronchoalveolar lavage, and saliva) after drug administration,[64,65] but these values might not truly reflect the concentration of drug at the surface of infected cells in the respiratory tract, and pharmacokinetic and pharmacodynamic (reduction in viral replication) correlates have not been established. Additionally, the IC_{50} value of a particular virus can differ substantially depending on the enzyme inhibition assay used.[54] Thus, a definition of resistance based on a suggested IC_{50} value is not a reliable description without further information about the context of the observation, and effort is needed to provide more precise definitions linking virus type and subtype with mean IC_{50} value and antiviral effectiveness in vivo. However, a growing body of clinical data suggests that, in children, oseltamivir is less effective in treating infections with influenza B than with influenza A,[66,67] providing preliminary indication that moderate reductions in in-vitro susceptibility might have clinical relevance, although the scaling of this observation and pharmacodynamic correlates need to be further explored to allow firm conclusions. Furthermore, moderate reductions in IC_{50} values might have greater relevance in immunocompromised patients unable to mount effective host immune responses. As a result of these practical uncertainties, use of a group of resistant and susceptible reference standards is recommended (panel 1).

By contrast with the oseltamivir-resistant seasonal H1N1 viruses from 2007–08, many of the oseltamivir-resistant His275Tyr-variant 2009 pandemic H1N1 viruses in clinical specimens have been present as mixed virus populations containing proportions of susceptible wild-type virus;[26,28,32,68,69] this finding has resulted in additional challenges in the laboratory diagnosis of resistance. Pure populations of either wild-type or His275Tyr variants yield a sigmoid-shaped inhibition curve in phenotypic assay. When the population is mixed, the inhibition curve can have a reduced slope gradient and three or more inflection (transition) points (rather than the standard two points for a sigmoid curve), which, after curve-fitting analysis, results in a lower IC_{50} value than that seen with a pure population of His275Tyr-variant viruses. As a result, the IC_{50} values of samples that contain small proportions of His275Tyr-variant virus in a mixed population might not differ from samples of pure wild-type viruses; therefore, resistance might not be easily detected unless the shape of the curve is also considered. For example, the proportion of His275Tyr variant needs to be greater than 25% in fluorescence-based assay,[70,71] and greater than 50% in chemiluminescence-based phenotypic assay, before its IC_{50} is above the mean IC_{50} plus three SD of wild-type viruses.

Various genotypic assays can be applied for the detection of previously characterised resistance-conferring mutations, such as those encoding the His275Tyr substitution. Technologies include short-read sequencing (pyrosequencing), mutation-dependent differential PCR amplification, traditional Sanger sequencing, and next-generation massively parallel sequencing.[70] Some of these technologies, such as pyrosequencing, not only detect single nucleotide polymorphisms but also estimate the relative proportion of variant and wild-type viruses in a mixed population sample,[72,73] and can be applied directly to clinical samples. Such technologies provide opportunities for rapid, local evaluation of antiviral susceptibility and rapid adjustment of antiviral therapies in individual patients. Genetic susceptibility testing can therefore provide qualitative and quantitative information, which allows for refining the information derived from a single sample and early alteration of therapy if indicated (panel 1). When virus mixture information is known and can be robustly validated, it should be included in the laboratory report—ie, by stating that a mixed virus population was detected and noting (when available) the estimated proportion of variant virus in the sample. As has been discussed for minority variants of drug-resistant HIV,[74] an understanding of the proportion of resistant virus in a mixed population that might result in treatment failure is essential. Guidelines regarding surveillance and clinical reporting of mixtures of resistant and sensitive viruses and methods used to detect them are needed (panel 1).

Analysis of the original clinical specimen, rather than an isolate derived from cell culture, is recommended when reporting the presence of a resistant variant in a mixed population, because the proportions of viruses in mixtures can change in cell culture (panel 1).[75,76] High-throughput automatable genotypic screening methods, such as RT-PCR assays covering the mutation encoding His275Tyr substitution, are becoming more popular in hospital laboratories. However, these methods do not recognise all possible substitutions conferring resistance and therefore might fail to detect novel mutations.

Clinical management

From a clinical perspective, the underlying concern relating to the detection of an antiviral-resistant virus or a virus with reduced susceptibility is whether the antiviral in question will be less effective than for an infection caused by a susceptible virus. A low threshold for testing for the emergence of resistance while on therapy should be considered for patients in priority risk groups (panel 2). Global resistance data from the recent pandemic showed that immunocompromised patients, particularly those with haematological malignancies or HSCT, were at the

Panel 2: Considerations for antiviral-resistance testing of specimens for the clinical management of patients infected with 2009 pandemic influenza A H1N1 virus

Infected patients who might benefit from antiviral resistance testing to inform clinical treatment decisions include:

- Immunocompromised patients, especially if symptomatic with evidence of ongoing virus replication. Issues in these patients include: clinical deterioration during or after antiviral treatment; monitoring for duration of infectiousness even after clinical improvement has occurred and antiviral treatment has been stopped, since ongoing asymptomatic infection with antiviral-resistant virus is possible and such patients could therefore pose a high transmission risk
- Patients with severe or progressive illness who do not clinically improve with neuraminidase-inhibitor treatment
- Any patients with evidence of ongoing influenza virus replication, whether they are receiving treatment with a neuraminidase inhibitor or treatment has ceased
- Patients on, or recently on, neuraminidase-inhibitor chemoprophylaxis who develop illness and have influenza-virus infection detected
- Patients who develop influenza-virus infection after exposure to a person with documented oseltamivir-resistant influenza-virus infection

Patients with influenza who receive treatment with a neuraminidase inhibitor for longer than the standard duration of 5 days should have periodic virus testing and antiviral-resistance screening where possible. If resources are available, influenza-virus-load monitoring as a possible indicator of treatment failure and the selection of drug resistance should be considered. In patients with lower respiratory tract infection, specimens from the lower respiratory tract should be obtained.

greatest risk for emergence of oseltamivir-resistant influenza, presumably because of the selective pressure evoked by prolonged exposure to oseltamivir during sustained, sometimes high levels of virus replication in such patients.[77,78] A high index of suspicion of resistance should be maintained in these patients. Suboptimum doses of antiviral therapy (eg, once daily prophylactic dosing in those with active influenza virus replication) or breaks in treatment while patients remain influenza positive should be avoided. Testing in a clinical setting requires different arrangements than those previously described for surveillance purposes. When testing for clinical management purposes, rapid analysis of samples with a molecular-based technique and prompt feedback to clinicians regarding the susceptibility of viruses to the antiviral agents being given is essential. Such rapid analysis requires that laboratories with relevant testing capabilities be in close proximity to patients, particularly since oseltamivir-resistant 2009 pandemic H1N1 viruses can emerge as early as 48 h after the start of treatment.[79,80] The implementation of several of these recommendations in the UK and an increased readiness to switch to zanamivir as a first-line treatment has led to a large reduction in oseltamivir-resistant cases in immunocompromised patients in 2010–11 compared with 2009–10.[36]

Combination antiviral therapy has been used in individual cases involving severely immunocompromised patients or those with severe illness due to 2009 pandemic H1N1 infection, to try and achieve rapid reduction in replication or avoid the selection of resistant viruses, or both.[32,81] However, interpretation of the efficacy of this combination therapy is limited by lack of adequately powered, randomised controlled trials. Some combinations of neuraminidase inhibitors have shown concentration-dependent additive or antagonistic effects for 2009 pandemic H1N1 in vitro.[82] One randomised controlled trial in ambulatory adults with predominately seasonal H3N2 illness reported that a combination of inhaled zanamivir and oral oseltamivir was less effective than oral oseltamivir alone,[83] and prolonged detection of 2009 pandemic H1N1 has been found in several cases series of severely ill patients given both oseltamivir and zanamivir.[84,85] Further preclinical studies are needed to assess available antiviral drug combinations (panel 1). On the basis of limited available data, intravenous zanamivir is the antiviral drug of choice when oseltamivir resistance is either proven or suspected in patients with severe illness due to 2009 pandemic virus infection.[69,86–89] WHO recommends that these and other investigational products, including combination therapies, only be used in accordance with relevant emergency use provisions or as part of a clinical trial.

The emergence of resistant virus in immunocompromised patients during or after cessation of antiviral treatment not only reduces the antiviral options for treatment, but can also be a source of resistant virus that transmits to other individuals in the hospital or community. Immunocompromised patients can excrete oseltamivir-resistant pandemic virus for prolonged periods (more than 60 days in some case reports),[90] and sometimes in the absence of apparent influenza-like symptoms (panel 1).[91,92] At least four patients in a haematology ward in Cardiff, UK, were infected with an oseltamivir-resistant His275Tyr-variant virus from other patients on the ward who were undergoing oseltamivir treatment at the time.[32] Detailed analysis revealed a mixture of resistant and sensitive virus in individuals given treatment, but a pure resistant virus population in the four secondary cases, showing that the resistant virus transmitted well, at least in this vulnerable group of patients.[32] Similar nosocomial transmission of oseltamivir-resistant 2009 pandemic H1N1 has been reported in patients in a haematology ward in North Carolina, USA,[31,93] and in an institution for disabled children in Israel.[94] Inhaled zanamivir is the first-line agent for chemoprophylaxis in managing influenza outbreaks due to suspected or proven oseltamivir resistance in such high-risk units, and has been suggested as a first-line treatment in highly immunocompromised hosts.[32] In addition to monitoring immunocompromised patients for influenza-virus infection and emergence of resistance, measures should be taken to prevent transmission to these patients.[95–97] This includes annual influenza vaccination in health-care workers, family members, and visitors, and strict adherence to recommended infection-control procedures.[98,99]

Conclusions

Although past experience shows that the emergence and global transmission of oseltamivir-resistant influenza A H1N1 viruses can occur unexpectedly and rapidly and can be unrelated to drug use, the frequency of resistance to neuraminidase inhibitors detected among 2009 pandemic H1N1 viruses globally has so far remained low. Sustained global monitoring for the emergence of resistance is important to underpin public health and guidance for clinical management. Sentinel or laboratory surveillance schemes should assess frequency of resistance in the community and in specific patient groups receiving treatment, such as severely immunocompromised, seriously ill patients in hospital, and patients not responding to antiviral therapy. Further studies to better understand influenza-virus infections in these patients and to improve antiviral treatment strategies are needed. Optimum antiviral treatment, especially for individuals in whom there are high risks for the emergence of influenza viruses resistant to neuraminidase inhibitors, can benefit from close collaboration between clinicians and testing laboratories. Specimens from such patients should be rapidly shipped and tested, and results communicated in a timely manner, to enable early action in the management of hospital inpatients if resistance emerges during treatment with a particular antiviral drug. Continued surveillance and detailed characterisation of viruses with unusual resistance profiles are necessary. Additionally, detailed studies of compensatory aminoacid substitutions that can overcome the fitness loss conferred by specific drug resistance-conferring substitutions are needed to improve prediction of emergence of transmissible drug-resistant variants.[44]

Community spread of oseltamivir-resistant 2009 pandemic H1N1 viruses remains a concern, particularly since data from animal studies suggest that the fitness of some His275Tyr variants is not substantially compromised, and the recent report of a widespread community cluster of resistant cases in Australia.[35] Therefore, surveillance programmes that can rapidly identify changes in the frequency of antiviral resistance among circulating influenza viruses in the community will benefit public health, enabling antiviral treatment and chemoprophylaxis guidelines to be revised in a timely manner to inform clinical management of patients with influenza.

Search strategy and selection criteria

References for this Review were identified by searches of PubMed and Scopus, for articles available before October, 2011, with the keywords "influenza", "pandemic", "A(H1N1) 2009", "H1N12009", "H1N1swl", "swine influenza", "neuraminidase inhibitors", "NA inhibitors", "NAIs", "oseltamivir", "zanamivir", "resistance", "sensitivity", "susceptibility", "mutant", and "mutation". References from relevant articles were also included. Abstracts from the Options for the Control of Influenza VII conference, Sept 3–7, 2010, Hong Kong SAR, China, were manually searched. Only reports published in English were included.

Contributors

All authors helped to formulate the public health, laboratory, and clinical questions addressed. ACH wrote the first draft with input from DSH, AL, TMU, and CP. All authors helped write the final draft.

Conflicts of interest

We declare that we have no conflicts of interest.

Acknowledgments

We thank Sara Martins of WHO, Geneva, for her input into the preparation of the manuscript.

References

1 Meijer A, Lackenby A, Hay A, Zambon M. Influenza antiviral susceptibility monitoring activities in relation to national antiviral stockpiles in Europe during the winter 2006/2007 season. *Euro Surveill* 2007; **12:** 3–4.

2 Reddy D. Responding to pandemic (H1N1) 2009 influenza: the role of oseltamivir. *J Antimicrob Chemother* 2010; **65** (suppl 2): 35–40.

3 Gubareva LV, Trujillo AA, Okomo-Adhiambo M, et al. Comprehensive assessment of 2009 pandemic influenza A (H1N1) virus drug susceptibility in vitro. *Antivir Ther* 2010; **15:** 1151–59.

4 Nicholson KG, Aoki FY, Osterhaus AD, et al. Efficacy and safety of oseltamivir in treatment of acute influenza: a randomised controlled trial. Neuraminidase Inhibitor Flu Treatment Investigator Group. *Lancet* 2000; **355:** 1845–50.

5 Gubareva LV, Kaiser L, Matrosovich MN, Soo-Hoo Y, Hayden FG. Selection of influenza virus mutants in experimentally infected volunteers treated with oseltamivir. *J Infect Dis* 2001; **183:** 523–31.

6 Treanor JJ, Hayden FG, Vrooman PS, et al. Efficacy and safety of the oral neuraminidase inhibitor oseltamivir in treating acute influenza. *JAMA* 2000; **283:** 1016–24.

7 Whitley RJ, Hayden FG, Reisinger KS, et al. Oral oseltamivir treatment of influenza in children. *Pediatr Infect Dis J* 2001; **20:** 127–33.

8 Kiso M, Mitamura K, Sakai-Tagawa Y, et al. Resistant influenza A viruses in children treated with oseltamivir: descriptive study. *Lancet* 2004; **364:** 759–65.

9 Stephenson I, Democratis J, Lackenby A, et al. Neuraminidase inhibitor resistance after oseltamivir treatment of acute influenza A and B in children. *Clin Infect Dis* 2009; **48:** 389–96.

10 Tamura D, Sugaya N, Ozawa M, et al. Frequency of drug-resistant viruses and virus shedding in pediatric influenza patients treated with neuraminidase inhibitors. *Clin Infect Dis* 2011; **52:** 432–37.

11 Gubareva LV, Matrosovich MN, Brenner MK, Bethell RC, Webster RG. Evidence for zanamivir resistance in an immunocompromised child infected with influenza B virus. *J Infect Dis* 1998; **178:** 1257–62.

12 Gubareva LV, Kaiser L, Hayden FG. Influenza virus neuraminidase inhibitors. *Lancet* 2000; **355:** 827–35.

13 Hurt AC, Holien JK, Parker M, Barr IG. Oseltamivir resistance and the H274Y neuraminidase mutation in seasonal, pandemic and highly pathogenic influenza viruses. *Drugs* 2009; **69:** 2523–31.

14 Meijer A, Lackenby A, Hungnes O, et al. Oseltamivir-resistant influenza virus A (H1N1), Europe, 2007–08 season. *Emerg Infect Dis* 2009; **15:** 552–60.

15 Dharan NJ, Gubareva LV, Meyer JJ, et al. Infections with oseltamivir-resistant influenza A(H1N1) virus in the United States. *JAMA* 2009; **301:** 1034–41.

16 Ciancio BC, Meerhoff TJ, Kramarz P, et al. Oseltamivir-resistant influenza A(H1N1) viruses detected in Europe during season 2007–08 had epidemiologic and clinical characteristics similar to co-circulating susceptible A(H1N1) viruses. *Euro Surveill* 2009; **14:** 19412.

17 Besselaar TG, Naidoo D, Buys A, et al. Widespread oseltamivir resistance in influenza A viruses (H1N1), South Africa. *Emerg Infect Dis* 2008; **14:** 1809–10.

18 Hurt AC, Ernest J, Deng Y, et al. Emergence and spread of oseltamivir-resistant A(H1N1) influenza viruses in Oceania, South East Asia and South Africa. *Antiviral Res* 2009; **83:** 90–93.

19 Collins PJ, Haire LF, Lin YP, et al. Structural basis for oseltamivir resistance of influenza viruses. *Vaccine* 2009; **27:** 6317–23.

20 Bloom JD, Gong LI, Baltimore D. Permissive secondary mutations enable the evolution of influenza oseltamivir resistance. *Science* 2010; **328:** 1272–75.

21 Carr J, Ives J, Kelly L, et al. Influenza virus carrying neuraminidase with reduced sensitivity to oseltamivir carboxylate has altered properties in vitro and is compromised for infectivity and replicative ability in vivo. *Antiviral Res* 2002; **54:** 79–88.

22 Herlöcher ML, Carr J, Ives J, et al. Influenza virus carrying an R292K mutation in the neuraminidase gene is not transmitted in ferrets. *Antiviral Res* 2002; **54:** 99–111.

23 Herlocher ML, Truscon R, Elias S, et al. Influenza viruses resistant to the antiviral drug oseltamivir: transmission studies in ferrets. *J Infect Dis* 2004; **190:** 1627–30.

24 WHO. Update on oseltamivir resistance to influenza A(H1N1)2009 viruses. http://www.who.int/influenza/2011_04_08_weekly_web_update_oseltamivir_resistance.pdf (accessed Dec 15, 2011).

25 IMS Health. IMS customer portal. https://websolutions.imshealth.com/EB2/User/Validate_UserID.aspx?TARGET=SMhttps://customerportal.imshealth.com/portal/site/imsportal (accessed Dec 1, 2011).

26 Ujike M, Ejima M, Anraku A, et al. Monitoring and characterization of oseltamivir-resistant pandemic (H1N1) 2009 virus from May 2009 to February 2010, Japan. *Emerg Infect Dis* 2011; **17:** 470–79.

27 Meijer A, Jonges M, Abbink F, et al. Oseltamivir-resistant pandemic A(H1N1) 2009 influenza viruses detected through enhanced surveillance in the Netherlands, 2009-2010. *Antiviral Res* 2011; **92:** 81–89.

28 Hurt AC, Deng Y, Ernest J, et al. Oseltamivir-resistant influenza viruses circulating during the first year of the influenza A(H1N1) 2009 pandemic in the Asia-Pacific region, March 2009 to March 2010. *Euro Surveill* 2011; **16:** 19770.

29 Leung TW, Tai AL, Cheng PK, Kong MS, Lim W. Detection of an oseltamivir-resistant pandemic influenza A/H1N1 virus in Hong Kong. *J Clin Virol* 2009; **46:** 298–99.

30 Zonis Z, Engelhard D, Hindiyeh M, et al. Community-acquired oseltamivir-resistant pandemic (H1N1) 2009 in child, Israel. *Emerg Infect Dis* 2010; **16:** 1045–46.

31 Wolfe C, Greenwald I, Chen L. Pandemic (H1N1) 2009 and oseltamivir resistance in hematology/oncology patients. *Emerg Infect Dis* 2010; **16:** 1809–11.

32 Moore C, Galiano M, Lackenby A, et al. Evidence of person-to-person transmission of oseltamivir-resistant pandemic influenza A(H1N1) 2009 virus in a hematology unit. *J Infect Dis* 2011; **203:** 18–24.

33 Oseltamivir-resistant 2009 pandemic influenza A (H1N1) virus infection in two summer campers receiving prophylaxis—North Carolina, 2009. *MMWR Morb Mortal Wkly Rep* 2009; **58:** 969–72.

34 Le QM, Wertheim HF, Tran ND, van Doorn HR, Nguyen TH, Horby P. A community cluster of oseltamivir-resistant cases of 2009 H1N1 influenza. *N Engl J Med* 2010; **362:** 86–87.

35 Hurt AC, Hardie K, Wilson NJ, et al. Community transmission of oseltamivir-resistant A(H1N1)pdm09 influenza. *N Engl J Med* (in press).

36 Lackenby A, Moran GJ, Pebody R, et al. Continued emergence and changing epidemiology of oseltamivir-resistant influenza A(H1N1)2009 virus, United Kingdom, winter 2010/11. *Euro Surveill* 2011; **16:** 19784.

37 Duan S, Boltz DA, Seiler P, et al. Oseltamivir-resistant pandemic H1N1/2009 influenza virus possesses lower transmissibility and fitness in ferrets. *PLoS Pathog* 2010; **6:** e1001022.

38 Hamelin ME, Baz M, Abed Y, et al. Oseltamivir-resistant pandemic A/H1N1 virus is as virulent as its wild-type counterpart in mice and ferrets. *PLoS Pathog* 2010; **6:** e1001015.

39 Kiso M, Shinya K, Shimojima M, et al. Characterization of oseltamivir-resistant 2009 H1N1 pandemic influenza A viruses. *PLoS Pathog* 2010; **6:** e1001079.

40 Seibert CW, Kaminski M, Philipp J, et al. Oseltamivir-resistant variants of the 2009 pandemic H1N1 influenza A virus are not attenuated in the guinea pig and ferret transmission models. *J Virol* 2010; **84:** 11219–26.

41 Memoli MJ, Davis AS, Proudfoot K, et al. Multidrug-resistant 2009 pandemic influenza A(H1N1) viruses maintain fitness and transmissibility in ferrets. *J Infect Dis* 2011; **203:** 348–57.

42 Chen H, Smith GJ, Li KS, et al. Establishment of multiple sublineages of H5N1 influenza virus in Asia: implications for pandemic control. *Proc Natl Acad Sci USA* 2006; **103:** 2845–50.

43 Jayaraman A, Pappas C, Raman R, et al. A single base-pair change in 2009 H1N1 hemagglutinin increases human receptor affinity and leads to efficient airborne viral transmission in ferrets. *PLoS One* 2011; **6:** e17616.

44 Bloom JD, Nayak JS, Baltimore D. A computational-experimental approach identifies mutations that enhance surface expression of an oseltamivir-resistant influenza neuraminidase. *PLoS One* 2011; **6:** e22201.

45 WHO. Influenza virological updates. http://www.who.int/influenza/gisrs_laboratory/updates/en/index.html (accessed Dec 1, 2011).

46 EUROFLU. EuroFlu weekly electronic bulletin, influenza season 2010–2011. http://www.euroflu.org/cgi-files/bulletin_v2.cgi?season=2010&inter=n&menu=y (accessed Oct 3, 2011).

47 ECDC. Weekly influenza surveillance overview. http://ecdc.europa.eu/en/publications/surveillance_reports/influenza/Pages/weekly_influenza_surveillance_overview.aspx (accessed Oct 3, 2011).

48 CNIC. Chinese National Influenza Center, surveillance. http://www.cnic.org.cn/eng/surveillance.php (accessed Oct 3, 2011).

49 CDC. Centers for Disease Control and Prevention, flu activity and surveillance. http://www.cdc.gov/flu/weekly/fluactivitysurv.htm (accessed Oct 3, 2011).

50 PHAC. Public Health Agency of Canada, FluWatch. http://www.phac-aspc.gc.ca/fluwatch/index-eng.php (accessed Oct 3, 2011).

51 Australian Government DoHaA. Influenza. http://www.health.gov.au/internet/main/publishing.nsf/Content/cda-surveil-ozflu-flucurr.htm (accessed Oct 3, 2011).

52 Lackenby A, Powers C, Elliott AJ, et al. Antiviral susceptibility of pandemic influenza in a self-sampling, oseltamivir treated community surveillance scheme in the UK. Options for the Control of Influenza VII; Hong Kong, SAR, China; Sept 3–7, 2010. Abstract P-186.

53 Lackenby A, Hungnes O, Dudman S, et al. Emergence of resistance to oseltamivir among influenza A(H1N1) viruses in Europe. *Euro Surveill* 2008; **13:** 5.

54 Nguyen HT, Sheu TG, Mishin VP, Klimov AI, Gubareva LV. Assessment of pandemic and seasonal influenza A (H1N1) virus susceptibility to neuraminidase inhibitors in three enzyme activity inhibition assays. *Antimicrob Agents Chemother* 2010; **54:** 3671–77.

55 Santos L, Correia V, Giria M, et al. Genetic and antiviral drug susceptbility profiles of pandemic A(H1N1)v influenza virus circulating in Portugal. Options for the Control of Influenza VII; Hong Kong, SAR, China; Sept 3–7, 2010. Abstract O-851.

56 Rousset D, Le Goff J, Abou-Jaoude G, et al. Emergence of successive mutations in the neuraminidase of the pandemic H1N1 virus respectively associated with oseltamivir resistance and reduced susceptbility to both oseltamivir and zanamivir under treatment with neuraminidase inhibitors. Options for the Control of Influenza VII; Hong Kong, SAR, China; Sept 3–7, 2010. Abstract P-198.

57 van der Vries E, Stelma FF, Boucher CA. Emergence of a multidrug-resistant pandemic influenza A (H1N1) virus. *N Engl J Med* 2010; **363:** 1381–82.

58 Nguyen HT, Fry AM, Loveless PA, Klimov AI, Gubareva LV. Recovery of a multidrug-resistant strain of pandemic influenza A 2009 (H1N1) virus carrying a dual H275Y/I223R mutation from a child after prolonged treatment with oseltamivir. *Clin Infect Dis* 2010; **51:** 983–84.

59 Hurt A, Lee R, Leang S, et al. Increased detection in Australia and Singapore of a novel influenza A(H1N1)2009 variant with reduced oseltamivir and zanamivir sensitivity due to a S247N neuraminidase mutation. *Euro Surveill* 2011; **16:** 19884.

60 Hurt AC, Leang SK, Speers DJ, Barr IG, Maurer-Stroh S. Mutations I117V and I117M and oseltamivir sensitivity of pandemic (H1N1) 2009 viruses. *Emerg Infect Dis* (in press).

61 Saito R, Sato I, Suzuki Y, et al. Reduced effectiveness of oseltamivir in children infected with oseltamivir-resistant influenza A (H1N1) viruses with His275Tyr mutation. *Pediatr Infect Dis J* 2010; **29:** 898–904.

62 Kawai N, Ikematsu H, Iwaki N, et al. Clinical effectiveness of oseltamivir for influenza A(H1N1) virus with H274Y neuraminidase mutation. *J Infect* 2009; **59:** 207–12.

63 Matsuzaki Y, Mizuta K, Aoki Y, et al. A two-year survey of the oseltamivir-resistant influenza A(H1N1) virus in Yamagata, Japan and the clinical effectiveness of oseltamivir and zanamivir. *Virol J* 2010; **7:** 53.

64 He G, Massarella J, Ward P. Clinical pharmacokinetics of the prodrug oseltamivir and its active metabolite Ro 64-0802. *Clin Pharmacokinet* 1999; **37:** 471–84.

65 Peng AW, Milleri S, Stein DS. Direct measurement of the anti-influenza agent zanamivir in the respiratory tract following inhalation. *Antimicrob Agents Chemother* 2000; **44:** 1974–76.

66 Sugaya N, Tamura D, Yamazaki M, et al. Comparison of the clinical effectiveness of oseltamivir and zanamivir against influenza virus infection in children. *Clin Infect Dis* 2008; **47:** 339–45.

67 Sugaya N, Mitamura K, Yamazaki M, et al. Lower clinical effectiveness of oseltamivir against influenza B contrasted with influenza A infection in children. *Clin Infect Dis* 2007; **44:** 197–202.

68 Speers DJ, Williams SH, Pinder M, Moody HR, Hurt AC, Smith DW. Oseltamivir-resistant pandemic (H1N1) 2009 influenza in a severely ill patient: the first Australian case. *Med J Aust* 2010; **192:** 166–68.

69 Suhaila M, Tang JW, Lee HK, et al. Mixtures of oseltamivir-sensitve and -resistant pandemic influenza A/H1N1/2009 viruses in immunocompromised hospitalized children. *Pediatr Infect Dis J* 2011; **30:** 625–27.

70 Lackenby A, Democratis J, Siqueira MM, Zambon MC. Rapid quantitation of neuraminidase inhibitor drug resistance in influenza virus quasispecies. *Antivir Ther* 2008; **13:** 809–20.

71 Deng YM, Caldwell N, Hurt A, et al. A comparison of pyrosequencing and neuraminidase inhibition assays for the detection of oseltamivir-resistant pandemic influenza A(H1N1) 2009 viruses. *Antiviral Res* 2011; **90:** 87–91.

72 Deyde VM, Okomo-Adhiambo M, Sheu TG, et al. Pyrosequencing as a tool to detect molecular markers of resistance to neuraminidase inhibitors in seasonal influenza A viruses. *Antiviral Res* 2009; **81:** 16–24.

73 Deyde VM, Sheu TG, Trujillo AA, et al. Detection of molecular markers of drug resistance in 2009 pandemic influenza A (H1N1) viruses by pyrosequencing. *Antimicrob Agents Chemother* 2010; **54:** 1102–10.

74 Gianella S, Richman DD. Minority variants of drug-resistant HIV. *J Infect Dis* 2010; **202:** 657–66.

75 Hurt AC, Holien JK, Parker M, Kelso A, Barr IG. Zanamivir-resistant influenza viruses with a novel neuraminidase mutation. *J Virol* 2009; **83:** 10366–73.

76 Okomo-Adhiambo M, Nguyen HT, Sleeman K, et al. Host cell selection of influenza neuraminidase variants: implications for drug resistance monitoring in A(H1N1) viruses. *Antiviral Res* 2010; **85:** 381–88.

77 Graitcer SB, Gubareva LV, Kamimoto L, et al. Characterization of patients with oseltamivir-resistant pandemic (H1N1) 2009, United States. *Emerg Infect Dis* 2011; **17:** 255–57.

78 Tramontana AR, George B, Hurt AC, et al. Oseltamivir resistance in adult oncology and hematology patients infected with pandemic (H1N1) 2009 virus, Australia. *Emerg Infect Dis* 2010; **16:** 1068–75.

79 Valinotto LE, Diez RA, Barrero PR, Farias JA, Lopez EL, Mistchenko AS. Emergence of intratreatment resistance to oseltamivir in pandemic influenza A H1N1 2009 virus. *Antivir Ther* 2010; **15:** 923–27.

80 Inoue M, Barkham T, Leo YS, et al. Emergence of oseltamivir-resistant pandemic (H1N1) 2009 virus within 48 hours. *Emerg Infect Dis* 2010; **16:** 1633–36.

81 Campbell AP, Jacob ST, Kuypers J, et al. Respiratory failure caused by 2009 novel influenza A/H1N1 in a hematopoietic stem-cell transplant recipient: detection of extrapulmonary H1N1 RNA and use of intravenous peramivir. *Ann Intern Med* 2010; **152:** 619–20.

82 Nguyen JT, Hoopes JD, Le MH, et al. Triple combination of amantadine, ribavirin, and oseltamivir is highly active and synergistic against drug resistant influenza virus strains in vitro. *PLoS One* 2010; **5:** e9332.

83 Duval X, van der Werf S, Blanchon T, et al. Efficacy of oseltamivir-zanamivir combination compared to each monotherapy for seasonal influenza: a randomized placebo-controlled trial. *PLoS Med* 2010; **7:** e1000362.

84 Petersen E, Keld DB, Ellermann-Eriksen S, et al. Failure of combination oral oseltamivir and inhaled zanamivir antiviral treatment in ventilator- and ECMO-treated critically ill patients with pandemic influenza A (H1N1)v. *Scand J Infect Dis* 2011; **43:** 495–503.

85 Fraaij PL, van der Vries E, Beersma MF, et al. Evaluation of the antiviral response to zanamivir administered intravenously for treatment of critically ill patients with pandemic influenza A (H1N1) infection. *J Infect Dis* 2011; **204:** 777–82.

86 Gaur AH, Bagga B, Barman S, et al. Intravenous zanamivir for oseltamivir-resistant 2009 H1N1 influenza. *N Engl J Med* 2010; **362:** 88–89.

87 Dulek DE, Williams JV, Creech CB, et al. Use of intravenous zanamivir after development of oseltamivir resistance in a critically ill immunosuppressed child infected with 2009 pandemic influenza A (H1N1) virus. *Clin Infect Dis* 2010; **50:** 1493–96.

88 Elbahlawan L, Gaur AH, Furman W, et al. Severe H1N1-associated acute respiratory failure in immunocompromised children. *Pediatr Blood Cancer* 2011; **57:** 625–28.

89 WHO. WHO guidelines for pharmacological management of pandemic influenza A(H1N1) 2009 and other influenza viruses. Revised February 2010. http://www.who.int/csr/resources/publications/swineflu/h1n1_guidelines_pharmaceutical_mngt.pdf (accessed Oct 3, 2011).

90 Renaud C, Boudreault AA, Kuypers J, et al. H275Y mutant pandemic (H1N1) 2009 virus in immunocompromised patients. *Emerg Infect Dis* 2011; **14:** 653–60.

91 Wiebe C, Reslerova M, Komenda P, Bueti J, Rigatto C, Sood MM. Atypical clinical presentation of H1N1 influenza in a dialysis patient. *Lancet* 2009; **374:** 1300.

92 Gooskens J, Jonges M, Claas EC, Meijer A, Kroes AC. Prolonged influenza virus infection during lymphocytopenia and frequent detection of drug-resistant viruses. *J Infect Dis* 2009; **199:** 1435–41.

93 Chen LF, Dailey NJ, Rao AK, et al. Cluster of oseltamivir-resistant 2009 pandemic influenza A (H1N1) virus infections on a hospital ward among immunocompromised patients—North Carolina, 2009. *J Infect Dis* 2011; **203:** 838–46.

94 Mandelboim M, Hindiyeh M, Meningher T, Mendelson E. Possible transmission of pandemic (H1N1) 2009 virus with oseltamivir resistance. *Emerg Infect Dis* 2010; **16:** 873–74.

95 Tomblyn M, Chiller T, Einsele H, et al. Guidelines for preventing infectious complications among hematopoietic cell transplantation recipients: a global perspective. *Biol Blood Marrow Transplant* 2009; **15:** 1143–238.

96 Ison MG. Epidemiology, prevention, and management of influenza in patients with hematologic malignancy. *Infect Disord Drug Targets* 2011; **11:** 34–39.

97 Kumar D, Morris MI, Kotton CN, et al. Guidance on novel influenza A/H1N1 in solid organ transplant recipients. *Am J Transplant* 2010; **10:** 18–25.

98 WHO. Epidemic- and pandemic-prone acute respiratory diseases: infection prevention and control in health care. http://www.who.int/csr/resources/publications/EPR_AM3_E3.pdf (accessed Oct 3, 2011).

99 CDC. Prevention strategies for seasonal influenza in healthcare settings: guidelines and recommendations. http://www.cdc.gov/flu/professionals/infectioncontrol/healthcaresettings.htm (accessed Oct 3, 2011).

Vaccine 29 (2011) 4156–4161

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Differences in the priming effect of various clades/subclades of inactivated H5N1 vaccine for booster injection with heterologous clades of vaccine strains

Daisuke Ikeno[a,b,*], Kazuhiko Kimachi[a], Kayo Ibaragi[a], Yasuhiro Kudo[a], Shuro Goto[a], Koichi Odoh[a], Shigeyuki Itamura[c], Takato Odagiri[c], Masato Tashiro[c], Yoichiro Kino[a]

[a] The Chemo-Sero-Therapeutic Research Institute, Kikuchi Research Center, Kawabe Kyokushi, Kikuchi, Kumamoto 869-1298, Japan
[b] Department of Biological Sciences, Graduate School of Science and Technology, Kumamoto University, Kumamoto 860-8555, Japan
[c] National Institute of Infectious Diseases, Gakuen 4-7-1, Musashi-Murayama, Tokyo 208-0011, Japan

ARTICLE INFO

Article history:
Received 2 August 2010
Received in revised form 15 March 2011
Accepted 22 March 2011
Available online 15 April 2011

Keywords:
H5N1 influenza
Heterologous prime–boost immunization

ABSTRACT

The prime–boost response induced by different combinations of four H5N1 vaccines (NIBRG-14 (clade 1), Indo05/2005(H5N1)/PR8-IBCDC-RG2 (clade 2.1), A/Bar-Headed Goose/Qinhai Lake/1A/05 SJ163222 (clade 2.2), and Anhui01/2005(H5N1)-PR8-IBCDC-RG5 (clade 2.3.4)) was evaluated in mice. Clade 1-primed BALB/c mice showed a booster response to all of the other three H5N1 vaccines. Clade 2.2 vaccine was also a good priming vaccine. However, mice primed with clade 2.1 or clade 2.3.4 vaccine did not respond to booster injection with clade 1 vaccine, suggesting that priming might actually inhibit the booster response with some combinations of vaccines belonging to different clades. Analysis of the mechanism involved showed that lymphocytes from primed mice secreted comparable amounts of cytokines with any combination of priming and booster vaccines. Therefore, impairment of B cell immunity specific to certain booster strains may have been involved.

1. Introduction

H5N1 influenza viruses have caused epidemics in bird populations throughout the world since 1997, and human infections transmitted from infected birds have continuously been reported in several Southeast Asian countries and Egypt. Although the number of human infections due to H5N1 influenza virus has been limited and no human-to-human transmission has been confirmed so far, the mortality rate is very high [1]. If the virus acquires the ability to be readily transmitted from human to human, it could lead to a pandemic. Therefore, H5N1 is considered to be a candidate virus for causing a possible pandemic. Although we have recently experienced a pandemic caused by H1N1 2009 virus, the fear of H5N1 virus has not diminished.

H5N1 influenza viruses are classified into 10 clades and several subclades, and 11 human vaccine strains have been selected by the WHO as part of the preparations for an influenza pandemic [2]. Although multiple vaccine strains have been prepared as a safeguard, it remains difficult to predict which influenza virus will actually cause a pandemic. In general, manufacturing an influenza vaccine takes longer than 4 months, so it is too late to start production after a pandemic strain has been identified. Under these circumstances, pre-pandemic vaccination is one of the possible solutions [3,4].

Pre-pandemic vaccination has two advantages. First, if the antigenic difference between the pre-pandemic vaccine strain and the pandemic viral strain is small, cross-protection can be expected against infection with the pandemic virus. Such cross-protection has been demonstrated in several animal studies [5–8]. Second, even if antigenic differences between the pre-pandemic vaccine strain and the pandemic viral strain are more important, a prime–boost effect could be expected upon administration of the pandemic vaccine that would lead to a rapid and enhanced antibody response against the pandemic virus. We have already reported the prime–boost effect of alum-adjuvanted whole H5N1 vaccines in mice [9], where priming with NIBRG-14 (clade 1) significantly enhanced the booster response induced by the antigenically heterologous clade of Indo05/2005(H5N1)/PR8-IBCDC-RG2 (clade 2.1). The clinical studies have also demonstrated that the same combination of the priming vaccine and the booster vaccine elicited an effective antibody response in humans [10–12].

A peculiar immunological phenomenon has been noted in humans infected with a seasonal influenza virus in which re-infection of immunologically primed individuals with a new variant induces antibodies that are predominantly specific for the earlier variant rather than the new one. This phenomenon is known

* Corresponding author at: The Chemo-Sero-Therapeutic Research Institute, Kikuchi Research Center, Kawabe Kyokushi, Kikuchi, Kumamoto 869-1298, Japan. Tel.: +81 968 37 4090; fax: +81 968 37 3616.
E-mail address: ikeno-da@kaketsuken.or.jp (D. Ikeno).

0264-410X/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2011.03.084