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Short communication

Limited adaptation of chimeric H9N2 viruses containing internal genes from bat influenza viruses in chickens

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ABSTRACT

Influenza virus-like sequences of H17N10 and H18N11 were identified in bats, despite there has been no live virus isolated. The genetic analysis indicated that they have distinct but relatively close evolutionary relationships to known influenza A viruses. However, the infectivity and adaptation of bat influenza viruses in avian species remain unclear. In this study, two modified bat influenza viruses cH9cN2/H17 and cH9cN2/H18 containing HA and NA coding regions replaced with those of H9N2 influenza A virus were generated in the background of the H17N10 or H18N11 viruses. These two modified viruses replicated less efficiently than wild type H9N2 virus in cultured chicken cells. The mini-genome assay showed that viral ribonucleoproteins (vRNPs) of H9N2 has significantly higher polymerase activity than that of bat influenza viruses in avian cells. In chicken study, compared with H9N2 virus, which replicated and transmitted efficiently in chickens, the cH9cN2/H17 and cH9cN2/H18 viruses only replicated in chicken tracheas with lower titers. Pathological examination showed that the H9N2 caused severer lesions in lung and trachea than the modified bat influenza viruses. Notably, the cH9cN2/H18 transmitted among chickens, but not cH9cN2/H17, and chicken IFN-β antagonism results showed that H18N11 NS1 protein inhibited chicken IFN-β response more efficiently than H17N10 NS1 protein in avian cells. Taken together, our data indicated that the internal genes of bat influenza viruses adapted poorly to chickens, while the internal genes of H18N11 seemed to adapt to chickens better than H17N10.

Bat is the natural reservoir of most of deadly zoonotic viruses, including rabies virus, Ebola virus, and SARS coronavirus (Buceta and Johnson, 2017; Cyranoski, 2017; Streicker et al., 2016). Recently, two novel influenza A-like virus genomes designated as H17N10 and H18N11 were identified from bat specimens, indicating that bat is a reservoir of a new group of influenza viruses (bat influenza virus) which are phylogenetically related to influenza A viruses (IAVs) (Tong et al., 2012, 2013). Recently, two more H18N11 strains have been identified from fruit bats, Brazil. These two H18N11 strains shared 93.5%–96.9% nucleotide identity among all 8 genomic segments with the previous H18N11 strain found in Peru, 2010, suggesting that bat H18N11 subtype virus is diverse and wide spread in bat species (Campos et al., 2019). However, no infectious virus has been isolated from bats. Studies have shown that neither hemagglutinin (HA) nor neuraminidase (NA) of bat influenza A-like viruses has similar functions as those of IAVs (Garcia-Sastre, 2012, 2013). On the other hand, the internal genes of bat influenza A-like viruses have been demonstrated to be functional to support virus replication by generating chimeric bat influenza viruses, which contain six internal genes of bat influenza A-like virus and two chimeric surface genes (coding regions of HA and NA genes flanked by bat virus packaging signals) of IAVs (Juozapaitis et al., 2014; Zhou et al., 2014).

Previous study showed that bat influenza virus has limited genetic and protein compatibility with influenza A and influenza B viruses, and the internal genes of bat influenza virus are well adapted to mammals, i.e. mice (Ciminisi et al., 2017; Tefsen et al., 2014; Yang et al., 2017; Zhao et al., 2016; Zhou et al., 2014). However, whether the bat influenza viruses would adapt to avian species and pose potential threat to birds remains largely unclear. H9N2 is one of the major subtypes of IAVs circulating in poultry in China since 1993, which has a wide host range from birds to mammals (Guo et al., 2000; Sun et al., 2010). Although H9N2 is a low pathogenic virus in birds, co-infection with other...
pathogens often causes significant economic loss to poultry industry (Chu et al., 2016; Kishida et al., 2004; Pan et al., 2012). In addition, the pathogens often causes significant economic loss to poultry industry (Chu et al., 2016; Kishida et al., 2004; Pan et al., 2012). In addition, the pathogens often causes significant economic loss to poultry industry (Chu et al., 2016; Kishida et al., 2004; Pan et al., 2012). In addition, the pathogens often causes significant economic loss to poultry industry (Chu et al., 2016; Kishida et al., 2004; Pan et al., 2012). In addition, the pathogens often causes significant economic loss to poultry industry (Chu et al., 2016; Kishida et al., 2004; Pan et al., 2012). In addition, the pathogens often causes significant economic loss to poultry industry (Chu et al., 2016; Kishida et al., 2004; Pan et al., 2012). In addition, the pathogens often causes significant economic loss to poultry industry (Chu et al., 2016; Kishida et al., 2004; Pan et al., 2012). In addition, the pathogens often causes significant economic loss to poultry industry (Chu et al., 2016; Kishida et al., 2004; Pan et al., 2012). In addition, the pathogens often causes significant economic loss to poultry industry (Chu et al., 2016; Kishida et al., 2004; Pan et al., 2012). In addition, the pathogens often causes significant economic loss to poultry industry (Chu et al., 2016; Kishida et al., 2004; Pan et al., 2012). In addition, the pathogens often causes significant economic loss to poultry industry (Chu et al., 2016; Kishida et al., 2004; Pan et al., 2012). In addition, the pathogens often causes significant economic loss to poultry industry (Chu et al., 2016; Kishida et al., 2004; Pan et al., 2012). In addition, the pathogens often causes significant economic loss to poultry industry (Chu et al., 2016; Kishida et al., 2004; Pan et al., 2012). In addition, the pathogens often causes significant economic loss to poultry industry (Chu et al., 2016; Kishida et al., 2004; Pan et al., 2012). In addition, the pathogens often causes significant economic loss to poultry industry (Chu et al., 2016; Kishida et al., 2004; Pan et al., 2012). In addition, the pathogens often causes significant economic loss to poultry industry (Chu et al., 2016; Kishida et al., 2004; Pan et al., 2012). In addition, the pathogens often causes significant economic loss to poultry industry (Chu et al., 2016; Kishida et al., 2004; Pan et al., 2012). In addition, the pathogens often causes significant economic loss to poultry industry (Chu et al., 2016; Kishida et al., 2004; Pan et al., 2012). In addition, the pathogens often causes significant economic loss to poultry industry (Chu et al., 2016; Kishida et al., 2004; Pan et al., 2012). In addition, the pathogens often causes significant economic loss to poultry industry (Chu et al., 2016; Kishida et al., 2004; Pan et al., 2012). In addition, the pathogens often causes significant economic loss to poultry industry (Chu et al., 2016; Kishida et al., 2004; Pan et al., 2012). In addition, the pathogens often causes significant economic loss to poultry industry (Chu et al., 2016; Kishida et al., 2004; Pan et al., 2012). In addition, the pathogens often causes significant economic loss to poultry industry (Chu et al., 2016; Kishida et al., 2004; Pan et al., 2012). In addition, the pathogens often causes significant economic loss to poultry industry (Chu et al., 2016; Kishida et al., 2004; Pan et al., 2012). In addition, the pathogens often causes significant economic loss to poultry industry (Chu et al., 2016; Kishida et al., 2004; Pan et al., 2012). In addition, the
Table 1

| Virus          | Time   | Trachea | Lung | Heart | Liver | Kidney | Pancreas | Intestine | Bursa of fabricius |
|---------------|--------|---------|------|-------|-------|--------|----------|-----------|-------------------|
| cH9cN2/H17    | 3 dpi  | 0/3(−)  | 2/3(0.28 ± 0.52) | 1/3(1.8) | 1/3(2.39 ± 0.15) | 1/3(2.02 ± 0.30) | 0/3(−) | 3/3(2.08 ± 0.23) | 0/3(−) |
| cH9cN2/H17    | 5 dpi  | 0/3(−)  | 2/3(1.49 ± 0.34) | 1/3(2.51 ± 0.40) | 1/3(2.42 ± 0.15) | 1/3(1.98 ± 0.12) | 0/3(−) | 2/3(1.63 ± 0.18) | 1/3(1.25) |
| H9N2-wt       | 3 dpi  | 3/3(4.33 ± 1.81) | 3/3(7.083 ± 0.52) | 1/3(2.5) | 0/3(−) | 1/3(5.75) | 1/3(1.5) | 1/3(1.5) | 1/3(3.75) |
| H9N2-wt       | 5 dpi  | 3/3(5.33 ± 1.52) | 3/3(7.39 ± 0.24) | 1/3(2.5) | 0/3(−) | 3/3(6.83 ± 0.52) | 1/3(1.5) | 2/3(2.38 ± 0.18) | 0/3(−) |

H9N2-wt was detected in all organs except liver of infected chickens, while cH9cN2/H17 was only detected in trachea, spleen, and pancreas, similarly cH9cN2/H18 was only detected in trachea and pancreas, suggesting that chimeric bat influenza virus showed limited replication in chickens compared with the systemic infection in chickens caused by H9N2-wt (Table 1).

Viral shedding results showed that all three chickens inoculated by H9N2-wt shed virus through oropharyngeal route at 2 and 4 dpi, and only two infected chickens shed virus through cloaca at 2 dpi (Fig. 2). In addition, two out of three contact chickens in H9N2-wt group shed virus through both oropharynx and cloaca at 2 dpi, which suggested that H9N2-wt is transmissible among chickens. Nevertheless, virus was undetectable in oropharyngeal and cloacal swabs from cH9cN2/H17 inoculated and contact chickens (Fig. 2). Notably, lower levels of cH9cN2/H18 virus were detected in oropharyngeal and cloacal swabs in one infected chicken at 2 dpi, and one to two contact chickens shed virus from oropharynx and cloaca at 2 dpi with lower titers (Fig. 2), suggesting that cH9cN2/H18 showed better transmission efficiency than cH9cN2/H17 in chickens.

To further explore the potential mechanism of the poor adaptation of chimeric bat influenza virus in chickens, polymerase activity and IFN inhibition activity of bat influenza viruses were evaluated in avian cells. The mini-genome assay of H17N10, H18N11, and H9N2-wt vRNPs was conducted in chicken DF-1 cells as described previously (Zhou et al., 2014). The results showed that polymerase activity of H9N2-wt vRNPs was significantly higher than those of H17N10 and H18N11 in avian cells (Fig. 1C). NS1 protein is a multifunctional protein that is responsible to antagonize host antiviral response during viral infection (Krug, 2015). Bat influenza virus NS1 protein has been reported to bind double-stranded RNA and antagonize IFN-β response in mammalian cells (Turkington et al., 2015). To compare the chicken IFN-β antagonism ability of NS1 proteins, DF-1 cells were transfected with the indicated pCDNA-H9N2-NS1, pCDNA-H17N10-NS1 or pCDNA-H18N11-NS1 expression plasmids (0.2 μg/well), together with plasmids expressing firefly luciferase under the control of the chicken IFN-β promoter (pGL-chIFNβ-LUC, 0.2 μg/well), Renilla luciferase expressing plasmid pRL-TK (0.07 μg/well) and 0.2 μg poly(I:C). 24 h post transfection, the cells were lysed and subjected to dual-luciferase reporter assay (Promega, USA). The results showed that all the three NS1 proteins showed strong chicken IFN-β antagonism ability, whilst H9N2 NS1 inhibited IFN-β response more efficiently than bat NS1 in chicken cells (Fig. 1D). Notably, H17N10 NS1 inhibited chicken IFN-β response relatively less efficiently than H18N11 NS1 in chicken cells, suggesting that the stronger IFN antagonist of H18N11 NS1 might help the virus to adapt to avian species better than H17N10.

In addition, studies showed that the compatibility between surface proteins and the internal proteins is important for IAVs replication (Lakdawala et al., 2011; Ma et al., 2012; Rossman and Lamb, 2011). Interaction between M1 with cytoplasmic tails of HA and NA is critical for viral genome packaging and assembly in infected cells (Enami and Enami, 1996; Zhang et al., 2000). While the M1 protein of bat influenza viruses only share 78% identity with that of H9N2-wt, more than 50 amino acid substitutions in the M1 proteins between the H9N2 and H17N10/H18N11 were observed. Nevertheless, how these mutations affect incompatibility between H9N2 surface proteins and the M1 proteins of bat viruses needs further study.
Fig. 2. Viral titers in oropharyngeal and cloacal swabs of infected and contact chickens. Nine chickens (6-week-old) were inoculated intranasally with the viruses at $10^{5.5}$ TCID$_{50}$/100 uL/chicken. Three naïve chickens were introduced into the isolators at 1 day post infection (dpi). Virus shedding in oropharyngeal (OP) and cloacal (CL) swabs of infected and contact groups was monitored at 2, 4, and 6 dpi/dpc.

Fig. 3. Haematoxylin and eosin (H&E) and IHC staining for microscopic trachea sections. Chickens were inoculated intranasally with the virus at $10^{5.5}$ TCID$_{50}$/100 uL/chicken. The trachea was collected at 5 dpi. A, E: mock group. B, F: H9N2-wt infected group. C, G: cH9cN2/H17 infected group. D, H: cH9cN2/H18 infected group.

Fig. 4. Haematoxylin and eosin (H&E) and IHC staining for microscopic lung sections. Chickens were inoculated intranasally with the virus at $10^{5.5}$ TCID$_{50}$/100 uL/chicken. The lungs were collected at 5 dpi. A, E: mock group. B, F: H9N2-wt infected group. C, G: cH9cN2/H17 infected group. D, H: cH9cN2/H18 infected group.
The identification of bat influenza virus expanded the host reservoir of IAVs, although the origin and the evolution of bat influenza virus still remain unclear. Serological surveys indicated that bat influenza viruses are widespread in various bat species in Central and South America (Tong et al., 2013). Bats are distributed worldwide and migrate over long distances, which enhance the opportunity of interaction and interspecies transmission of viruses. Waterfowl are considered as reservoirs of IAVs, including 16 HA and nine NA subtypes (Yoon et al., 2014). However, the potential of bat influenza virus infects birds needs to be explored. Zhou’s studies have shown that the packaging signals of most gene segments of the bat viruses are not compatible with those of canonical IAVs (Zhou et al., 2014). In this study, the H9N2 is unable to reassort with H17N10 or H18N11 using reverse genetic system which confirmed the previous finding (Data not shown). Nevertheless, the chimeric ch9 and c2 genes containing packaging signals of H17N10 or H18N11 were compatible with the six internal genes of bat influenza viruses. Both of ch9cncH2/H17 and ch9cncH2/H18 replicated to significantly lower titer than H9N2-wt virus on CEF cells, suggesting the internal genes of bat influenza virus showed limited adaptation to avian species.

In chickens, H9N2-wt virus replicated efficiently in respiratory tract and other tissues, however, only lower level of virus was detected in trachea and pancreas of chimeric ch9cncH2/H18 and ch9cncH2/H17 viruses infected chickens. The H9N2-wt was shed and transmitted efficiently among chickens, and ch9cncH2/H18 also showed transmissibility though limited, while ch9cncH2/H17 was not transmissible in chickens. In addition, NS1 protein of H18N11 showed stronger IFN-β activation activity than H17N10 in avian cells. Taken together, all the present data indicated that the chimeric ch9cncH2/H17 and ch9cncH2/H18 showed limited adaptation to chickens, while the internal genes of H18N11 seemed to adapt better to chickens than H17N10.

Declaration of interest

The authors declare that they have no competing interests.

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