Veterinary Microbiology 163 (2013) 351–357

Contents lists available at SciVerse ScienceDirect

Veterinary Microbiology

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

Short communication

Characterization of three H5N5 and one H5N8 highly pathogenic avian influenza viruses in China

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ARTICLE INFO

Article history:
Received 10 July 2012
Received in revised form 18 December 2012
Accepted 18 December 2012

Keywords:
Avian influenza virus
H5N5
H5N8
Reassortant

ABSTRACT

One H5N8 and three H5N5 highly pathogenic avian influenza (HPAI) viruses which derived their HA genes from the Asian H5N1 lineage were isolated from poultry during 2009–2010 in mainland China. Pathogenicity studies showed that these viruses were all highly virulent to chickens, while they varied from moderate to high virulence in mice and from mild to intermediate virulence in mallards. Phylogenetic analyses showed that these viruses were reassortants bearing the H5N1 backbone while acquiring PB1, NP and NA genes from unidentified non-H5N1 viruses, and had developed into three distinct genotypes (B–D). Molecular characterization indicated that all these viruses might resist to antiviral agents. Our findings highlight the emergence and development of HPAI H5 viruses of other NA subtypes in H5N1 endemic areas and their potential threat to poultry industry and public health.

1. Introduction

The Asian H5N1 (Gs/GD-lineage) highly pathogenic avian influenza (HPAI) viruses have caused considerable damage to poultry industry in over 64 countries and have posed great threat to public health on several continents since their first appearance in 1996. According to the World Health Organization (http://www.who.int/csr/disease/en/), these viruses have caused 608 human infections and 359 deaths by 10 August 2012. H5N1 HPAI viruses have become endemic in Southern China since 2003, which have given rise to multiple genotypes or sublineages in this region based on comprehensive phylogenetic analysis of the whole genomes (Chen et al., 2006; Duan et al., 2008; Li et al., 2010). Despite the extensive genetic reassortment of Gs/GD-lineage H5 viruses by incorporating numerous non-H5N1 internal gene segments to generate novel variants, almost all those H5 HPAI viruses derive their NA genes from Gs/GD-like viruses, while viruses of other NA subtypes or from other N1 origins are rarely detected except for the recently reported HPAI H5N5 combination (Alexander and Brown, 2009; Duan et al., 2008, 2007; Gu et al., 2011; Hoffmann et al., 2000; Li et al., 2010; Zou et al., 2012). Domestic ducks have always been thought to play as the interface between the natural gene pool of wild aquatic birds and land-based poultry in the ecology of influenza viruses in southern China. The traditional animal production pattern makes it possible for domestic ducks to contact with wild waterfowls and terrestrial poultry simultaneously, providing the opportunity to transmit viruses asymptomatically from the former to the latter at the same time. Therefore, systematic and persistent surveillance of influenza viruses in domestic ducks can provide valuable information for the

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http://dx.doi.org/10.1016/j.vetmic.2012.12.025

prevention and control of HPAI in poultry. In addition to domestic ducks, other poultry like quails and geese should also be monitored constantly because of their huge populations on hand and their vulnerability to HPAI.

2. Materials and methods

2.1. Study design

A monthly avian influenza virus survey was performed in a wholesale live bird market (LBM) in Eastern China from December 2009 through December 2010. The approximate daily size of the poultry population of the market was 50,000 for chicken (1000–1500 per flock), 10,000 for goose (200–300 per flock) and 5000 for duck (200–300 per flock), with quails and pigeons occasionally be tradable. In the LBM, the poultry were mainly transported from Shandong, Jiangsu, Zhejiang, Anhui, Guangdong, Fujian, Hubei and Hunan provinces. About 300–500 cloacal swabs, 25–30 swabs per flock, were collected monthly from apparently healthy ducks, geese and chickens and occasionally from quails and pigeons. The sampling data were listed in Table 1. Virus isolation and identification were carried out as previously described (Liu et al., 2010), and prevalence of influenza A viruses in each avian species were listed in Table 1.

2.2. Viruses

Three H5N5 avian influenza viruses, A/goose/Shandong/k1204/2009 (Gsk1204), A/goose/Guangdong/k0103/2010 (Gsk0103), A/quail/Jiangsu/k0104 (Qak0104) and one H5N8 virus, A/duck/Jiangsu/k1203/2010 (Dkk1203) were isolated from swab samples from goose, quail and mallard, respectively. Viruses were purified by three consecutive rounds of plaque purification in Madin-Darby Canine Kidney (MDCK) cells as documented previously (Hayden et al., 1980). All experiments involving live viruses were executed in animal biosafety level 3 laboratory facilities.

2.3. Animal experiments

2.3.1. Pathogenicity in chickens

Groups of ten 6-week-old SPF chickens (Beijing Experimental Animal Center, Beijing, China) were intravenously inoculated with 0.1 ml of a 1:10 dilution of allantoic fluid containing each virus to determine the corresponding intravenous pathogenicity index (IVPI) according to the recommendation of the World Organization for Animal Health (OIE) (http://www.oie.int/en/international-standard-setting/terrestrial-manual/access-online/).

The 50% tissue culture infectious dose ($TCID_{50}$) and 50% egg infectious dose (EID_{50}) of each virus were determined by serial titration of viruses in MDCK cells and Specific-pathogen-free (SPF) eggs, respectively, using the Reed and Muench method (Reed and Muench, 1938).

2.3.2. Pathogenicity in mice

Groups of six 6-week-old female BALB/c mice (Beijing Experimental Animal Center, Beijing, China) were lightly anesthetized with CO_2 and inoculated intranasally (i.n.) with $10^{6.0}$ $TCID_{50}$ of each virus suspending in 50 μl of phosphate buffered saline (PBS), while $10^{6.0}$ EID_{50} was for A/quail/Jiangsu/k0104/10(H5N5) due to its inefficient growth in MDCK cells. Control mice were mock-infected with PBS. On 1, 3 and 5 days post inoculation (dpi), two mice from each group were killed, respectively, for observation of gross lesions and virus titration. Tissue samples including the heart, liver, spleen, lung, kidney, brain and pancreas were collected. The 50% mouse lethal dose (MLD_{50}) was determined for viruses that caused lethal infection of mice by the intranasal inoculation of groups of five mice. Groups of five 6-week-old female BALB/c mice were lightly anesthetized and inoculated intranasally with 10^{0}–10^{7} EID_{50} of each virus in diluted in 50 μl PBS to evaluate the MLD_{50} (Lu et al., 1999; Reed and Muench, 1938).

2.3.3. Pathogenicity in ducks

Groups of four 6-week-old mallard ducks (*Anas platyrhynchos*) were intranasally inoculated with

Table 1
Sampling data of surveillance and prevalence of influenza A viruses in each species during December 2009–December 2010.

Date[a]	No. samples/flocks/prevalence (%)[b]				
	Chicken	Goose	Duck	Quail	Pigeon
Dec. 2009	100/4/0	100/4/1	135/5/3.7	–[c]	–
Jan. 2010	110/4/13.6	130/5/11.5	205/8/24.4	25/1/40	–
Feb. 2010	140/5/14.3	180/7/2.8	180/7/5.6	–	–
Mar. 2010	140/5/0	180/7/2.8	180/7/22.2	–	–
Apr. 2010	180/7/2.8	160/6/3.1	160/6/12.5	–	–
May 2010	60/2/0	260/10/1.9	180/7/11.1	–	–
Jun. 2010	60/2/0	100/4/0	150/6/4	–	–
Jul. 2010	100/4/0	120/4/0	160/6/0	–	25/1/0
Aug. 2010	80/3/5	240/9/0	120/4/30	–	–
Sep. 2010	118/4/1.7	120/4/0	162/6/1.2	–	–
Oct. 2010	100/4/4	30/1/0	278/11/4.3	–	–
Nov. 2010	100/4/2	90/3/2.2	228/9/12.3	–	–
Dec. 2010	110/4/0	120/4/0	190/7/17.9	–	–

[a] Five swab samples collected from same flock during Dec. 2009 and May 2010 were pooled together as one specimen for handing.
[b] Numbers of samples and flocks and prevalence of influenza A viruses in each species were listed, respectively.
[c] Not sampled.