Original Article

Pathogenesis and Immunogenicity of an Avian H9N2 Influenza Virus Isolated from Human*

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Abstract

Objective To investigate the pathogenesis and immunogenicity of H9N2 influenza virus A/Guangzhou/333/99 (a reassortant of G1 and G9 viruses isolated from a female patient in 1999) in a mouse model of infection.

Methods Mice were infected with increasing virus titers. Viral load in the lungs and trachea was determined by EID50 assay. Pulmonary histopathology was assessed by hematoxylin-eosin staining. Anti-HI antibody titers and T-cell responses to viral HA were determined by ELISPOT and confirmed by flow cytometry.

Results Mice presented a mild syndrome after intranasal infection with A/Guangzhou/333/99 (H9N2) influenza virus. Virus was detected in the trachea and lungs of mice harvested on days 3, 6, and 9 post-infection. A T-cell response to viral HA was detected on day 6 and H9 HA-specific CD^{4+} T-cells predominated. Seroconversion was detected after 14 days and antibody persisted for at least 28 weeks.

Conclusion Our results suggest that H9N2 (A/Guangzhou/333/99) can replicate in the murine respiratory tract without prior adaptation, and both humoral and cell-mediated immunity play an important role in the immune response.

Key words: Avian influenza H9N2; Pathogenic characteristics; Host immune responses

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INTRODUCTION

Influenza A viruses are classified based on the hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins. To date, 16 HA and nine NA subtypes have been identified[1]. The avian H9N2 virus was first isolated from turkey[2]. In mainland China, H9N2 avian influenza virus was first isolated from chicken in Guangdong province in 1994[3]. Influenza viruses generally have a narrow host range, but recent reports have indicated transmission to a number of mammalian species, including human[4-5]. Several cases of H9N2 infecting humans have been reported in southern China and Hong Kong[6-7]. Patients presented with mild clinical signs but no deaths have occurred. The seroprevalence of anti-H9N2 antibodies in apparently healthy individuals has been reported to be around 2%[5,8-9]. Although H9N2 is thought to be of low pathogenicity compared with H5N1, it remains a potential threat to humans.

Avian H9N2 viruses are subdivided into three major genetic lineages: G1, G9, and Korean. In Asia, these are represented by: A/Quail/Hong Kong/G1/97(G1), A/Chicken/Hong Kong/G9/97(G9) and A/Duck/Hong

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Kong/Y439/97(Korean)[10]. The six internal genes of the G1 virus, and the PB1 and PB2 genes of the G9 virus share a high degree of nucleotide homology with those of the highly pathogenic H5N1 viruses isolated from humans in 1997[11-12]. In China, all H9N2 strains isolated from humans in 1998 were of the G9 lineage. However, in 1999, a novel H9N2 strain, A/Guangzhou/333/99(H9N2), was isolated from a child with an influenza-like illness. Unlike other Chinese H9N2 isolates, this virus was a G9 and G1 reassortant in which four genes (encoding the HA, NA, NP, and NS proteins) were derived from the G9 lineage, while the genes encoding the M and the three polymerase proteins (PB1, PB2, and PA), were derived from the G1 lineage[13].

We used a mouse model to investigate the pathogenesis and immunogenicity profiles the naturally reassortant virus A/Guangzhou/333/99(H9N2), to further understand the mechanism of cross-species infection of the H9N2 virus.

MATERIALS AND METHODS

Viral Strains And Culture Conditions

The virus used in this study was A/Guangzhou/ 333/99(H9N2), isolated originally from a 22-month-old female. Virus was passaged in Madin-Darby canine kidney (MDCK) twice, and in allantoic cavities of 10-day-old embryonated specific-pathogenic-free (SPF) eggs once. Viral stocks were propagated in allantoic cavities of 10-day-old embryonated SPF eggs at 35 °C for 48 h, aliquoted, and stored at -70 °C. Fifty-percent tissue culture infectious dose ($TCID_{50}$) and 50% egg infectious dose (EID_{50}) titers were determined by serial titration in MDCK cells and eggs, respectively. Titers were calculated using the method of Reed and Muench[14].

Mouse Model

Female BALB/c mice (Beijing Colab Bio-technology Ltd., Beijing, China), six to eight weeks old, were divided into seven groups with 12 mice per group. Mice were anesthetized with CO_2 and infected intranasally with 10^9, 10^8, 10^7, 10^6, 10^5, and 10^4 EID_{50} (50 μL in PBS). Sterile PBS was used as a control. Mouse 50% infectious doses (MID_{50}), and 50% lethal doses (LD_{50}) were determined.

For determination of viral loads and investigation of pathogenesis, three mice from each group were euthanized at 3, 6, and 9 days post-infection, and tracheas and lungs were collected and homogenized in cold PBS (1 mL). Homogenates were stored at -70 °C. Homogenates were clarified by centrifugation and viral loads determined by egg culture. The remaining mice in each group were checked daily for clinical symptoms, or death, for 14 days post-infection. MID_{50} and LD_{50} titers were calculated by the method of Reed and Muench, and expressed as the EID_{50} value corresponding to one MID_{50} or LD_{50}. Lung virus titers were used for the determination of MID_{50}. Virus endpoint titers are expressed as $\log_{10}$ EID_{50}/mL.

For assessment of viral immunogenicity, hemagglutination inhibition (HI) antibody assays were performed on sera from each group harvested on day zero (pre-infection), and on days 7, 14, and 28 post-infection. Serum from mice infected with 10^8 and 10^6 EID_{50} was harvested 28 weeks post-infection, and subjected to HI assays. Spleens were harvested from the 10^7 EID_{50} group on day 6 post-infection and ELISPOT assays and flow cytometric analysis were performed.

HI Antibody Assays

Serum collected from the orbital plexus of mice was treated with a receptor-destroying enzyme. Samples were then serially diluted in two-fold steps, mixed with an identical volume of virus, and incubated for 30 min. Turkey red blood cells (1%; 50 μL) were added to each well and the plates incubated at RT for 30 min. The $\log_2$ of the dilution that completely inhibited agglutination was defined as the HI antibody titer. A serum HI titer $\geqslant$5.32 was taken as indicative of a humoral response to infection.

IFN-γ ELISPOT Assay

Spleens were harvested from infected mice on day 6 post-infection and cells collected for ELISPOT assay (ELISPOT Mouse IFN-γ Set, and ELISPOT AEC Substrate Set, BD, Franklin Lakes, NJ). Briefly, whole spleens were removed and forced into a single cell suspension using a cell strainer. Cells were treated with lymphocyte separation medium and washed in RPMI. Cells were centrifuged and resuspended in 1 mL 10% RPMI/FBS (GIBCO). Cell viability was determined by trypan blue exclusion staining. Pre-coated anti-IFN-γ plates were incubated at 25 °C for 2 h in RPMI/FBS, and incubated with splenocytes. Spleen-derived T-lymphocytes that released IFN-γ upon stimulation with H9 HA-derived peptide pools were assayed by ELISPOT. Splenocytes were stimulated for 48 h with peptides (provided by MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital,

University of Oxford, United Kingdom). H9 HA peptide pools (18 total; each peptide an 18-mer with a 10-residue overlap) were used to stimulate cells. Additional wells were stimulated with PMA/ionomycin (20 ng/mL and 1 μg/mL, respectively) or mock stimulated. Plates were washed with PBS-Tween (3×) and incubated with anti-IFN-γ antibody at RT for 2 h. Plates were then washed and incubated at RT for 1 h with HRP-conjugated streptavidin. Following extensive washing, complexes were incubated at RT for 15 min, rinsed with H_2O, and air-dried. Spots were counted by ELISPOT (ImmunoSpot, Cellular Technology Ltd, Shaker Heights, OH, USA).

Flow Cytometry

Peptides positive by ELISPOT were subjected to flow cytometric analysis. Lymphocytes (10^6) were incubated with peptides and PMA/ionomycin at 37 °C for 1 h, followed by FBS/RPMI with BFA (10 μg/mL) and incubation at 37 ℃ for 8 h. Cells were washed with FACS buffer and stained with anti-mouse APC conjugated with anti-CD^{3+}, APC/cy7 conjugated anti-CD^{4+}, and FITC conjugated anti-CD^{8+} (Biolegend, San Diego, CA, USA). Cells were fixed and permeabilized using BD Cytofix/Cytoperm solution (BD Biosciences, San Jose, CA, USA), and washed in Perm/Wash buffer (BD Biosciences). Following staining with intracellular PE conjugated anti-IFN-γ (Biolegend), cells were fixed in 1% formalin/PBS and data acquired using a FACSAria III Cell Sorter (BD Biosciences). The gates were set to analyze lymphocytes, and 50 000 cells were analyzed.

Hematoxylin-eosin (HE) Staining

Lung tissue from infected mice was prepared by formalin-fixation, paraffin-embedding, and sectioning. Specimens were then stained with hematoxylin followed by eosin.

Statistical Analysis

Weight data were analyzed using the two-sided Student's-test and differences were considered significant at $P<0.05$.

RESULTS

Clinical Signs of Infection

All mice infected with A/Guangzhou/333/99 (H9N2) survived for at least 14 days post-infection. Transient body weight reduction was observed only in the high titer infection groups (10^9, 10^8, and 10^7 EID_{50}; Figure 1). Control animals showed an overall upward trend in body weight. In groups that received 10^9, 10^8, or 10^7 EID_{50}, body weight began to decline after 1 day, and reached its lowest point 6 days post-infection, before starting a slow recovery. Weight loss was about 10% in the 10^9 EID_{50} group (SD=7.02, $P<0.05$), about 5% in the 10^8 (SD=0.83, $P<0.05$), and 10^7 (SD=7.18, $P<0.05$) EID_{50} groups, respectively, on day 6 post-infection. Body weight in groups that received 10^6, 10^5, and 10^4 EID_{50} showed a slow increase, similar to the control group.

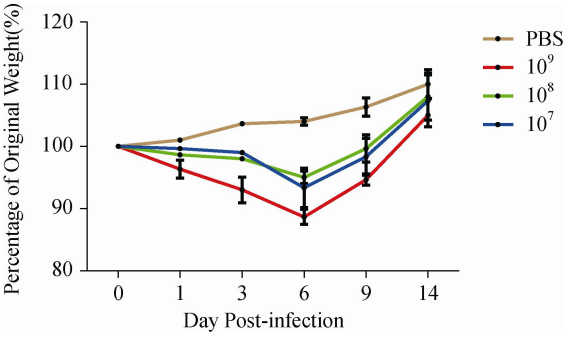

Figure 1. Mice were infected intranasally with 50 μL of 10^9, 10^8, or 10^7 EID_{50} H9N2 virus. PBS was used as a control.

Viral Loads in the Trachea and Lungs

Virus was detected in pulmonary and tracheal tissues in the 10^9, 10^8, 10^7, 10^6, and 10^5 EID_{50} groups on days 3 and 6 post-infection (Table 1). On day 9, virus was detected only in the 10^9 EID_{50} group, and at a lower level than on days 3 or 6. Virus was not detected in the 10^4 EID_{50} group at 3 days post-infection. Viral loads in lung were generally higher than those in trachea. The MID_{50} titer, determined by detection of viral load in the lungs at 3 days post-infection, was 1 MID_{50}=$10^{5.5}$$EID_{50}$/mL.

Humoral Immune Response Post Infection

H1N1 (A/Guangdong/51/2008), H3N2 (A/Anhui/137/2008), and H9N2 (A/Guangzhou/333/99) virus were used for HI assays. No serum antibody could be detected to either of the currently circulating human influenza viruses, or the H9N2 virus, prior to experiment. Antibody responses were evaluated using an HI assay at days 7, 14, and 28 post-infection, and at days 84 and 96 post-infection in mice infected with 10^8, 10^6, and 10^4 EID_{50}. No HI antibody was detected at day 7 post-infection. Viral doses of 10^9, 10^8, 10^7, and 10^6 EID_{50} induced HI antibody titers of 5.32 or higher at day 14 post-infection (Table 2). The

Table 1. Virus Loads in Lung and Trachea of Mice

Group Virus Titer (EID_{50}/mL)	Animal No.	Virus Load[a]					
		Lung			Trachea		
		3d	6d	9d	3d	6d	9d
10^9	1	8	7.3	3.3	5.33	6.66	<
	2	7.8	7.3	2.6	6	7	3.16
	3	6.8	6	<	6.16	6.83	<
10^8	1	9.1	7	<	6.5	8.16	<
	3	8.6	7.3	<	5.83	6.16	<
	4	8.3	7	<	6.5	4	<
10^7	1	9.1	3.6	<	6	5	<
	2	8.3	7	<	6	4.75	<
	3	6.6	7	<	6.33	6.16	<
10^6	1	8	3.9	<	7.5	4.5	<
	2	4.5	7.8	<	<	5.25	<
	3	4.8	<	<	4.83	<	<
10^5	1	6.5	3.5	<	6.83	3.16	<
	2	6	<	<	7.75	<	<
	3	6.1	6.3	<	7	3.16	<
10^4	1	<	<	<	<	<	<
	2	<	<	<	<	<	<
	3	<	<	<	<	<	<

Note. [a]Virus titer is given as $\log_{10}EID_{50} \cdot mL^{-1} \cdot g^{-1}$. <, Virus was not detected in the undiluted samples.

Table 2. Antibody Response Induced by the H9N2 Virus

Group (EID_{50}/mL)	HI Antibody Titer A/Guangzhou/333/99(H9N2)			
	14d	28d	84d	196d
10^9	7.32	8.32	ND	ND
	7.32	10.32	ND	ND
	8.32	10.32	ND	ND
10^8	7.32	8.32	10.32	10.32
	8.32	8.32	10.32	10.32
	6.32	7.32	10.32	10.32
10^7	7.32	8.32	ND	ND
	8.32	7.32	ND	ND
	6.32	7.32	ND	ND
10^6	6.32	8.32	9.32	9.32
	5.32	5.32	9.32	9.32
	5.32	5.32	9.32	9.32
10^5	5.32	7.32	ND	ND
	4.32	6.32	ND	ND
	3.32	4.32	ND	ND
10^4	4.32	5.32	5.32	5.32
	4.32	3.32	5.32	5.32
	3.32	3.32	5.32	5.32

Note. Various doses (10^9, 10^8, 10^7, 10^6, 10^5, and 10^4 EID_{50}/mL) were used to infect mice. Intranasally and antibody responses were evaluated by HI assay with samples taken at days 7, 14, 28, 84, and 196 post-infection. ND, not done.

highest antibody titers were induced by the highest viral doses (10^9 and 10^8 EID_{50}). The mean HI titer was 5.84 in the 10^5 EID_{50} group. HI titers in mice that received 10^9, 10^8, 10^7, and 10^6 EID_{50} increased in a dose-dependent manner. The highest titer recorded was 10.32 in the 10^9 EID_{50} group at day 28 post-infection. Antibody was not detected until day 28 post-infection in the 10^4 EID_{50} group. In the 10^8 EID_{50} group, HI titers increased to 10.32 at day 84 post-infection, and were maintained at this level until at least day 196. Similarly, serum antibody titers in mice infected with 10^6 EID_{50} were 9.32 on days 84 and 196 post-infection. However, administration of 10^4 EID_{50} induced only antibody titers of 5.32 at days 84 and 196 post-infection. Thus, serum antibody responses to H9N2 persisted for at least 196 days post-infection.

Cellular Immune Response Post Infection

Splenic lymphocytes of mice infected with 10^7 EID_{50} at 6 days post-infection were exposed to H9HA peptides, and subjected to IFN-γ ELISPOT assays. Based on the IFN-γ ELISPOT data, we selected six H9HA peptides that induced anti-mouse IFN-γ (Table 3). These peptides consisted of H9HA residues HA71-88, HA88-104, HA95-109, HA323-340, HA331-348, and HA349-356. These six peptides were used to stimulate

spleen-derived lymphocytes *in vitro*. Intracellular cytokine staining revealed that these peptides induced IFN-γ and CD^{4+} T-cells (Figure 2), suggesting that this T-cell subset may play a role in H9N2 pathogenesis. Peptides of HA10, 12, and 13 induced greater levels of IFN-γ than did HA46, 47, or 48.

Table 3. T Cell Peptides* and Their Corresponding T Cell Subsets

Name of Peptide	Peptide Sequence	Amino Acid Position	T Cell Subset
HA10	DTCTIEGLIYGNPSCDLL	71–88	CD^{4+}
HA12	LLLGGREWSYIVERPSAV	88–104	CD^{4+}
HA13	SYIVERPSAVNGLCY	95–109	CD^{4+}
HA46	LKLAVGLRNVPARSSRGL	323–340	CD^{4+}
HA47	NVPARSSRGLFGAIAGFI	331–348	CD^{4+}
HA48	GLFGAIAGFIEGGWPGLV	349–356	CD^{4+}

Note. *Sequences were based on the consensus of H9HA, "_"Represented identical sequences among peptides.

Pathogenesis Investigation

HE staining on day 6 post-infection was used to visualize lung-tissue histology in mice infected with 10^6 EID_{50}. Bronchiole epithelial cells were partly desquamated, with significant infiltration by inflammatory cells, mainly lymphoid cells, as well as a small number of macrophages (Figure 3). These data suggest that the mice developed a mild pneumonia after infection with H9N2 virus.

DISCUSSION

Although avian H9N2 viruses have circulated globally since the late 1990s[15-17], they have been categorized as low pathogenic avian influenza viruses. Only China (mainland and Hong Kong) have reported cases of H9N2 infection[4-7]. These influenza viruses, therefore, have been regarded as being restricted to avian species. However, some H9N2 strains have acquired human influenza virus-like receptor specificity, and show continuous reassortment within all genetic groups and form multiple genotypes[18-19]. Such traits may lead to increased incidence of human infection.

We investigated the pathogenesis and immunogenicity of a H9N2 strain (A/Guangzhou/333/99)(H9N2) in mice. Mice are not a natural host for influenza. However, some studies suggest that H9N2 viruses replicate in the murine respiratory tract without prior adaptation, and may even prove lethal[10,20]. Our results showed that infection with H9N2 did not kill the mice, even at high doses. Although viral replication occurred, no weight loss was observed in mice infected with 10^6 or 10^5 EID_{50}. This suggests that this virus is only mildly pathogenic in mice without prior adaptation, but is capable of replication in the murine lung and trachea.

Specific antibody was detected on day 14 post-infection by HI assay, and long-term antibody production was induced by a 10^6 or higher EID_{50} infectious dose. We also evaluated cell-mediated immunity. Since specific antibody had not developed at day 6 post-infection, H9HA peptides were used to stimulate splenocytes harvested at day 6 post-infection to evaluate the immune response. Viral infections are thought to induce predominantly CD^{8+} T-cell responses[21], but our data suggests that H9HA peptides stimulated T-cells to produce IFN-γ and that these cells were predominantly CD^{4+} T-cells. We hypothesize that during influenza virus infection, CD^{4+} T-cells not only assist B-cell antibody production, but also participate in antiviral immunity through cytokine production, such as IFN-γ. Thus, our data suggest that both humoral and cell-mediated immunity play an important role in the immune response to H9N2 influenza infection.

Many factors influence viral transmissibility, including virus specific factors, and a variety of environmental and host factors. Studies have stated that the mutations PB2 E627K or 627E/701N are implicated in expanding the host range of avian viruses and increasing their transmissibility[22-24]. However, the A/Guangzhou/333/99 virus, first isolated from a 22-month-old girl, did not possess such mutations. This suggests that other factors allow the H9N2 virus to replicate efficiently in mice. HA leucine-226 is thought to increase the ability of the virus to bind to SA-2,6 Gal[25], a receptor expressed in both the human and murine respiratory tract[26-27]. Recently, Bussey et al. reported that a T271A mutation in PB2 plays a key role in enhancing the polymerase activity of influenza A viruses in mammalian host cells[28]. The H9N2 virus used in this study possesses such a mutation. We hypothesized that the HA and PB2 genes contribute to the ability of the virus to infect mice without adaptation, and to replicate in the trachea and lung.

An investigation carried out in 2003-2004 in gu-

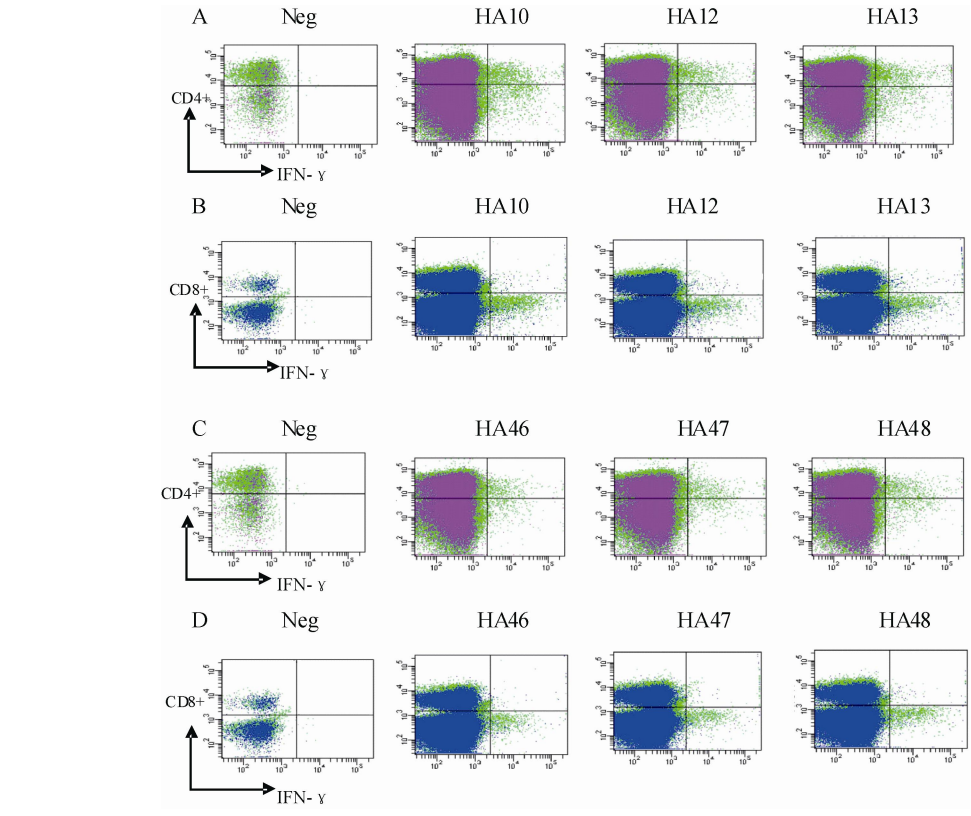

Figure 2. Flow cytometric analysis. A Association of CD^{4+} cells and IFN-γ by stimulation with HA10, 12, and 13 peptides. B Association of CD^{8+} cells and IFN-γ by stimulation with HA 10, 12, and 13 peptides. C Association of CD^{4+} cells and IFN-γ by stimulation with HA46, 47, and 48 peptides. D Association of CD^{8+} cells and IFN-γ by stimulation with HA46, 47, and 48 peptides.

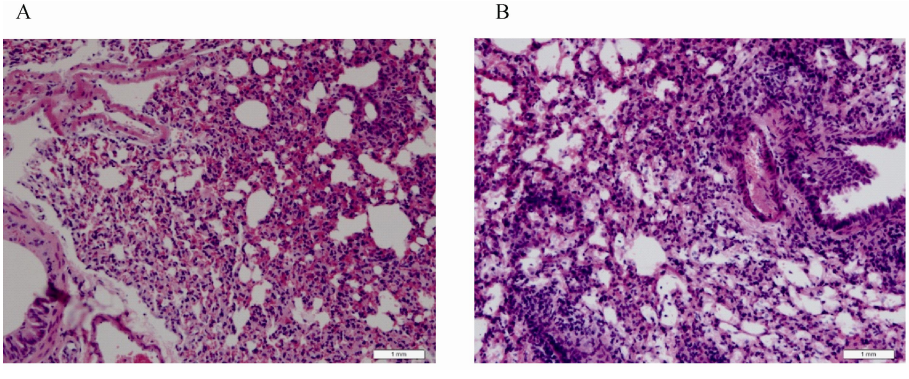

Figure 3. Photomicrographs of lung sections (HE stained). A, Negative control. Original magnification ×200. B, Infected lungs harvested on day six post-infection. Original magnification ×200.

angdong Province revealed an H9N2 seropositivity rate of 9.52% due to occupational exposure, and 3.76% in the general population[29]. These data imply that subclinical H9N2 avian influenza infections occur in the southern region of China. Although no significant human-to-human spread of the viruses has been documented, a virus able to transmit among humans may arise either by mutation of the avian H9N2 virus genome and/or by reassortant between an avian and human influenza A virus. This in turn suggests that influenza surveillance should be strengthened and more attention paid to viral strains able to cross species. The data presented herein provide clues as to the most appropriate direction

for future avian/human H9N2 virus research.

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