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The PB2 and M genes of genotype S H9N2 virus contribute to the enhanced fitness of H5Nx and H7N9 avian influenza viruses in chickens

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ABSTRACT

Genotype S H9N2 viruses frequently donate their internal genes to facilitate the generation of novel influenza viruses, e.g., H5N6, H7N9, and H10N8, which have caused human infection. Genotype S was originated from the replacement of F/98-like M and PB2 genes of the genotype H with those from G1-like lineage. However, whether this gene substitution will influence the viral fitness of emerging influenza viruses remains unclear. We found that H5Nx and H7N9 viruses with G1-like PB2 or M gene exhibited higher virulence and replication than those with F/98-like PB2 or M in chickens. We also determined the functional significance of G1-like PB2 in conferring increased polymerase activity and improved nucleus transportation efficiency, and facilitated RNP nuclear export by G1-like M. Our results suggest that G1-like PB2 and M genes optimize viral fitness, and thus play a crucial role in the genesis of emerging influenza viruses that cause rising prevalence in chickens.

1. Introduction

The H5Nx, H7N9 and H9N2 subtype avian influenza viruses (AIVs) have co-circulated and become endemic in poultry flocks in many areas in China (Su et al., 2015a). Genotype S H9N2 subtype viruses have become predominant in chicken flocks in China since 2010, and frequently donate all their internal genes as a whole cassette to facilitate the generation of novel reassortants such as H5N6, H10N8 and H7N9 viruses that pose great threat to poultry and humans. Phylogenetic analysis revealed that H9N2 viruses have evolved into multiple lineages in China, mainly represented by A/chicken/Beijing/1/1994 (BJ/94-like), A/quail/Hong Kong/G1/1997 (G1-like), A/chicken/Shanghai/F/1998 (F/98-like) (Xu et al., 2007b; Zhang et al., 2008). According to the constellation of eight viral gene segments, H9N2 subtype AIVs have also categorized into twenty-three distinct series (A-W) in poultry in China (Gu et al., 2017). For instance, since the first isolation of genotype A viruses (with the gene constellation from BJ/94-like) in China in the mid-1990s (Gu et al., 2014). In 1998, genotype H viruses (with F/98-like PB2, PB1, PA and NP substitutions in the backbone of BJ/94-like viruses) epizootics occurred in eastern China, and the genotype H viruses had become predominant from 2000 onward (Sun et al., 2010; Zhang et al., 2009). Subsequently, during the co-circulation of F/98-like and G1-like viruses in poultry, genetic reassortments occurred between the two lineages (Bi et al., 2010; Gu et al., 2014; Liu et al., 2016). The M and PB2 from G1-like viruses have been consecutively introduced into F/98-like viruses since 2007, which promoted the generation of genotype S virus (with G1-like M and PB2 substitution in the backbone of F/98-like viruses) (Gu et al., 2014). Previous studies have demonstrated that genotype G57 (generally equivalent to genotype S) virus shows greater infectivity than other genotypes (Pu et al., 2015), and H9N2 virus with G1-like M gene, relative to BJ/94-like M gene, showed enhanced virus prevalence in chickens in China (Pu et al., 2017). The stable replacement of F/98-like M and PB2 genes with G1-like M and PB2 genes is a key feature in the generation of genotype S H9N2

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viruses. Thus, whether H9N2 viruses with G1-like M and PB2 genes, relative to F/98-like M and PB2 genes, confer survival advantage to the novel reassortants and subsequently contribute to their increasing the fitness in poultry remains uncertain.

The PB2 gene of AIVs plays a crucial role during the virus infection cycle (Fislova et al., 2010). The PB2 subunit of influenza virus RNA polymerase participates in viral transcription and replication in the nucleus of infected cells (Thierry et al., 2016). Moreover, a previous study has reported that the level of PB2 accumulation in the nucleus of infected cells correlates with the virulence of AIV (Gabriel et al., 2008). According to the literature, PB2 is a major determinant of both host range and pathogenicity of influenza virus (Gao et al., 2009; Sediri et al., 2016), and it is found to alter viral polymerase activity in cells that results in changes in virus replication and pathogenicity in animal model (Bi et al., 2015; Su et al., 2015b). Likewise, the M gene plays multiple roles in the life cycle of AIV through the M1 and M2 proteins (Cao et al., 2012; Takeda et al., 2002). M1, as a multifunctional protein, is not only the major structural component of the virion, but also plays an important role in many steps in the replication of AIV (Liu and Ye, 2002). Prior researches generally showed that the association of M1 with ribonucleotide protein (RNP) leads to translocation of RNP from the nucleus to the cytoplasm and affects viral replication (Liu et al., 2002; Martin and Helenius, 1991). Change in the M gene has been found to be critical to viral replication and the pathogenicity of AIV in avian cells and hosts (Fan et al., 2009; Nao et al., 2015).

In this study, we investigated the selective advantage that the PB2 and M gene segments of genotype S viruses confer to H5Nx and H7N9 viruses in chickens. Additionally, we examined the functional significance of the PB2 and M genes from different H9N2 lineages, including polymerase activity, nuclear accumulation and translocation of RNP to cytoplasm *in vitro*. Our findings may help understand the mechanism underlying the continuous donation of internal genes by the genotype S H9N2 viruses to novel influenza reassortants, including H5N6 and H7N9, which have caused prevalence in chickens.

2. Materials and methods

2.1. Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China. All animal experiments were approved by the Jiangsu Administrative Committee for Laboratory Animals (Permission number: SYXK-SU-2017-0007), and by the Institutional Biosafety Committee of Yangzhou University and complied with the guidelines of Jiangsu laboratory animal welfare and ethics of Jiangsu Administrative Committee of Laboratory Animals. All experiments involving live virulent H5 and H7 viruses and animals were housed in negative-pressure isolators with HEPA filters in animal biosecurity level-3 facilities in accordance with the institutional bio-safety manual.

2.2. Cells and viruses

Madin-Darby canine kidney (MDCK) cells, human embryonic kidney (293T) cells, chicken embryo fibroblasts (CEFs) and chicken fibroblast cell line (DF-1) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Life technologies, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Gibco). Viruses A/chicken/Guangdong/GD15/2016 (GD/15, H7N9), A/chicken/Jiangsu/YB7/2015 (YB/7, clade 2.3.2.1 H5N1), A/goose/Jiangsu/QD5/2014 (QD/5, H5N8), A/chicken/Anhui/QD1/2014 (QD/1, clade 2.3.4.4 H5N1), A/Chicken/Jiangsu/CZ/73/2014 (CZ/73, genotype S H9N2) and A/chicken/Shanghai/14/2001 (SH/14, genotype H H9N2), which have been described previously (Hao et al., 2017; Liu et al., 2018; Zhang et al., 2008), were used in this study. The complete genome sequences used in this study were all deposited to NCBI (Table S1). Viruses were propagated in specific pathogen-free (SPF) embryonated chicken eggs.

2.3. Phylogenetic analysis

The PB2 and M gene sequences of H9N2 viruses isolated from chickens (1997–2017) in China were downloaded from the Influenza Virus Resource (NCBI; https://www.ncbi.nlm.nih.gov/genomes/FLU), the EpiFlu Database (GISAID; http://platform.gisaid.org/epi3), and the Influenza Research Database (FluDB; https://www.fludb.org). MEGA 7 software (http://megasoftware.net/) was used to separately perform multiple nucleotide sequence alignments. Phylogenetic trees were constructed by a distance-based neighbor joining method in MEGA 7 with a bootstrap value of 1000.

2.4. Generation of reassortant H5 and H7 viruses by reverse genetics

The reassortant H5 and H7 viruses were generated by reverse genetics as described previously (Hao et al., 2017). Briefly, MDCK and 293T cells were co-cultured and transfected with the plasmids carrying all eight influenza virus genes using PolyFect transfection reagent (Qiagen, GmBH, Germany). At 48 h, the cell supernatants were harvested and inoculated into 10-day-old SPF embryonated chicken eggs to produce stock viruses. All virus stocks were sequenced to confirm the absence of unwanted mutations. The genotypes of the original internal genes of H7N9, H5Nx or H9N2 viruses were listed in Table S2.

2.5. Growth curve and virus titration

The stock virus titers and infectivity were determined by 50% egg infective dose (EID_{50}) in chicken embryos and 50% tissue culture infective dose ($TCID_{50}$) in CEFs as described previously (Hu et al., 2013). To evaluate growth kinetics of the viruses, CEFs were inoculated with the viruses at a multiplicity of infection (MOI) of 0.01. After 1.5 h of incubation at 37 °C, cells were washed twice with phosphate-buffered saline (PBS) and further incubated in the appropriate medium containing 1% FBS. The supernatants were sampled at 12, 24, 48, 72 and 96 h post-inoculation (hpi), and the virus titers were determined by $TCID_{50}$ assay in CEFs (Reed, 1938). Three independent experiments were performed.

2.6. Western blotting

Total protein lysates were extracted from infected CEFs or transfected DF-1 cells with RIPA lysis buffer. Protein samples derived from cell lysates were heated at 95 °C for 5 min and subsequently used for SDS-PAGE electrophoresis and then transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad), which were then probed with primary antibodies. Primary antibodies were specific for β-actin (Santa Cruz Biotechology, TX), PB2, PB1, PA, NP, M1 and M2 (GeneTex). The secondary antibodies used were horseradish peroxidase (HRP)-conjugated goat-*anti*-mouse or goat-*anti*-rabbit secondary antibodies (Sigma, St.Louis, MO), the blotted proteins were detected using enhanced chemiluminescence (ECL) system (Thermo).

2.7. Chicken study

To determine viral pathogenicity in chickens, the intravenous pathogenicity index (IVPI) was determined as described previously (Hao et al., 2017). Groups of ten 6-week-old SPF White Leghorn chickens housed in isolators were infected intravenously (i.v.) with 0.1 ml of a 1:10 dilution of the allantoic fluids and were observed for clinical signs for 10 days. Fourteen additional chickens per group were also inoculated intranasally (i.n.) with $10^{6.0}$ EID_{50} of each virus in 0.1 ml, and three chickens per group were euthanized at 3 and 5 days post-inoculation (dpi), tissue samples, including lungs, kidneys, spleens and

brains were harvested for virus titration. Tracheal swabs from the chickens were also collected at 3 and 5 dpi for virus titration. The remaining 8 birds were monitored for mortality and morbidity for 14 days.

2.8. Polymerase activity

A dual-luciferase reporter assay system (Promega, Madison, WI) was utilized to detect luciferase activities of ribonucleoprotein (RNP) complexes as described previously (Hao et al., 2017). Briefly, DF-1 cells were transfected with four pcDNA3.1 + expression plasmids encoding the PB2, PB1, PA and NP genes (300 ng each) from of H9N2 viruses (CZ/73 or SH/14), together with the firefly luciferase reporter plasmid paviPolIT-Luc (300 ng) and internal control Renilla plasmid (30 ng) using Polyfect Transfection Reagent (Qiagen). After 48 h, cell lysates were processed to measure firefly and Renilla luciferase activities using GloMax 96 microplate luminometer (Promega) The ratio of the firefly luciferase activity value and Renilla luciferase activity value was used to represent RNP activity of the virus. All results are the means with the standard ceviation (SD) from three independent experiments.

2.9. Confocal microscopy

CEFs were grown on sterilized glass coverslips in 24-well plates and infected with the indicated viruses at 2.0 MOI. At specified time points post-infection, cells were fixed with 4% paraformaldehyde in PBS for 30 min and permeabilized with 0.1% Triton X-100 for 30 min, blocked with 1% bovine serum albumin in PBS for 1 h at room temperature. Primary antibodies [rabbit antiserum against PB2, M1 (GeneTex, Irvine, CA) or monoclonal antibody against NP] were incubated with the cells for 1 h at 37 ˚C. Cells were then washed three times with PBS and incubated with secondary antibodies [Alexa Fluor 488 Goat anti-Rabbit IgG (H + L) and Alexa Fluor 594 Goat anti-Mouse IgG (H + L)] (Invitrogen) at 37 ˚C for 1 h. Cells were then stained with DAPI (4′,6-diamidino-2-phenylindole) for 10 min. Cells were examined by using a Leica SP8 confocal microscope. The accumulation of PB2, NP and M1 in the nuclei of infected cells was determined using cell counting by ImageJ software (n = 100).

2.10. Statistical analysis

Statistically significant differences between experimental groups were determined using the Independent-Samples *t*-Test in the SPSS statistics software (IBM company, SPSS 19.0). *P* values of < 0.05 were considered statistically significant.

3. Results

3.1. Predominance of G1-like PB2 and M genes in H9N2 and H7N9 viruses in chicken in China

To better understand the circulation of G1-like and F/98-like lineages with the PB2 and M genes in H9N2 and emerging influenza viruses in chickens in China, we performed a phylogenetic analysis of all available sequences from 1997 to 2017. For the PB2 gene, the G1-like lineage was dominant at the beginning, but decreased in the following years. Since 2007, G1-like PB2 gene of H9N2 viruses in chickens has increased gradually and has become dominant (~92.9%) in 2010 (Fig. 1A). As shown in Fig. 1B, chicken H9N2 viruses with G1-like M gene were first detected in China in 1997, and have since become more prevalent. G1-like M gene in H9N2 viruses of chicken-origin increased sharply, replaced F/98-like M gene and has become dominant (~93.0%) since 2007. These data suggest that the G1-like PB2 and M genes have gradually replaced F/98-like PB2 and M genes and become dominant H9N2 viruses in chickens.

We next examined the prevalence of G1-like and F/98-like PB2 and M genes in avian-derived H5N6/H7N9/H10N8 strains. As shown in Fig. 1C–D, we found that G1-like PB2 and M genes are present in 99.8% of avian-derived H7N9 strains, while 59.6% of the avian H10N8 viruses carry G1-like M gene (Fig. 1C) and all avian H10N8 viruses harbor the G1-like PB2 gene (Fig. 1D). These results suggest the high prevalence of the G1-like PB2 and M genes in avian H7N9 and H10N8 viruses.

3.2. G1-like PB2 and M genes increase viral replication and protein expression of H5 reassortants in vitro

A panel of H5 and H7 reassortants with the internal genes from different lineages of H9N2 viruses, whereas the surface protein genes were from H5 or H7 viruses, i.e., YB/7 (H5N1), QD/5 (H5N8), QD1 (H5N1), and GD/15 (H7N9) (Table 1), were rescued. Reassortants carrying the internal genes of H9N2 viruses, with G1-like M and PB2 genes were named G15/S, Y7/S, Q5/S and Q1/S, respectively. Accordingly, the M and PB2 genes of these recombinants were replaced by M and PB2 genes from the F/98-like lineage to generated recombinants G15/H, Y7/H, Q5/H and Q1/H, respectively. Furthermore, we constructed Q1/M recombinant with the surface protein genes from QD/1 and M gene was from F/98-like lineage, while the remaining genes were from the H9N2 virus with G1-like PB2 and M genes. Similarly, another recombinant Q1/PB2, whose surface protein genes were from QD/1 virus, PB2 gene was from F/98-like lineage, and the remaining genes were from the H9N2 virus with G1-like M and PB2 genes, was also generated. The viral titer of each reassortant virus was determined. As shown in Table 1, all H5 and H7 reassortant viruses replicated efficiently in embryonated eggs.

To explore their impacts on viral replication and protein expression *in vitro*, the growth curves of Q1/S, Q1/M, Q1/PB2 and Q1/H were determined in CEFs. Q1/S grew to significantly (20-fold) higher titers than Q1/H at each time point ($P < 0.01$) (Fig. 2A). In addition, Q1/S had 4-fold higher titers when compared to that of Q1/M at 48 hpi ($P < 0.05$) and 15-fold higher than that of Q1/PB2 at each time point ($P < 0.01$) (Fig. 2A). Next, we examined if the replacement of G1-like with F/98-like PB2 or M gene affects viral protein expression. The expression of M1, M2, NP and PB2 was determined in CEFs at 3, 6, 9 and 12 hpi. As shown in Fig. 2B, at all the tested time points, Q1/S displayed higer M1, M2, NP, and PB2 expression when compared to those of Q1/H in CEFs. The substitution of PB2 and M genes of Q1/S with those from F/98-like lineage resulted in decreased expression of viral proteins (Fig. 2B). Taken together, these data highlight that G1-like PB2 or M conferred higher replication capacity of H5 virus and increased viral protein expression in avian cells.

3.3. G1-like PB2 and M genes enhance virulence and replication of H5 and H7 reassortants in chickens

Since G1-like PB2 or M endowed the recombinant viruses with high replication abilities in CEFs, we next investigated the impact of G1-like PB2 and M genes on the pathogenicity of reassortant viruses in chickens by determining IVPI values of these viruses. Our previous study has shown that Y7/S, Q5/S and Q1/S reassortants were highly pathogenic for chickens, with IVPI values of 2.9, 2.7 and 2.4, respectively (Hao et al., 2017). In the current study, IVPI values for G15/S, G15/H, Y7/H, Q5/H and Q1/H were 2.9, 2.7, 1.5, 2.4 and 0.3, respectively (Table 1), indicating that viruses that carry G1-like PB2 and M genes were more pathogenic in chickens than those harboring F/98-like PB2 and M genes. Furthermore, Q1/S also showed higher virulence than Q1/M and Q1/PB2, with IVPI values of 2.4, 1.8 and 0.4, respectively (Table 1), suggesting that the G1-like PB2 gene played a more critical role in the high virulence of Q1/S in chickens than the G1-like M gene.

To further investigate the pathogenesis of these reassortants in chickens, groups of 14 SPF chickens were i.n. infected with $10^{6.0}$ EID_{50} of each virus. Lungs, spleens, kidneys, brains, trachea swabs were collected at 3 and 5 dpi from 3 birds for virus titration and the other 8

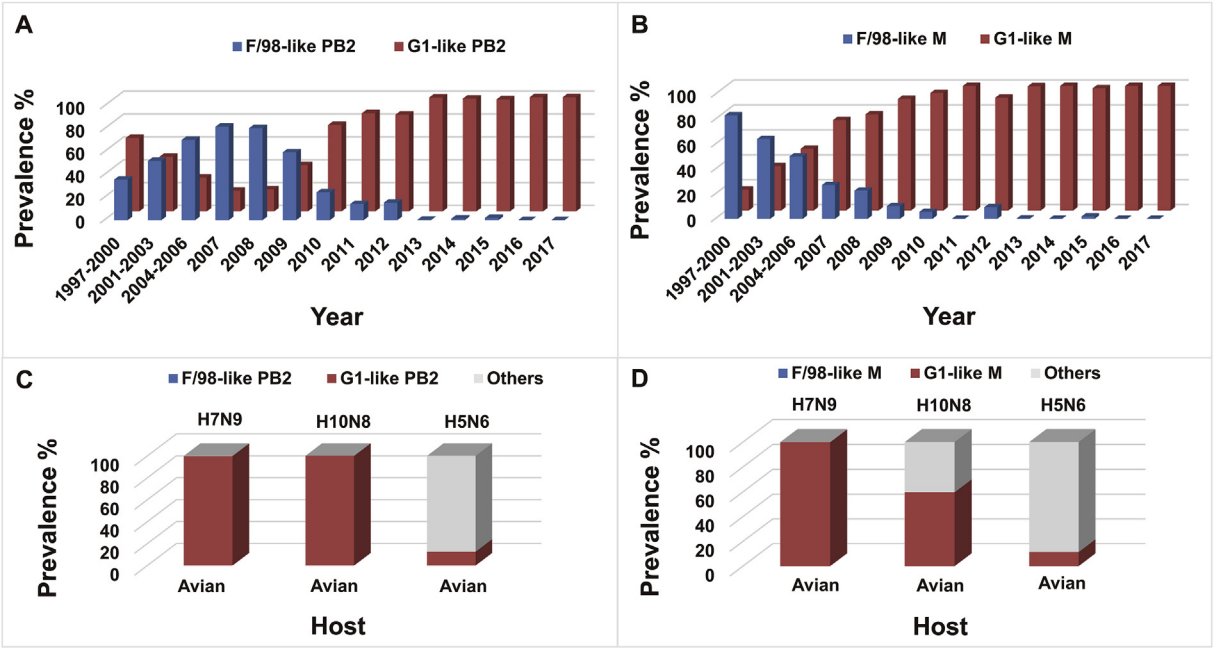

Fig. 1. Prevalence of the F/98- and G1-like PB2 and M genes in chicken H9N2 viruses in China during 1997–2017 (A–B), and the prevalence of F/98- and G1-like M and PB2 genes in avian H5N6/H10N8/H7N9 viruses during 2013–2017 (C–D). For each column from left to right, the actual number of viruses is 45, 148, 165, 44, 36, 37, 37, 78, 118, 325, 85, 54, 15 and 12 for PB2 gene (A), and 95, 179, 185, 44, 44, 39, 36, 70, 127, 325, 90, 54, 18 and 12 for M gene (along x-axis) respectively in B. For each column from left to right, the actual number of viruses is 547, 46 and 662 for PB2 gene (C), and 541, 47 and 661 for M gene (along x-axis) respectively in D. In A and B, the proportion of viruses with F/98-like M (0.3%) or F/98-like PB2 (0.3%) is too low to be seen in 2013. In C and D, others refer to viruses without the F/98-like and G1-like lineages.

Table 1
Characteristics of the H5 and H7 reassortants used in this study.

Virus[a]	Genotype[b]								Rescue times[c]	EID_{50}[d]	IVPI[e]
	PB2	PB1	PA	HA	NP	NA	M	NS			
Group I											
G15/S	CZ	CZ	CZ	G15	CZ	G15	CZ	CZ	1	$10^{8.5}$	2.9
G15/H	SH	CZ	CZ	G15	CZ	G15	SH	CZ	2	$10^{8.0}$	2.7
Group III											
Y7/S[f]	CZ	CZ	CZ	Y7	CZ	Y7	CZ	CZ	1	$10^{8.3}$	2.9
Y7/H	SH	CZ	CZ	Y7	CZ	Y7	SH	CZ	2	$10^{6.6}$	1.5
Group II											
Q5/S[f]	CZ	CZ	CZ	Q5	CZ	Q5	CZ	CZ	3	$10^{7.2}$	2.7
Q5/H	SH	CZ	CZ	Q5	CZ	Q5	SH	CZ	3	$10^{7.0}$	2.4
Group IV											
Q1/S[f]	CZ	CZ	CZ	Q1	CZ	Q1	CZ	CZ	2	$10^{8.8}$	2.4
Q1/M	CZ	CZ	CZ	Q1	CZ	Q1	SH	CZ	2	$10^{7.3}$	1.8
Q1/PB2	SH	CZ	CZ	Q1	CZ	Q1	CZ	CZ	3	$10^{7.1}$	0.4
Q1/H	SH	CZ	CZ	Q1	CZ	Q1	SH	CZ	3	$10^{7.0}$	0.3

[a] Internal genes derived from A/Chicken/Jiangsu/CZ/73/2014 (CZ/73, genotype S H9N2) and A/chicken/Shanghai/14/2001 (SH/14, genotype H H9N2), surface genes derived from A/chicken/Guangdong/GD15/2016 (GD/15, H7N9), A/chicken/Jiangsu/YB7/2015 (YB/7, clade 2.3.2.1 H5N1), A/goose/Jiangsu/QD5/2014 (QD/5, H5N8), A/chicken/Anhui/QD1/2014 (QD/1, clade 2.3.4.4 H5N1), the reassortants were categorized into groups I, II, III, IV based on different parent viruses.

[b] CZ indicates genes derived from the CZ/73; SH indicates genes derived from the SH/14; G15, Y7, Q5 and Q1 indicate genes derived from the GD/15, YB/7, QD/5 and QD/1, respectively.

[c] Data is representative of each virus independent rescues.

[d] Value for the EID_{50} is per 0.1 ml.

[e] IVPI, intravenous pathogenicity index.

[f] Three H5 recombinant strains with associated Rescue times, EID_{50} and IVPI data that have been reported in our previously research, which are just used to compare with those of new recombinants here.

birds were observed daily for mortality and morbidity. As shown in Fig. 3, the survival rates of chickens infected with G15/S, Y7/S, Q5/S and Q1/S were 12.5%, 25%, 12.5% and 50%, lower than those observed with G15/H, Y7/H, Q5/H and Q1/H, which were 62.5%, 37.5%, 50%, 100%, respectively ($P < 0.01$) (Fig. 3A–B). Additionally, the survival rates for Q1/M- and Q1/PB2-inoculated chickens were 62.5% and 100% ($P < 0.01$), respectively, higher than that of birds infected with Q1/S (50%) (Fig. 3B). At both 3 and 5 dpi, higher viral titers were detected in chickens infected with G15/S, Y7/S and Q5/S compared with birds infected with G15/H, Y7/H and Q5/H (Fig. 3C). For Q1/S, Q1/M, Q1/PB2 and Q1/H, we also found that on 3 and 5 dpi, viral titers in Q1/PB2- and Q1/H -infected birds were significantly lower than

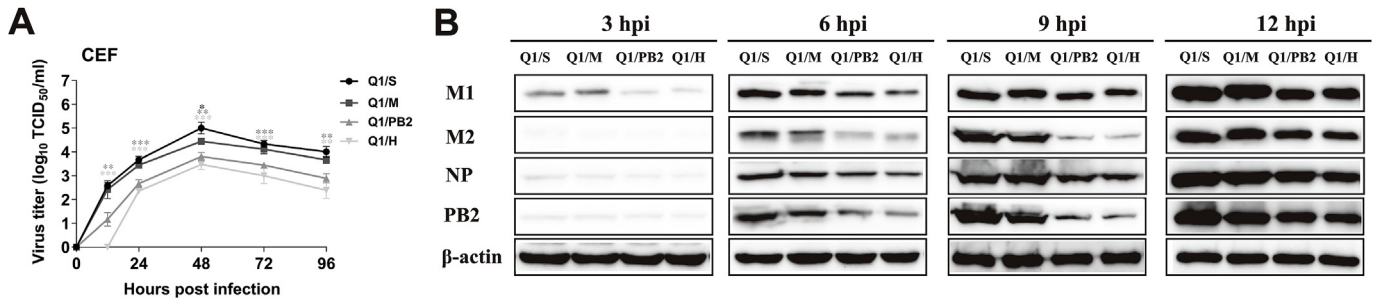

Fig. 2. Replication and expression of viral proteins of reassortant H5 viruses in cell cultures. The multiple-cycle growth of each virus in CEFs (A) was examined by infecting cells at an MOI of 0.01. Virus titers in supernatants harvested at indicated time points were measured in CEF cells. Data represent the mean ± SD of three independent infections. Statistical difference was analyzed against Q1/S virus. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. CEFs (B) were infected with Q1/S, Q1/M, Q1/PB2 and Q1/H virus at an MOI of 0.1. The infected cells were collected at the indicated time points and analyzed by Western blot. Protein bands of M1, M2, NP and PB2 detected by western blotting on CEF cells.

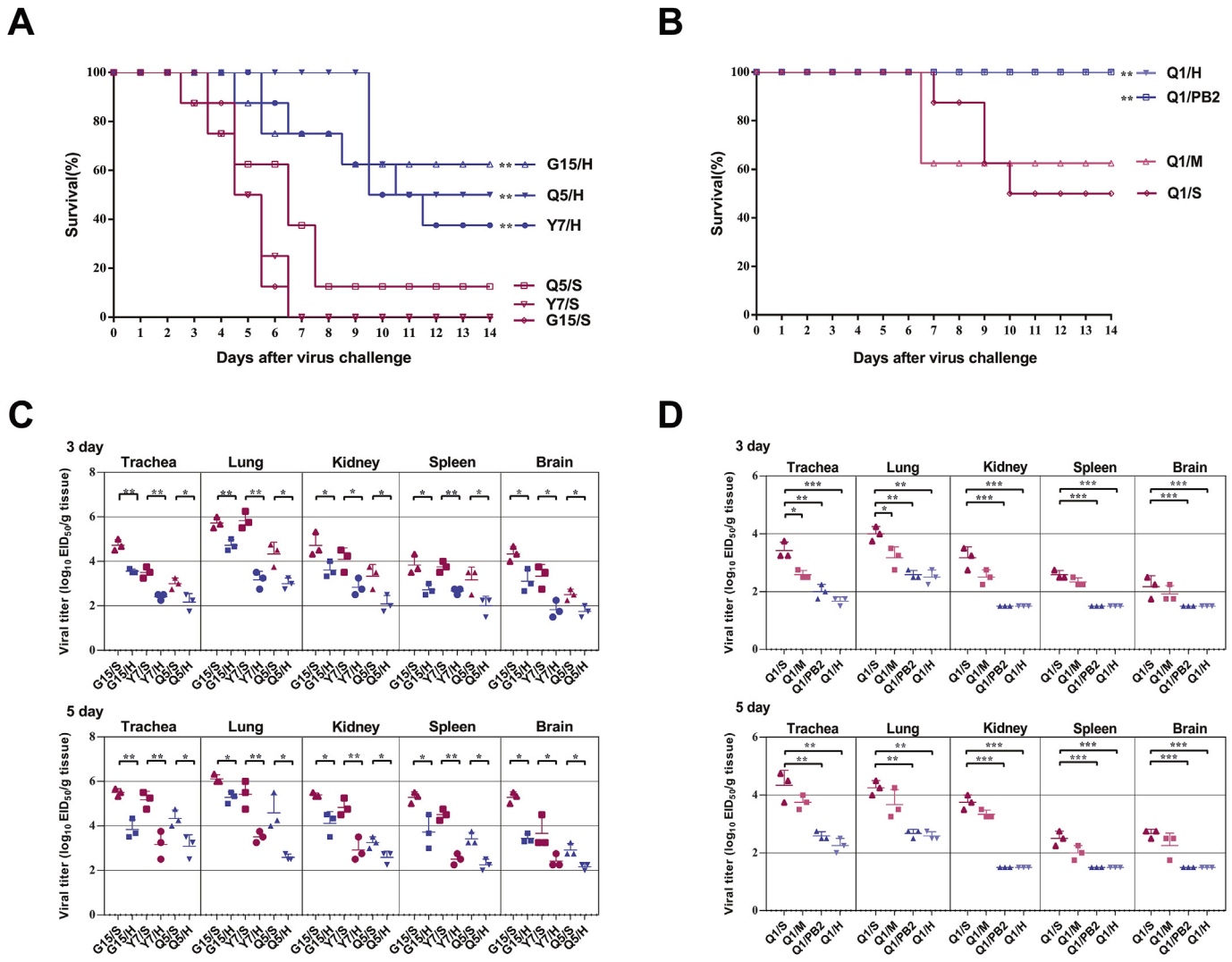

Fig. 3. Virulence and replication of reassortant H5 and H7 viruses in chickens. Chickens were infected i.n. with $10^{6.0}$ EID_{50} of each virus; tracheal swabs and organs were taken at 3 and 5 dpi for virus titration in eggs and the remaining birds were observed for survival. Shown are survival (A and B) and viral titers in organs (C and D) from chickens. Viral titers are expressed as the mean ± standard deviation (SD). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (treatment results differed significantly from those for the G15/S, Y7/S, Q5/S or Q1/S).

those that were infected with Q1/S virus (Fig. 3D). However, lower viral titers in the lung and trachea from chickens infected with Q1/M were detected compared to birds infected with Q1/S at 3 dpi (Fig. 3D). Taken together, these results indicate that the G1-like M or PB2 gene segments contribute to the increased virulence and replication of H5 and H7 reassortants in chickens, with the G1-like PB2 gene playing a more significant role.

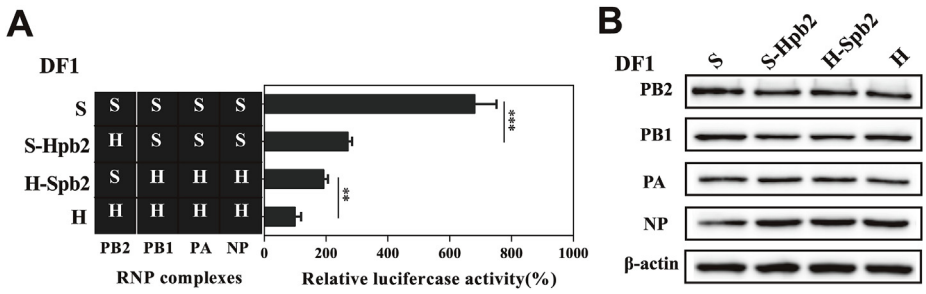

Fig. 4. The polymerase activity of RNPs bearing components from genotypes S and/or H viruses. The activity was determined by minigenome assay. Chicken DF-1 cells (C) were co-transfected with plasmids expressing PB2, PB1, PA and NP gene from SH/14 or CZ/73 virus, together with a firefly luciferase reporter plasmid, and a Renilla luciferase reporter plasmid (internal control). After 48 h, cell lysates were used to measure firefly and Renilla luciferase activities. S and H represent the gene segments from CZ/73 and SH/14. Values shown are mean ± SD of results from three independent experiments and are standardized to values of SH/14 (100%). The value of each recombinant virus was compared with that of the corresponding parental virus (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Western blot was performed on RNP components expression from DF-1 cells (B) transfected with plasmids expressing PB2, PB1, PA and NP from SH/14 or CZ/73 virus. The density of each band on the immunoblot was normalized to that of β-actin.

3.4. G1-like PB2 gene enhances the polymerase activity of genotype S H9N2 viruses in avian cells

Previous studies demonstrated that the viral RNP complex is pivotal for viral replication and pathogenicity (Hu et al., 2013; Sun et al., 2015). We analyzed the impact of different PB2 lineages on RNP polymerase activity in avian DF-1 cells. As shown in Fig. 4, the polymerase activity of CZ/73 virus with G1-like PB2 was significantly higher than that of SH/14 virus with F/98-like PB2. Replacing the G1-like PB2 gene of CZ/73 by F/98-like PB2 gene markedly decreased the RNP activity in DF-1 cells ($P < 0.001$) (Fig. 4A). The RNP complex of S ($S_{PB2}S_{PB1}S_{PA}S_{NP}$, "S" stands for CZ/73 virus) exhibited higher activity than that of S-Hpb2 RNP ($H_{PB2}S_{PB1}S_{PA}S_{NP}$; "H" stands for SH/14 virus) in DF-1 cells. By contrast, exchanging the F/98-like PB2 gene of SH/14 by G1-like PB2 gene remarkably increased the viral polymerase activity in DF-1 cells ($P < 0.01$) (Fig. 4A). The RNP complex of H ($H_{PB2}H_{PB1}H_{PA}H_{NP}$) showed lower activity than that of H-Spb2 RNP ($S_{PB2}H_{PB1}H_{PA}H_{NP}$). To determine whether the difference in polymerase activity was associated with the protein expression level of the RNP complexes, we compared the expression of viral proteins in DF-1 cells transfected with RNP plasmids. The results showed that RNP proteins PB2, PB1, PA, and NP were expressed at similar levels (Fig. 4B). Therefore, the difference between the CZ/73 and SH/14 polymerase activities detected with PB2 replacement was likely attributed to the changed enzymatic activity rather than changed viral protein expression. These results demonstrate that G1-like PB2 enhanced the polymerase activity of genotype S H9N2 viruses.

3.5. G1-like PB2 gene accelerates the translocation into the nucleus in avian cells

It has been reported that the efficacy of transport and accumulation of PB2 in the nucleus correlates with the adaptation and virulence of AIV (Gabriel et al., 2008). We therefore tested whether Q1/S, Q1/M, Q1/PB2 and Q1/H differ in their polymerase accumulation in the nucleus. CEFs were infected with viruses at 2.0 MOI, and the localization of PB2 and NP in the infected cells was determined at different time points post infection. By the confocal microscopy, we found that in Q1/S-infected CEFs, PB2 displayed significantly higher rate of nuclear accumulation than that of the Q1/H infected cells at each time points (Fig. 5A). At 9 hpi, PB2 was detected in the nuclei of approximately 69% of the Q1/S-infected CEF cells (Fig. 5C), whereas only 12% of the Q1/H-infected CEFs displayed PB2 nuclear localization. By contrast, the accumulation of NP in the nuclei of CEFs infected with Q1/S and Q1/H (Fig. 5A) was comparable at the time points tested. A larger proportion of the CEFs (Fig. 5B–C) infected with Q1/S and Q1/M than those infected with Q1/PB2 and Q1/H showed PB2 nuclear accumulation. These findings indicate that G1-like PB2 accelerates the translocation into the nucleus in avian cells.

3.6. G1-like M gene improves RNP nuclear export in avian cells

Previous studies demonstrated that the association of M1 with RNP results in translocation of RNP from nucleus to cytoplasm and affects viral replication (Liu et al., 2002; Martin and Helenius, 1991). To further study the spatiotemporal relationship between influenza RNP and M1, CEFs infected with Q1/S, Q1/M, Q1/PB2 and Q1/H at 2.0 MOI. Infected-CEFs were performed for immunostaining with *anti*-NP and *anti*-M1 antibodies at 15 hpi. By a confocal microscope, we found that M1 and NP were predominantly present in the nuclei in Q1/H or Q1/M-infected CEFs, while in Q1/S or Q1/PB2 virus infected-CEFs, NP and M1 were distributed throughout the cytoplasm of infected cells and were co-localized around the plasma membrane (Fig. 6A). Further, co-localization of NP and M1 was analyzed by using ImageJ software (Fig. 6B). As shown in Fig. 6B, in Q1/H or Q1/M infected cells, NP (red) and M1 (green) were both distributed in the central area of cell nucleus with a high degree of co-localization. However, NP and M1 proteins distributed throughout the cytoplasm of infected cells and co-localized around the plasma membrane in Q1/S or Q1/PB2 infected cells. These results indicate G1-like M improves RNP nuclear export in avian cells.

4. Discussion

In the present study, our results clearly demonstrated that the G1-like PB2 and M genes in avian H9N2 virus has markedly contributed to the fitness of the H5Nx and H7N9 viruses. We found that the G1-like PB2 or M gene in H5Nx and H7N9 virus was able to confer increased infectivity and severity of infection in CEFs and chickens. Furthermore, H5 virus harboring the G1-like PB2 or M, but not F/98-like PB2 or M gene, exhibited high polymerase activity, accelerated the translocation into the nucleus, enhanced protein expression and improved NP nuclear export from avian cells. Crucially, the PB2 gene has a dominant role.

The establishment of G1-like PB2 and M genes in H9N2 viruses has important evolutionary implications. In the late 1990s, two distinct H9N2 virus lineages (F/98-like and G1-like) emerged in chickens and quail in China (Xu et al., 2007b; Zhang et al., 2008). Phylogenetic analysis revealed two-way transmission of F/98-like and G1-like H9N2 viruses between chickens and quail (Bi et al., 2010; Liu et al., 2016). Xu et al. predicted that two-way transmission between different poultry species can increase the threat of H9N2 virus to humans by direct infection or contributing their internal genes to novel influenza virus subtypes (Xu et al., 2007b). Additionally, H9 and H5 viruses have a two-way exchange of gene segments to generate new genotype viruses that have pandemic potential (Xu et al., 2007a). In the present study, our results revealed that G1-like M and PB2 genes, relative to F/98-like M and PB2 genes, confer an increased fitness of H5 and H7 viruses in chickens (Fig. 3). Our findings along with others on the emergence of H7N9 and H5N6 viruses in poultry have in part confirmed these predictions.

As previously reported, H5 AIV reassortants with the 6 internal genes of a genotype S H9N2 AIV (H5/S) are attenuated both in chickens

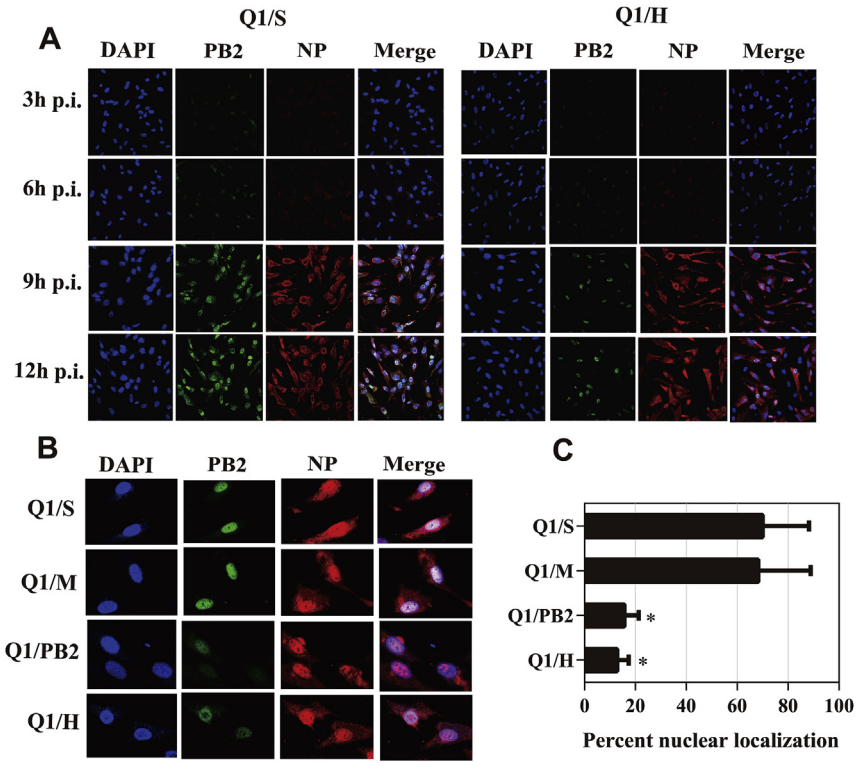

Fig. 5. Kinetics of the nuclear transport of PB2 and NP of H5 viruses in infected cells. CEF cells (A) were infected at an MOI of 2.0 with Q1/S and Q1/H respectively, and the localization of PB2 (green) and NP (red) was determined by immunofluorescence. Cell nuclei were stained with DAPI. CEF cells (B) were infected with Q1/S, Q1/M, Q1/PB2 and Q1/H virus at an MOI of 2.0 respectively, and the localization of PB2 (green) and NP (red) was determined by immunofluorescence 9 hpi. CEF cells (C) exhibiting nuclear PB2 were quantified by counting virus-infected cells (n = 100) under the microscope. Shown are mean ± SD for three independent experiments. Statistical difference was analyzed against Q1/S virus. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

and mice compared to wild type viruses (four H5 strains) (Hao et al., 2017). Our data revealed that the reassortant H5 viruses with genotype H internal gene cassette (H5/H) displayed lower virulence than those of the H5/S viruses in chicken models (Fig. 3). The results presented in the current study seem contradictory to our previously published findings. In fact, this study seeks to evaluate the virulence of the H5 reassortants containing genotype S or H H9N2 subtypes AIV whole set of internal gene in chickens, but the parental H5 viruses carry H5 subtypes AIV or part of H9N2 subtype internal gene in the previously study (Table S2). It is possible that changes in the biological properties of the virus

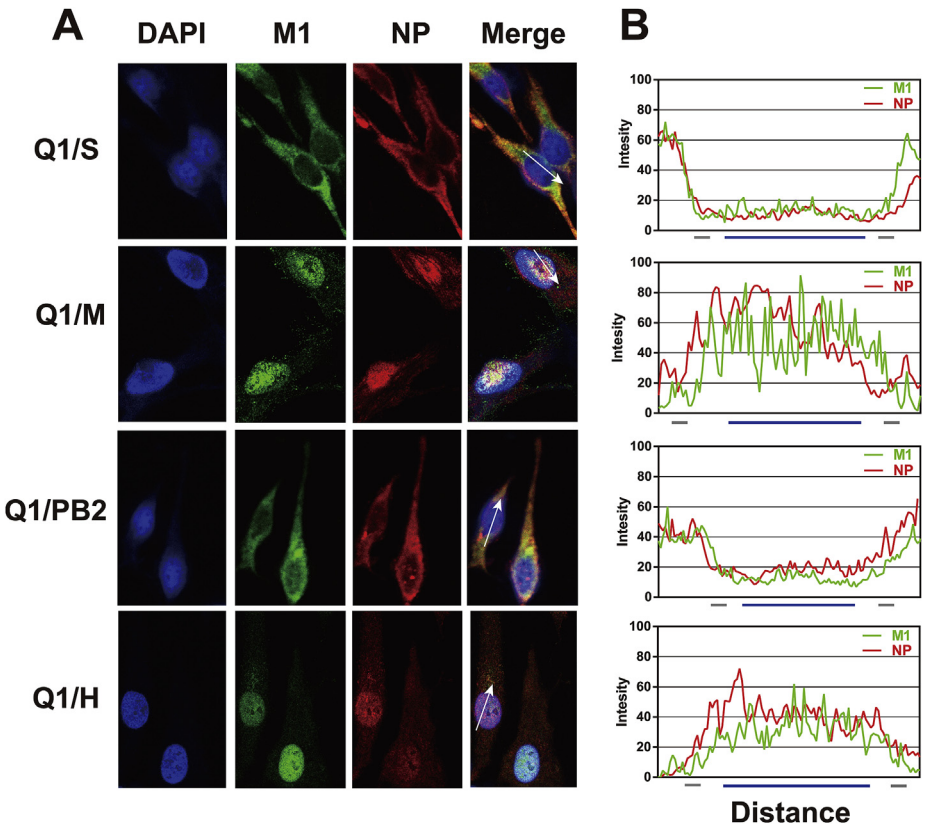

Fig. 6. Co-localization of NP and M1 in infected cells. CEFs (A and B) were infected with the indicated viruses at an MOI of 2.0, and the localization of M1 (green) and NP (red) was determined by immunofluorescence at 15 hpi. All images were obtained using a Leica SP8 confocal microscope. An intensity plot over a cellular distance (white arrow) of these merged images is shown to the right. The blue lines indicate the nuclear margin and the grey line indicates the plasma membrane margin.

depend on the specific genetic background (Bi et al., 2015; Sediri et al., 2016). In addition, pathogenicity studies demonstrated that the effect of a single gene segment on pathogenicity was influenced by the other seven segments of AIV. For example, Sun et al. reported that the H9 reassortants containing single PA gene from the pandemic H1N1/2009 virus could enhance the virulence in mice, whereas the ones possessing the whole internal gene cassette from the pandemic H1N1/2009 virus displayed a lower pathogenicity than the parental H9 viruses (Sun et al., 2011). Similarly, although the single PA or NP gene of genotype S H9N2 virus enhanced the virulence of the H5N1 recombinants in mice (Hao et al., 2016), the H5 reassortants possessing the internal gene cassette from genotype S H9N2 virus displayed lower virulence in both chickens and mice than their parental H5 viruses (Hao et al., 2017). We surmise that the attenuating effect of the internal gene of the genotype S H9N2 virus on the H5 reassortants may not be determined by a single gene but by a joint effect of multiple genes. Indeed, the pathogenesis of influenza virus includes polygenic virulence factors. Su W. et al. evaluated the pathogenicity of 63 reassortants derived from avian H7N9 and H9N2 viruses and found that 13 reassortants were more virulent than parental viruses (Su et al., 2015b). Significantly, H5 reassortants possessing genotype S H9N2 subtypes but not genotype H contributed to their increasing fitness in chickens. Furthermore, H5/S reassortants showed obviously longer MDTs than the corresponding parental H5 viruses, which may facilitate the longer virus shedding and circulation in chickens (Hao et al., 2017). Thus, these findings conveyed that H5 recombinants contained genotype S H9N2 subtypes AIV all internal genes showed improved adaptation in chickens, which might contribute to their the circulation in the field and pose a substantial threat to poultry industry.

In the present study, our results illustrated that G1-like PB2 and M genes contribute to the high virulence of H5 and H7 viruses in chickens and the PB2 gene has a greater effect (Fig. 3). The role of the PB2 gene in determining host range and virulence is well documented (Gao et al., 2009; Sediri et al., 2016), and it is found to alter viral polymerase activity and result in altered virus replication and pathogenicity in animal model (Bi et al., 2015; Su et al., 2015b). Su et al. reported that the heterogeneous PB2 gene significantly decreased the genesis of the H7N9 virus. In addition, the incompatibility of the wild bird PB2 gene with the H7N9 virus may result from its adverse effect on polymerase activity (Su et al., 2015b). The H7N9 PB2 gene has proven to be a key gene contributing to replication of the H7N9 virus in humans (Bi et al., 2015). In this study, our results attributed that inclusion of the G1-like PB2 gene in the F/98-like context significantly increased the polymerase activity, and expression of the F/98-like PB2 gene in the G1-like background resulted in a dramatic attenuation of polymerase activity from avian cells (Fig. 4). The level of polymerase accumulation in the nucleus of influenza virus-infected cells may affect the virulence of the viruses. For example, Hu et al. demonstrated that the PA mutation increased the nuclear accumulation of the PA protein in DEFs (Hu et al., 2013). In this study, we found that the accumulation of polymerase PB2 in the nucleus of CEFs infected with H5 virus containing G1-like PB2 was much higher than that of PB2 in cells infected with H5 virus containing F/98-like PB2 (Fig. 5). In addition, such G1-like PB2 gene regulated high polymerase activity and efficient PB2 nucleus accumulation may further contribute to the increased virulence of viruses in vivo. Thus, our results showed that the G1-like PB2 gene may be more adaptable than the PB2 gene derived from the F/98-like lineage among poultry.

Previous studies demonstrated the binding of M1 with RNP causes the transport of M1/RNP complex from the nucleus to the cytoplasm (Martin and Helenius, 1991). Our results show that the Q1/S and Q1/PB2 had higher efficiency in translocating M1/RNP from the nucleus to the cytoplasm in infected-CEFs than Q1/M and Q1/H (Fig. 6). Prior research reported that association of influenza virus M protein with RNP regulates viral growth and morphology (Liu et al., 2002). Thus, the G1-like M might contribute to enhance virus replication by facilitating RNP nuclear export in avian cells.

To better understand the natural selection of G1-like PB2 and M genes, we investigated their emergence and prevalence in avian viruses. We found that G1-like PB2 and M genes have become predominant in chicken flocks such as H9N2, H7N9 viruses (Fig. 1). The H9N2 virus subtype has been postulated to provide the internal genes including G1-like M and PB2 to H5N1(Guan et al., 1999), and H5N2 (Zhao et al., 2012) subtypes other than newly emerged viruses of H5N6, H7N9 and H10N8 posing great threat to poultry industry and public health. Therefore, the threat of H5 and H7 viruses harboring the G1-like PB2 and M gene segments in chickens to human health should be considered seriously.

In summary, our study indicated that the G1-like PB2 and M genes of H9N2 viruses confer increased fitness to H5 and H7 viruses in chickens. The G1-like PB2 contribute to improve the viral fitness through increasing viral polymerase activities and improving the transportation into the nucleus, while the G1-like M facilitates the translocation of RNP to cytoplasm. Our study provided further information for understanding why the S genotype H9N2 viruses have frequently donated their internal genes to emerging reassortant viruses, such as H7N9 and H5N6 that cause rising prevalence in chickens.Acknowledgements

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Conflicts of interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2019.07.001.

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