M gene reassortment in H9N2 influenza virus promotes early infection and replication: contribution to rising virus prevalence in chickens in China

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Abstract

Segment reassortment and base mutagenesis of influenza A viruses are the primary routes to the rapid evolution of high fitness virus genotypes. We recently described a predominant G57 genotype of avian H9N2 viruses that caused country-wide outbreaks in chickens in China during 2010-2013 which led to the zoonotic emergence of H7N9 viruses. One of the key features of the G57 genotype is the substitution of the earlier BJ/94-like M gene with the G1-like M gene of quail origin. We report here on the functional significance of the G1-like M gene in H9N2 viruses in conferring increased infection severity and infectivity in primary chicken embryonic fibroblasts and chickens. H9N2 virus housing the G1-like M gene, in place of BJ/94-like M gene, showed early surge in viral mRNA and vRNA transcription that were associated with enhanced viral protein production, and with early elevated release of progeny virus comprising largely spherical rather than filamentous virions. Importantly, H9N2 virus with G1-like M gene conferred extrapulmonary virus spread in chickens. Five highly represented signature amino acid residues (37A, 95K, 224N and 242N in M1 protein, and 21G in M2 protein) encoded by the prevalent G1-like M gene were demonstrated as prime contributors to enhanced infectivity. Therefore, the genetic evolution of M gene in H9N2 virus increases reproductive virus fitness, indicating its contribution to rising virus prevalence in chickens in China.

43 **Importance**

44 We recently described the circulation of a dominant genotype (G57) of H9N2
45 viruses in country-wide outbreaks in chickens in China, which was responsible
46 through reassortment for the emergence of H7N9 viruses that cause severe human
47 infections. A key feature of the G57 genotype H9N2 virus is the presence of quail
48 origin G1-like M gene which had replaced the earlier BJ/94-like M gene. We found
49 that H9N2 virus with G1-like M gene, but not BJ/94-like M gene, showed early surge
50 in progeny virus production, more severe pathology and extrapulmonary virus spread
51 in chickens. Five highly represented amino acid residues in M1 and M2 proteins
52 derived from G1-like M gene were shown to mediate enhanced virus infectivity.
53 These observations enhance what we currently know about the roles of reassortment
54 and mutations on virus fitness and have implications for assessing the potential of
55 variant influenza viruses that can cause rising prevalence in chickens.
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58 **Introduction**

59 Avian H9N2 and H5N1 influenza A viruses are two major globally circulating
60 subtypes in poultry populations (1, 2). Unlike highly pathogenic H5N1 viruses, the
61 low pathogenicity nature of H9N2 viruses had attracted less attention in disease
62 management and public health controls (3). In 2013, a novel reassortant H7N9 virus,
63 carrying six internal genes from avian H9N2 influenza virus, caused serious outbreaks
64 in humans in China (4, 5) which led to intense scrutiny of the evolution of H9N2
65 viruses. We previously demonstrated that H9N2 viruses of genotype G57 have
66 become predominant in chickens since 2010, and that G57 type viruses, with
67 enhanced infectivity and antigenic drift, had caused nationwide outbreaks in chicken
68 flocks during 2010-2013 (6). The increased prevalence of H9N2 viruses in chickens
69 has directly contributed to the emergence in human of H7N9 and other novel
70 reassortants with H9N2-like segments (6). Furthermore, prevalent chicken H9N2
71 isolates are able to preferentially bind to the human-type sialic acid receptor, and
72 transmit between ferrets by respiratory droplets (3). Presently, H9N2 viruses continue
73 to cause mild infections in humans in China and other countries based on etiological
74 and serological evidence (7-14). In 2013-2016, 18 human cases are
75 laboratory-confirmed in China, while only 10 cases are reported during the previous
76 fourteen years of 1999-2012
77 (http://www.who.int/influenza/human_animal_interface/HAI_Risk_Assessment/en/).
78 These evidences suggest that the prevalent H9N2 virus poses increasing threat to
79 human health.

H9N2 influenza viruses are enzootic in poultry in several Asian and Middle Eastern countries (http://www.who.int/influenza/vaccines/virus/characteristics_virus_vaccines/en/). Phylogenetic analysis revealed that multiple lineages of H9N2 viruses have been circulating, including A/chicken/Beijing/1/1994-like (BJ/94-like), A/quail/Hong Kong/G1/1997-like (G1-like), and A/duck/Hong Kong/Y439/1997 (Y439-like) viruses (15,16). Since the first isolation of BJ/94-like and G1-like viruses in China in the mid-1990s, the two lineages have become predominant in chickens and quail respectively, indicating relative host restriction (17, 18). During co-circulation of BJ/94-like and G1-like H9N2 viruses in poultry, genetic reassortments were observed between the two lineages (15). Several virus segments including PB2, PB1, PA and M from G1-like viruses have been introduced into BJ/94-like viruses, but only G1-like M gene is firmly established in BJ/94-like viruses (6, 15). The stable replacement of BJ/94-like M gene with G1-like M gene since 2004 is one key change in the generation of G57 genotype of H9N2 influenza viruses (6). Thus, the main question is whether G1-like M gene confers replication advantage to H9N2 viruses to contribute to their increasing prevalence in chickens in China since 2010.

M gene performs multiple roles in the life cycle of influenza A virus through encoding the matrix protein (M1) and the proton channel protein (M2) (19, 20). M1 is the most abundant viral protein responsible for the structural shell of the virus linking the viral envelope with the nucleocapsid (19); it is involved in the shuttling of viral ribonucleotide protein (vRNP) complex between the nucleus and cytoplasm during

viral replication (21-23). M2 is an integral membrane protein inserted into the viral envelope and possesses proton channel activity (20); it is thought to function at an early stage of the virus life cycle (24-26). Once virions have undergone endocytosis, the M2 proton channel is believed to permit protons to flow from the endosome into the virion interior which promotes vRNP release into the cytoplasm (24). In addition, M1 and M2 proteins play an important role in influenza virus assembly and budding, and are key determinants of virus morphology (19). Changes in M1 and M2 genes have been found to be critical to viral replication and the pathogenicity of avian H5N1, seasonal human H1N1, H3N2 and pandemic H1N1/2009 viruses in avian or mammalian cells and hosts (27-29).

In this study, we examined the preferential selection of M gene from the G1-like lineage in place of the BJ/94-like lineage in avian H9N2 viruses in chickens. H9N2 virus with G1-like M gene, relative to the presence of BJ/94-like M gene, showed early and enhanced virus replication in chicken embryo fibroblasts (CEFs) and chickens, extrapulmonary virus spread, and changed viral morphology, which collectively indicate increased virus fitness. Five dominant amino acid residues in M1 and M2 proteins encoded by G1-like M genes are identified as critical contributors to enhanced virus replication.

Materials and Methods

Ethical approval

All animal studies were performed in compliance with the recommendations in the Guide for the Care and Use of Laboratory Animals of China Agricultural

University (CAU) (ID: SKLAB-B-2010-003) and with approval of the Beijing Association for Science and Technology of China (approval ID SYXK, Beijing, 2007-0023).

Phylogenetic analysis

All available M gene sequences of H9N2 viruses isolated from various hosts in China during 1994-2015 were downloaded from the Influenza Virus Resource at the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/genomes/FLU). Multiple sequence alignment was carried out using MUSCLE (30), guided by amino acid sequence alignment. The final alignment covered 804 (position 79-882, reference A/Turkey/California/189/66) nucleotides in length and contained 1145 sequences. The aligned sequences were then used to generate a maximum likelihood (ML) tree by employing FastTree (version 2.1.7) (31). GTR+ gamma model was used and phylogenetic uncertainty was assessed by the Shimodaira-Hasegawa (SH) test for each split in the tree and was resampled 1,000 times. The final tree was viewed and edited in FigTree (version 1.4.2) (http://tree.bio.ed.ac.uk/software/figtree/) and rooted using A/Turkey/California/189/66.

Viruses, plasmids and cells

The use of wild type H9N2 viruses, A/chicken/Shandong/lx1023/2007 (lx1023) and A/chicken/Jiangsu/TS/2010 (TS), was previously described (6, 32). Lx1023 and TS house the BJ/94-like M gene and G1-like M gene respectively. M gene expression plasmids derived from H9N2 virus strainslx1023 and TS were generated by separately

146 inserting each M gene coding sequence into pcDNA3.1 vector. CEFs were isolated
147 from 10-day-old embryonated chicken eggs by trypsin digestion (33). Human
148 embryonic kidney (293T) cells, Madin-Darby canine kidney (MDCK) cells and CEFs
149 were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco)
150 supplemented with 10% fetal bovine serum (FBS, Gibco), 100 units/ml of penicillin,
151 and 100 μg/ml of streptomycin at 37 °C in 5% CO_2 atmosphere.

152 **Generation of recombinant and mutant H9N2 viruses by reverse genetics**

153 All eight gene segments of lx1023 and M gene of TS (G1-like M gene) were
154 amplified by reverse transcription-PCR (RT-PCR) and individually cloned into a
155 dual-promoter plasmid pHW2000 (34). Reverse genetic virus, rCK1023:M-BJ/94,
156 containing all eight genes from lx1023, and reassortant virus, rCK1023:M-G1, with M
157 gene from TS virus and remaining seven genes from lx1023 virus were generated in
158 293T cells as previously described (34). In the backbone of rCK1023:M-BJ/94,
159 mutations of T37A, R95K, S224N and K242N were separately introduced into M1
160 protein, and D21G was introduced into M2 protein, by using a site-directed Quik
161 Change mutagenesis kit (Agilent, Santa Clara, CA) according to the manufacturer's
162 instructions. Primer sequences are available upon request. The rescued viruses
163 possessing single mutation were designated as rM1-T37A, rM1-R95K, rM1-S224N,
164 rM1-K242N and rM2-D21G respectively. All viruses were propagated in 9-day-old
165 specific pathogen-free (SPF) chicken embryos and sequence verified prior to use.

166 **Quantitative real-time PCR (qRT-PCR)**

167 Levels of mRNA and vRNA were determined in CEFs infected with different

168 H9N2 viruses at a multiplicity of infection (MOI) of 0.01 or 0.001. Total RNA was
169 extracted from infected CEF cells using TRIzol reagent according to the
170 manufacturer's instructions (Invitrogen). For the detection of mRNA and vRNA, oligo
171 dT primer and uni-12 primer (5'-AGCAAACGACC-3') were respectively used to
172 generate cDNAs by reverse transcription with 1 μg of total RNA per sample using
173 Superscript III First-Strand Synthesis SuperMix (Invitrogen). The qRT-PCR mixture
174 for each reaction sample consisted of 10 μl of 2 × SYBR green PCR master mix
175 (Applied Biosystems), 7 μl of nuclease-free water, 0.5 μl of each primer and 2 μl of
176 cDNA template (diluted 1:100). Messenger RNA and vRNA of M1, M2 and
177 nucleoprotein (NP) genes as well as β-actin mRNA were quantified using the 7500
178 real-time PCR system (Applied Biosystems) with the following program: 1 cycle at
179 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.
180 Expression values of each gene, relative to β-actin, were calculated using the $2^{-\triangle\triangle CT}$
181 method. Each experiment comprised three technical replicates for each sample, and
182 two experimental replicates were performed. Primers for amplification of β-actin, M1,
183 M2 and NP genes are listed as follows: β-actin, Forward,
184 5'-AGAGCTACGAGCTGCCTGAC-3', Reverse,
185 5'-CGTGGATGCCACAGGACT-3'; M1, Forward,
186 5'-CCATCAGGCCCCCTCAAAGCCGAGA-3', Reverse,
187 5'-ACGGTGAGCGTGAACACGAACCCTA-3'; M2, Forward, 5'
188 -TTTCTTCAAATGCATTTATCGTCGC-3', Reverse,
189 5'-AAAATGACCATCGTCAACATCCACA-3'; NP, Forward,

5'-AGAGACGGAAAATGGGTGAGAGAGC-3', Reverse, 5'-GGATCCATTCCAGTACGCACGAGAG-3'.

Western blotting

Total cell protein lysates were extracted from transfected 293-T cells or infected CEFs with RIPA lysis buffer and total protein concentration was determined with a BCA protein assay kit (Beyotime, China). Protein samples derived from cell lysates were heated at 100 °C for 10 min and separated on a 12% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, USA), and subsequently incubated with an appropriate primary antibody. Primary antibodies were specific for β-actin (Beyotime, China), influenza A virus M1 (GeneTex, USA), M2 (Thermo Fisher Scientific, USA), and NP (Biorbyt, UK). Horseradish peroxidase (HRP)-conjugated anti-rabbit or -mouse secondary antibody was used (Beyotime, China). HRP presence was detected using a Western Lightning chemiluminescence kit (Amersham, USA) following the manufacturer's protocol.

Virus titration and replication kinetics

Fifty percent tissue culture infectious dose ($TCID_{50}$) assays were performed on MDCK cells inoculated with 10-fold serially diluted viruses and incubated at 37 °C in 5% CO_2 atmosphere for 72 h. $TCID_{50}$ values were calculated by the Reed-Muench method (35). Multistep replication kinetics assays were determined by infecting CEF sat 0.001 MOI. After 1 h incubation at 37°C, the cells were washed twice and further incubated in serum-free DMEM containing 0.5μg/ml TPCK trypsin. Supernatants

were sampled at 12, 24, 36, 48, 60 and 72h post-inoculation (hpi). Single-replication-cycle kinetics assays were similarly conducted except with starting virus inoculations at 0.01 MOI. Supernatants were sampled at 2, 4, 6, 8, 10 and 12hpi.

Chickens challenge study

Nine six-week-old SPF White Leghorn chickens were inoculated intranasally with 10^6 50% egg infective doses (EID_{50}) of each stock virus. Three chickens per group were euthanized at each time of 3 and 5 days post-inoculation (dpi), and lungs and kidneys were collected for virus titration and histopathological examination. Tracheal and cloacal swabs from the remaining three chickens of each group were collected at 1, 3, 5 and 7 dpi. Virus titer detection limit was at $0.75\log_{10}EID_{50}$/ml.

Histopathology and immunohistochemistry (IHC)

The lungs and kidneys collected at 3 dpi were fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E). The tissue sections were also immunostained for viral nucleoprotein (NP) with a monoclonal primary antibody (AA5H, Abcam, Hong Kong). Secondary antibody (Millipore, Billerica, MA, USA) used was conjugated to HRP, and the color reaction was based on the use of a HRP reaction kit (diaminobenzidine-tetrahydrochloride, Sigma, St. Louis, MO, USA). Two independent pathologists scored all slides from blinded experimental groups.

Transmission electron micrographs

For imaging of virions, samples were prepared as previous described with some modification (36). Briefly, CEFs were infected at 3.0 MOI. At 15 hpi, cells were fixed

with 2.5% glutaraldehyde in 0.1M cacodylate buffer for 2 to 3 h at room temperature or overnight at 4°C. Cells were then embedded in Eponate 12 resin, cut into 80-nm sections, and stained with 5% uranylacetate and 2% lead citrate. After sample preparation, grids were imaged at 75 kV using a JEOL1200EX transmission electron microscope.

Statistical analysis

All statistical analyses were performed using GraphPad Prism software version 5.00 (GraphPad Software Inc., San Diego, CA, USA). Statistically significant differences between experimental groups were determined by analysis of variance (ANOVA). Differences were considered statistically significant at $P< 0.05$.

Results

1. Increased prevalence of G1-like M gene segment in chicken H9N2 viruses in China

Previous studies revealed that H9N2 viruses with G1-like M genes were mainly present in quails and to lesser extent in other minor poultry, but few G1-like M genes were isolated from chicken H9N2 viruses (15, 16). To better understand the current circulation of G1-like M genes in different poultry populations in China, we performed a phylogenetic analysis of all available sequences from 1997 to 2015. As shown in Fig. 1A, H9N2 viruses with G1-like M genes were predominantly present (95.52%) in quail and other minor poultries in 1997-2003, but were uncommon (4.48%) in chickens. In 2004-2009, G1-like M genes had reassorted with BJ/94-like M genes in chicken H9N2 viruses (HA gene belonged to BJ/94-like lineage) leading

256 to the proportion of G1-like M genes rising to 77.05%. Since 2010, G1-like M genes
257 of H9N2 viruses from chickens account for 87.79%, but those of quail and minor
258 poultry isolates were only found in 12.21% of the H9N2 isolates with G1-like M
259 genes; G1-like M gene remained the predominant lineage in quail and minor poultry.

260 We further examined the dynamic prevalence of BJ/94-like and G1-like M genes in
261 chicken H9N2 viruses. In 1994, H9N2 viruses with BJ/94-like M gene segment were
262 first isolated from chickens; it remained dominant at around 97% frequency in this
263 host until 2004 (Fig. 1B). H9N2 virus with G1-like M gene was found in some
264 chickens in 1997-2004, but by 2005, the year after reassortment, detection of G1-like
265 M gene in chicken H9N2 viruses had risen sharply. Since 2007, G1-like M gene
266 containing H9N2 virus has replaced BJ/94-like M gene as the dominant (94.95%)
267 genotype in chickens. These findings suggest that the segmental replacement of
268 BJ/94-like with G1-like M gene could be a significant adaptation of H9N2 viruses
269 that confers improved infection fitness in chickens.

270 **2. G1-like M gene conferred early elevated viral mRNA transcription, vRNA**
271 **production and protein expression**

272 To address the viral fitness hypothesis, we first determined if the replacement of
273 BJ/94-like with G1-like M gene affects viral infection in terms of viral messenger
274 RNA transcription and viral genomic (vRNA) production. We produced virus
275 rCK1023:M-BJ/94 from a wild type H9N2 isolate (A/chicken/Shandong/1x1023/2007,
276 Lx1023) that contained a BJ/94-like M gene, and another virus rCK1023:M-G1 based
277 the same H9N2 virus backbone with only the M gene substituted with the G1-like M

segment from A/chicken/Jiangsu/TS/2010 (TS). Levels of viral transcription (mRNA) and genomic replication (vRNA) were determined in CEFs separately infected with rCK1023:M-BJ/94 and rCK1023:M-G1 viruses for 1, 2, 4, 6 and 24 hpi by real-time PCR. As shown in Fig. 2, rCK1023:M-G1 virus produced significantly higher levels of viral M1, M2 and NP mRNA and vRNA, from as early as 2 hpi onwards, than with rCK1023:M-BJ/94 virus ($P < 0.05$). Thus, compared with BJ/94-like M gene, G1-like M gene in H9N2 virus enhanced early viral mRNA and vRNA transcription in CEFs.

We next examined viral protein expression in CEFs separately infected with rCK1023:M-BJ/94 and rCK1023:M-G1 viruses at 4, 6, 8 and 12 hpi (Fig. 3A). M1 and M2 proteins in rCK1023:M-G1 were detected at 4 and 6 hpi respectively, while those of rCK1023:M-BJ/94 were found 2 hours later. At each time point, rCK1023:M-G1 produced more proteins of M1, M2 and NP compared with rCK1023:M-BJ/94. Thus, the accumulation of M1, M2 and NP proteins was earlier and higher with rCK1023:M-G1 virus than with corresponding rCK1023:M-BJ/94 virus infection. To examine if differences between G1-like and BJ/94-like M genes alone could affect protein production, 293T cells were transfected with individual M gene expression plasmids derived from BJ/94-like and G1-like M gene segments. Western blotting at 12, 24, 36 and 48 h post-transfection or at 36 h post-transfection with plasmid doses of 2μg or 4μg found no significant difference in M1 and M2 protein expression between the two M genes (Fig. 3B). Taken together, the enhanced viral mRNA transcription conferred by the G1-like M gene segment was translated into earlier and higher accumulation of H9N2 viral proteins.

3. G1-like M gene resulted in increased frequency of spherical virions released from CEFs

Influenza M1 and M2 protein has been implicated in the determination of variation in virus morphology (19). Shorter spherical virus particles has been associated with higher avian progeny virus output (37). We compared the morphology of nascent rCK1023:M-BJ/94 and rCK1023:M-G1 viruses from CEFs by transmission electron microscopy (Fig. 4). About 61.90% of rCK1023:M-BJ/94 virus particles were morphologically filamentous, whereas rCK1023:M-G1 virions were predominantly spherical or ovoid (96.05%) with only 3.95% filamentous particles. Thus, the G1-like M gene segment specified a spherical morphological phenotype which might have facilitated virus replication and shedding of rCK1023:M-G1 virus from CEFs.

4. G1-like M gene in H9N2 virus conferred early replication in CEFs and increased virus output

To compare the effect of different M gene lineages on viral replication *in vitro*, multistep replication kinetics of rCK1023:M-BJ/94 and rCK1023:M-G1 viruses were performed at 0.001 MOI in CEFs over a duration of 72 h. rCK1023:M-G1 virus production was up to10-fold higher than that of rCK1023:M-BJ/94 virus from 12 to 36 hpi ($P < 0.05$) (Fig. 5A, left). We further conducted single-replication-cycle kinetics at an MOI of 0.01 over 12 h of infection (Fig. 5B, left). rCK1023:M-G1 virus generated progeny at around 60-fold higher than that of rCK1023:M-BJ/94 virus from 6 to 12 hpi. Notably, rCK1023:M-G1 progeny virus output was detected earlier by 6 hpi, about 4 h ahead of rCK1023:M-BJ/94 virus. Taken together, G1-like M gene in

322 H9N2 virus conferred earlier release and higher overall output of progeny virus from
323 CEFs.

324 **5. G1-like M gene in H9N2 virus conferred increased severity and early onset of**
325 **infection in chickens**

326 Typically, avian H9N2 virus is mildly pathogenic in chickens; its replication is
327 largely confined to the upper respiratory tract causing little or no overt clinical signs
328 especially in SPF chickens (6). The pathogenicity and replication of
329 rCK1023:M-BJ/94 and rCK1023:M-G1 H9N2 viruses were evaluated in SPF
330 chickens. All chickens infected with rCK1023:M-BJ/94 or rCK1023:M-G1 virus
331 showed no overt clinical signs. Lung and kidney tissues were collected at 3 dpi for
332 histopathological examination. rCK1023:M-BJ/94 virus infected lungs showed mild
333 inflammatory changes and bronchitis (Fig. 6A). rCK1023:M-G1 virus, on the other
334 hand, showed evidence of more severe inflammation with interstitial pneumonia and
335 bronchopneumonia, characterized by alveolar interstitial consolidation, extensive
336 infiltration of inflammatory cells, and sloughing of mucous epithelial lining (Fig. 6B).
337 The kidneys from rCK1023:M-BJ/94 virus infected chickens appeared normal (Fig.
338 6C). However, renal congestion was evident from rCK1023:M-G1 virus infected
339 chickens (Fig. 6D). Expectedly, viral NP was extensively detected in the lungs
340 (bronchioles, terminal bronchioles and alveoli) of chickens infected with each H9N2
341 virus type (Fig. 6E and F). However, NP was only detected in the tubular renal
342 epithelial cells of chickens infected with rCK1023:M-G1 virus (Fig. 6G and H),
343 indicating extrapulmonary infection.

344 At 1, 3, 5 and 7 dpi, tracheal and cloacal virus titers were determined from three
345 chickens per virus group; virus titers from lung and kidney tissues were ascertained at
346 3 and 5 dpi. At each time point of tracheal sampling, rCK1023:M-G1 virus produced
347 more progeny than rCK1023:M-BJ/94 virus; from 3 to 7 dpi, the difference in virus
348 output was over 500-fold ($P<0.001$) (Fig. 7A, left). rCK1023:M-G1 virus shedding
349 from the trachea lasted at least 7 days while rCK1023:M-BJ/94 virus shedding lasted
350 for 5 days (Fig. 7A, left). rCK1023:M-G1 virus also showed cloacal virus shedding in
351 one infected chicken while no virus was found in the cloaca of H9N2-M-BJ/94
352 infected chickens (data not shown). High recovery of rCK1023:M-G1 virus was made
353 from the lungs of all infected chickens at both 3 and 5 dpi (Fig. 7B, left).
354 rCK1023:M-BJ/94 virus recovery, however, was lower (up to 100 fold less, $P< 0.05$)
355 and only successful in 1 out of 3 infected chickens at each time point. A similar
356 contrast of high rCK1023:M-G1 virus recovery but no rCK1023:M-BJ/94 virus
357 detection from the kidneys of infected chickens (Fig. 7B, left). Collectively, G1-like
358 M gene in H9N2 virus conferred higher severity of infection, more progeny virus, and
359 extrapulmonary virus spread in chickens.

360 **6. Highly represented amino acid residues encoded by prevalent G1-like M genes**
361 **from chicken H9N2 viruses**

362 We sought to identify critical amino acid residues represented in the M1 and M2
363 proteins derived from prevalent G1-like M genes that could explain the increased
364 severity and early onset of H9N2 virus production in chickens. In the M phylogenetic
365 tree (Fig. 1A), the prevalent M genes circulating in chickens since 2010 are mostly

366 from the major group of G1-like lineage, while outside the major group, the M genes
367 are mainly from the earlier period. We compared the amino acid sequences of M1 and
368 M2 proteins between the major group and non-major group of chicken-origin M genes
369 within the G1-like lineage (Fig. 1A). The alignment identified five amino acid
370 residues highly represented in the major group of G1-like lineage: M1-37A, M1-95K,
371 M1-224N, M1-242N and M2-21G (Table 1); these residues were also present in the
372 rCK1023:M-G1 virus. They were uncommon in the M genes of G1-like non-major
373 group (0.96-5.77%) and in the BJ/94-like lineage (0-2.23%). Therefore, the five
374 amino acid residues are distinct molecular markers of G1-like M genes of prevalent
375 H9N2 viruses.

376 We further investigated the dynamic prevalence of the five amino acid residues in
377 the G1-like M genes of chicken H9N2 viruses in China. As shown in Fig. 8 and Table
378 1, all five residues first emerged in 2006-2008 followed by a sharp rise in their
379 detection in the subsequent years that coincided with the country wide H9N2 virus
380 chicken outbreaks of 2010-2013. This finding suggests that the five distinct residues
381 encoded by the prevalent G1-like M gene could be involved in the recent increase in
382 reproductive fitness of the H9N2 virus in chickens.

383 **7. Dominant G1-like M gene amino acid residues conferred increased and early**
384 **onset of virus replication in CEFs and chickens**

385 We constructed five virus mutants (rM1-T37A, rM1-R95K, rM1-S224N,
386 rM1-K242N and rM2-D21G) each with a single amino acid substitution in the M gene
387 of the rCK1023:M-BJ/94 virus. Compared with rCK1023:M-BJ/94 virus, mutant

388 viruses exhibited higher (Fig. 5A, right) and earlier (Fig. 5B, right) production of
389 progeny virus from CEFs. rM1-T37A mutant virus, in particular, showed significantly
390 greater virus output between 6 and 12 hpi, and earlier virus output, about 4 h ahead,
391 than parental rCK1023:M-BJ/94 virus ($P<0.001$).

392 SPF chickens infected with the five mutant H9N2 viruses variously showed
393 elevated virus output from tracheal, lung and kidney samples, relative to the parental
394 rCK1023:M-BJ/94 virus (Fig. 7A and B, right). rM1-R95K, rM1-K242N and
395 rM2-D21G mutants but not parental virus remained detectable in tracheal samples at 7
396 dpi (Fig. 7A, right). rM1-T37A mutant consistently showed elevated tracheal release
397 of virus at 1, 3 and 5 dpi (Fig. 7A, right). All chickens inoculated with each of the five
398 mutant viruses significantly produced higher virus loads in the lung and kidney than
399 those infected with the parental virus (Fig. 7B, right). For both sampling time points
400 (3 and 5 dpi), virus recovery was successful from the lungs of all chickens infected
401 with each mutant virus but only 1 in 3 infections with the parental virus was recovery
402 from the lung successful at each time point (Fig. 7B, right). All five mutant viruses
403 could also be isolated at raised levels from the kidneys of infected chickens at 3 dpi,
404 albeit at differing frequency. At 5 dpi, 3 out of 5 mutant viruses could still be isolated
405 at differing frequency from the kidneys of infected chickens (Fig. 7B, right). No virus
406 was recovered from kidneys of chickens infected with the parental virus. Collectively,
407 these results demonstrated that each of the five amino acid residues, identified in the
408 major group of G1-like M genes, introduced into parental rCK1023:M-BJ/94 H9N2
409 virus was able to increase virus replication, elicit an earlier onset of virus release and

410 confer extrapulmonary spread in chickens.

411 **Discussion**

412 In the present study, our combined *in vitro* and *in vivo* findings clearly
413 demonstrated that the recent evolution of M gene through reassortment and mutations
414 has significantly contributed to the fitness of the H9N2 virus in chickens which could
415 account for its increased prevalence in chicken flocks in China during 2010-2013. We
416 found that the G1-like M gene in H9N2 virus was able to confer increased infectivity
417 and severity of infection in primary CEFs and chickens. Crucially, H9N2 virus
418 housing the G1-like M gene, but not BJ/94-like M gene, exhibited early surge
419 (detected by 2 hpi) in viral mRNA and vRNA synthesis that were associated with
420 enhanced viral protein production, and with early and elevated release of progeny
421 virus from infected cells (by up to 4 h earlier) and chickens. Five signature amino acid
422 residues (37A, 95K, 224N and 242N in M1 protein, and 21G in M2 protein) were
423 demonstrated to be functionally important for the enhanced virus fitness effect of the
424 G1-like M gene.

425 The establishment of G1-like M gene, in place of BJ/94-like M gene, in chicken
426 H9N2 viruses has important evolutionary implications. During the 1990s, two distinct
427 H9N2 virus lineages (BJ/94-like and G1-like) were established in chickens and quail
428 respectively in China (16). Subsequently, phylogenetic analysis revealed two-way
429 transmissions of BJ/94-like and G1-like H9N2 viruses between chickens and quails in
430 the country (15, 16, 38). Over the past 20 years, the G1-like M gene is the only
431 segment from quail G1-like viruses to be established in chickens. Based on

epidemiological predictions by Yi Guan and colleagues (16), two-way transmissions between different types of poultry can increase the risk of H9N2 virus mutants to humans by direct infection or indirectly through contribution of their internal genes to promote novel subtypes (16). Our present findings along with others on the emergence of H7N9 viruses (4, 6, 39) have in part corroborated this prediction. Namely, through viral transmission from quails to chickens, G1-like M gene was reassorted into chicken H9N2 virus to generate the G57 genotype which subsequently provided six internal genes to the novel H7N9 viruses causing severe outbreaks in humans. The ability of the G1-like M gene to increase and initiate early virus replication would be enormously advantageous to virus genotype dominance.

Reassortment and subsequent mutation is an important strategy of influenza viruses in host adaptation (40). Here, we identified five highly represented amino acid residues in M1 and M2 proteins encoded by prevalent G1-like M genes from chicken H9N2 viruses in China. With the exception of M1-37A, the remaining four residues (M1-95K, M1-224N, M1-242N and M2-21G) are sited at or close to known functional domains. M1-224 and M1-242 are located in binding site of M1 protein for vRNP (41). M1-95 is sited close to the M1 nuclear localization signal sequence (22) which is also a potential binding site for NEP (42). M2-21 is located close to the transmembrane domain of M2 proton channel protein (26). Each of the five amino acid residues (M1-37A, M1-95K, M1-224N, M1-242N and M2-21G) introduced into parental rCK1023:M-BJ/94 H9N2 virus was variously able to increase virus replication, elicit an earlier onset of virus release and confer extrapulmonary spread in

454 chickens. These mutations may play functions through the above domain-mediated
455 effects, especially through the effects on M2 proton channel activity and shuttling of
456 vRNP between nucleus and cytoplasm, to facilitate early production of viral genome,
457 proteins and virus particles. We surmise that these residues in concert could have a
458 greater and more consistent promotional impact on virus propagation.

459 M1 and M2 proteins play important roles at the later stages of virus life cycle
460 through participating in virus assembly and budding of nascent virions. The influenza
461 virion is pleomorphic, forming spherical and filamentous virions, but little is known
462 about the functional significance of influenza virus morphology (19). It was thought
463 that human infection produces predominantly filamentous virions (43, 44). Several
464 groups demonstrated that the M segment of the 2009 pandemic influenza virus
465 confers increased filamentous morphology, and efficient contact transmissibility in
466 mammalian hosts (29, 45). Interestingly, serial passage of filamentous isolates of
467 influenza virus in eggs caused loss of filamentous morphology (36, 46), and led to
468 improved growth in eggs (36). Low pathogenicity avian H2N3 virus infected CEFs
469 were found to produce mainly spherical virions whereas the same infection in duck
470 embryonic fibroblasts (which are inherently more resistant to virus replication)
471 generated largely filamentous virions (37). In our study, CEFs infected with H9N2
472 virus housing the G1-like M gene produced mainly spherical/ovoid virus particles
473 whereas H9N2 virus harboring the BJ/94-like M gene generated mainly filamentous
474 virions. The amino acid change from K to R at the position 95 in M1 protein has a
475 critical role in filamentous particle formation (47, 48). G1-like M1 protein possesses a

476 reverse mutation of R95K which may account for the increased spherical particles of
477 corresponding H9N2 virus. In summary, the change from filamentous to spherical
478 morphology correlated with the more efficient infection of rCK1023:M-G1 virus in
479 CEFs and chickens, suggesting spherical virions are better adapted at virus
480 replication.

481 Relative to other lineages of H9N2, G1-like H9N2 viruses or the viruses with
482 G1-like genes appear to show growing infectivity towards humans (7, 17, 49, 50). We
483 found that all of the human H9N2 virus isolates with available M gene sequences
484 from databases have G1-like M genes (data not shown). The H9N2 virus subtype has
485 contributed its internal genes including G1-like M gene to H5N1, H7N9 and H10N8
486 subtypes with ensuing human infections (4, 17, 51). The H9N2 virus origin of PB2, M
487 and NP genes are key virulence genes in human cases of H7N9 virus infection (52).
488 Experimentally, H9N2 viruses are shown to have extensive reassortment compatibility
489 with pH1N1 (34, 53), human H3N2 (54), or avian H5N1 viruses (55). Therefore, the
490 threat of H9N2 viruses harboring the G1-like M gene segment in chickens to human
491 health should be taken seriously.

492 Our data indicate that the early virus replication and more severe infection acquired
493 by genetic reassortment is critical in conferring virus fitness to better counter host
494 defenses and increasingly cause outbreaks in avian or mammalian populations.

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666 **Figure legends**

667 **FIG 1** Prevalence of G1-like M gene in chicken, quail and minor poultry (A), and the
668 prevalence of BJ/94-like and G1-like M genes in chicken H9N2 viruses in China (B).
669 The phylogenetic tree of M gene was generated with all available M sequences from
670 H9N2 viruses isolated from various hosts in China during the period of 1994-2015. In
671 the tree, the names of viruses from chicken, quail, minor poultry and others are shown
672 in red, green, blue and black color respectively. For each column from left to right, the
673 actual number of virus isolates is 67, 183 and 475 in FIG 1A, and 13, 141, 21, 41, 16,
674 30, 40, 53, 53, 64, 88, 189, 33 and 6 (along x-axis) in FIG 1B. In FIG 1B, all HA
675 genes of chicken H9N2 viruses belonged to BJ/94-like lineage; the G1-like M gene
676 has become predominant in the BJ/94-like viruses through reassortment.

677

678 **FIG 2** Relative expression of viral M1, M2 and NP mRNA and vRNA of
679 CK1023:M-G1 and rCK1023:M-BJ/94 H9N2 viruses in CEFs. CEFs were infected
680 with the indicated H9N2 viruses at 0.01 MOI for 1, 2, 4, and 6 h, or at 0.001 MOI for
681 24 h. Messenger RNA and vRNA expression are presented as fold change relative to
682 rCK1023:M-BJ/94virus. Data are presented as means±SD of three independent
683 experiments. Statistical significance was based on two-way ANOVA (***$p<0.001$).

684

685 **FIG 3** Replacement of BJ/94-like M gene with G1-like M gene in avian H9N2 virus
686 increased viral protein expression. (A) CEFs were infected with rCK1023:M-BJ/94 or
687 rCK1023:M-G1 virus at an MOI of 0.1. Cells were harvested at the indicated time

688 points and Western blotting performed on cell lysates to detect viral NP, M1 and M2
689 proteins. The panel showing the viral protein expression at 4, 6 and 8 hpi and that at
690 12 hpi were the photos taken from different gels. (B) 293T cells were separately
691 transfected with M gene expression plasmids derived from field H9N2 isolates lx1023
692 and TS which housed BJ/94-like and G1-like M gene segments respectively. At 12, 24,
693 36 and 48 h post-transfection, cell lystes were harvested for Western blotting.
694 Comparative M gene protein expression was conducted at 36 h post-transfection with
695 2μg or 4μg of individual plasmids.
696
697 **FIG 4** Transmission electron micrographs of negatively stained H9N2 virus particles
698 housing BJ/94-like (A) or G1-like (B) M gene. CEFs were infected at 3.0 MOI for
699 15h. rCK1023:M-BJ/94 progeny was mainly filamentous and rCK1023:M-G1
700 particles were primarily spherical/ovoid in shape.
701
702 **FIG 5** Virus output of rCK1023:M-G1, rCK1023:M-BJ/94 and M gene point mutants
703 of rCK1023:M-BJ/94 H9N2 viruses from infected CEFs. (A) Multi-step-growth
704 curves of H9N2 viruses from CEFs inoculated at MOI of 0.001. (B)
705 Single-replication-cycle of H9N2 viruses in CEFs inoculated at MOI of 0.01. Virus
706 titers were determined from supernatants collected at the indicated time points.
707 Statistical significance was based on two-way ANOVA (*p<0.05, **p<0.01,
708 ***p<0.001).
709

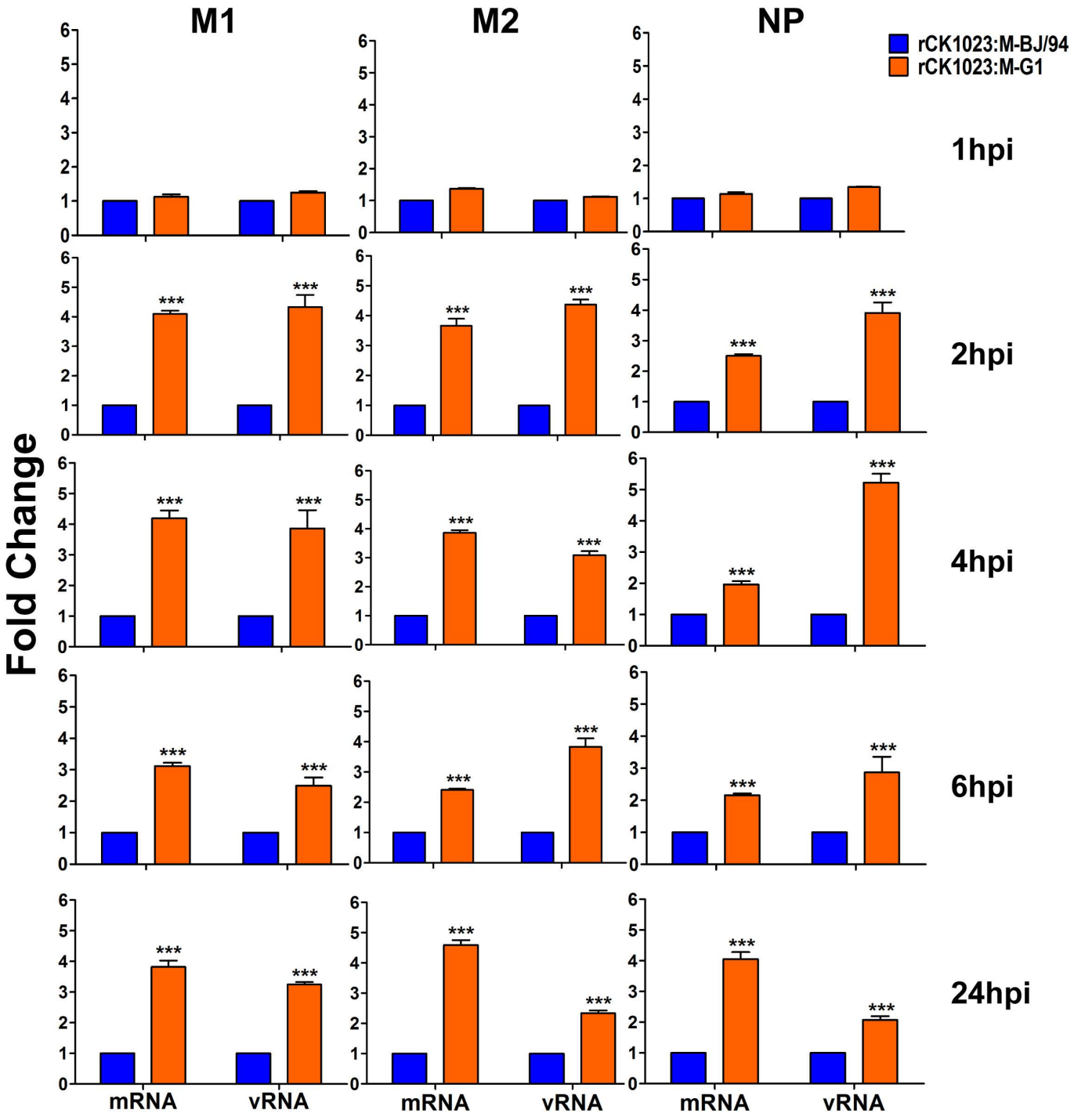
710 **FIG 6** Histological examination of lungs and kidneys from chickens infected with
711 H9N2 virus housing BJ/94-like or G1-like M gene. Representative H&E (A to D) and
712 immunohistochemical (E to H) staining of lung and kidney sections at 3 dpi.
713 rCK1023:M-BJ/94 virus infection caused mild bronchitis and inflammatory changes
714 (A), and no apparent pathological changes in the kidney (C). rCK1023:M-G1 virus
715 infection caused severe interstitial pneumonia and bronchopneumonia (B) and
716 hyperemic renal congestion (D). There was extensive viral NP distribution in the
717 pulmonary tissues with each virus type (E, F). However, only with rCK1023:M-G1
718 virus was viral NP detected in the kidney (G, H). Magnification, A, B, E, F taken at ×
719 100; C, D, at × 200 magnification; G, H at × 400 magnification.
720
721 **FIG 7** Virus titers of rCK1023:M-G1, rCK1023:M-BJ/94 and M gene point mutants
722 of rCK1023:M-BJ/94 H9N2 viruses recovered from chicken trachea (A), and lung and
723 kidney (B). Nine six-week-old SPF White Leghorn chickens per group were
724 inoculated with $10^6 EID_{50}$ of indicated viruses; tracheal swabs from three chickens per
725 group were taken at 1, 3, 5, 7 dpi, and lungs and kidneys were harvested from three
726 chickens per group at 3 and 5 dpi for virus titration. Virus titers are means ± SD.
727 Dashed line indicates the lower limit of detection. Statistical significance was based
728 on two-way ANOVA (*p<0.05, **p<0.01, ***p<0.001).
729
730 **FIG 8** Prevalence of the five distinct amino acid residues encoded by G1-like M
731 genes of chicken H9N2 viruses in China. Shortly after 2004, the G1-like M segment

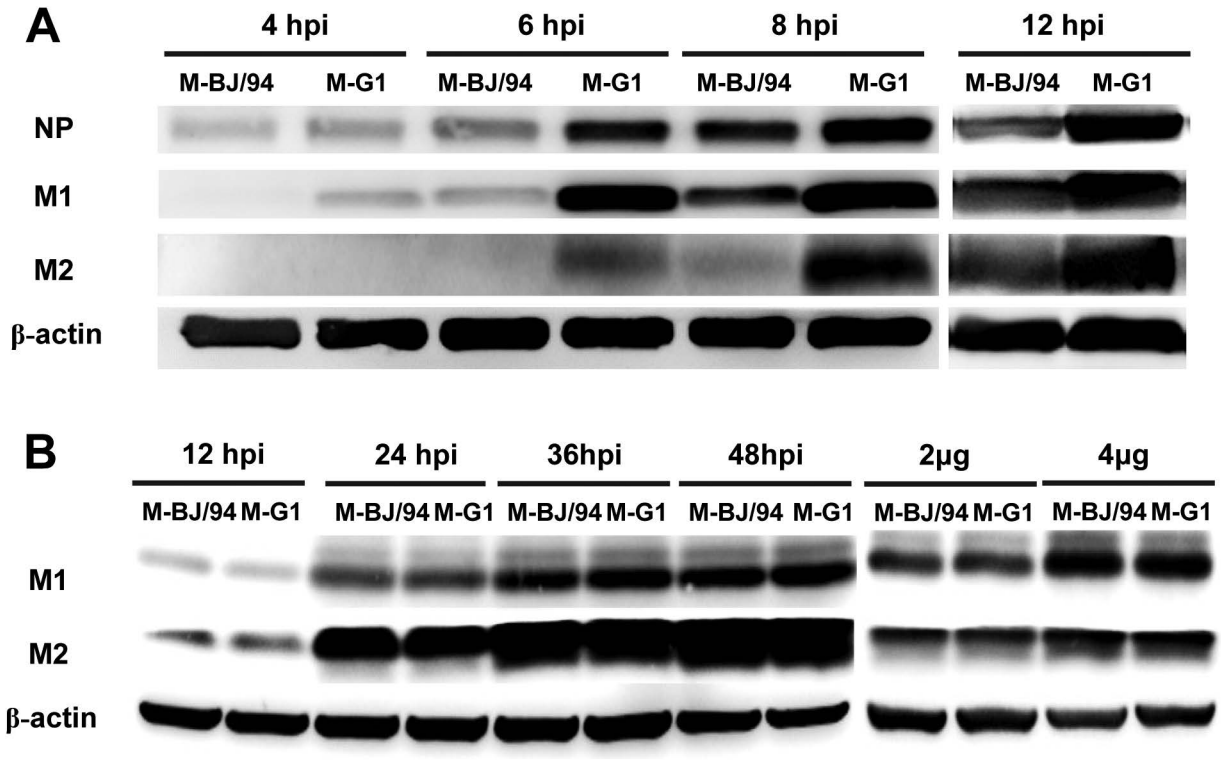
was introduced into and established in the BJ/94-like H9N2 viruses in chickens. G1-like M gene residues M1-37A, M1-95K, M1-224N, M1-242N and M2-21G were first detected in 2007, 2008, 2008, 2006 and 2007 respectively. All M1 and M2 sequences were downloaded from NCBI (www.ncbi.nlm.nih.gov/genomes/FLU). The total number of virus isolates in each year from 2006 to 2015 was 6, 20, 35, 52, 51, 63, 77, 189, 32 and 6 respectively.

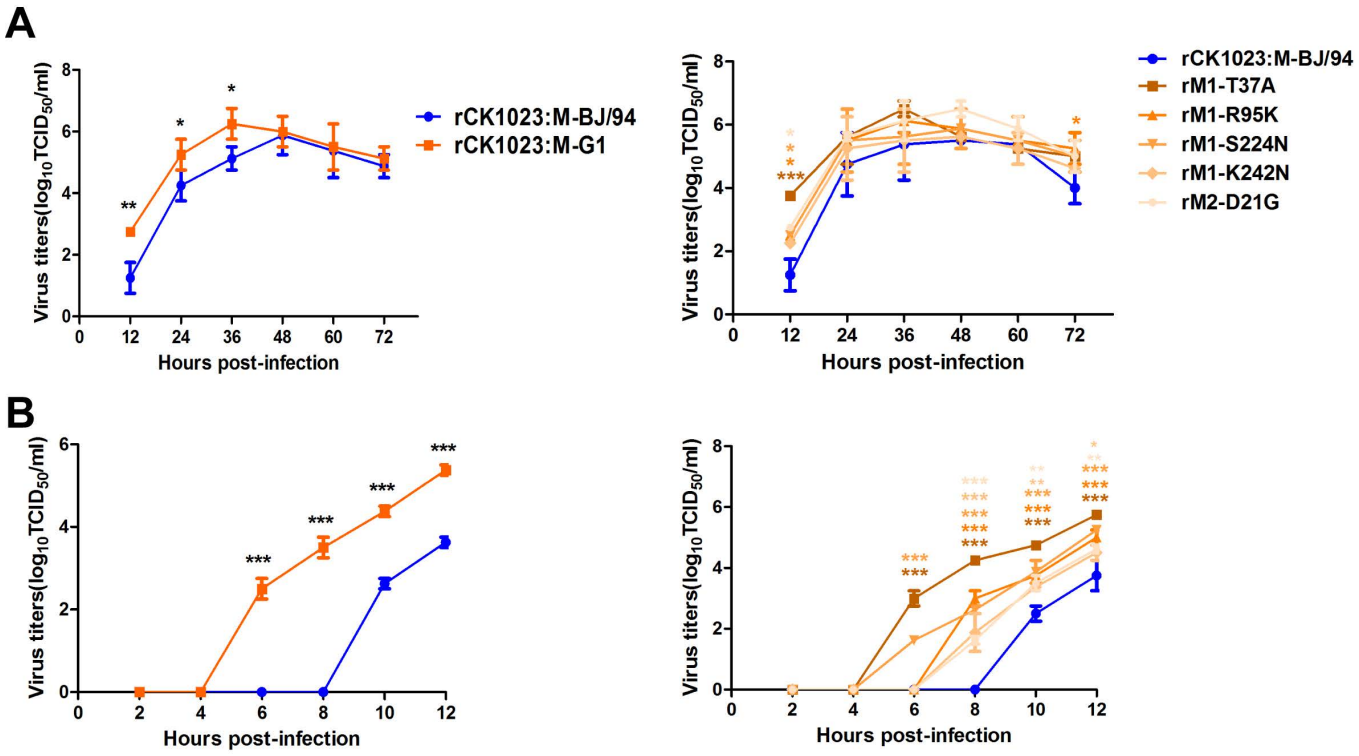
TABLE 1 Highly represented amino acid residues in prevalent G1-like M gene from chicken H9N2 viruses in China

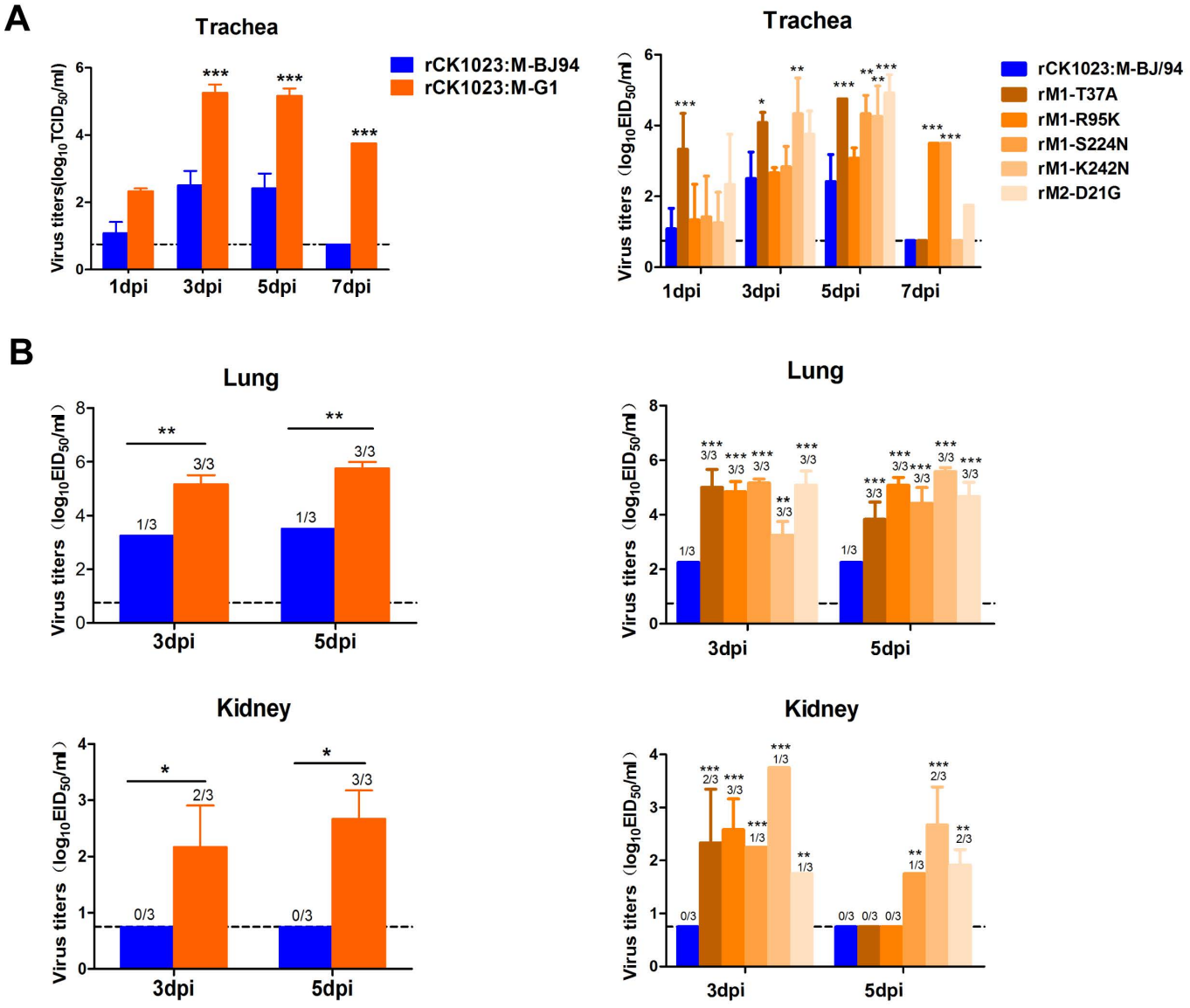
Mutation	Emerging time (year)	Prevalence in chicken H9N2 influenza viruses (%)[a]		
		With G1-like M gene from major group	With G1-like M gene from non-major group	With BJ/94-like M gene
M1-37A	2007	73.54	5.77	1.34
M1-95K	2008	96.25	4.81	0.45
M1-224N	2008	93.44	0.96	0.45
M1-242N	2006	96.02	4.81	0
M2-21G	2007	85.48	2.38	2.23

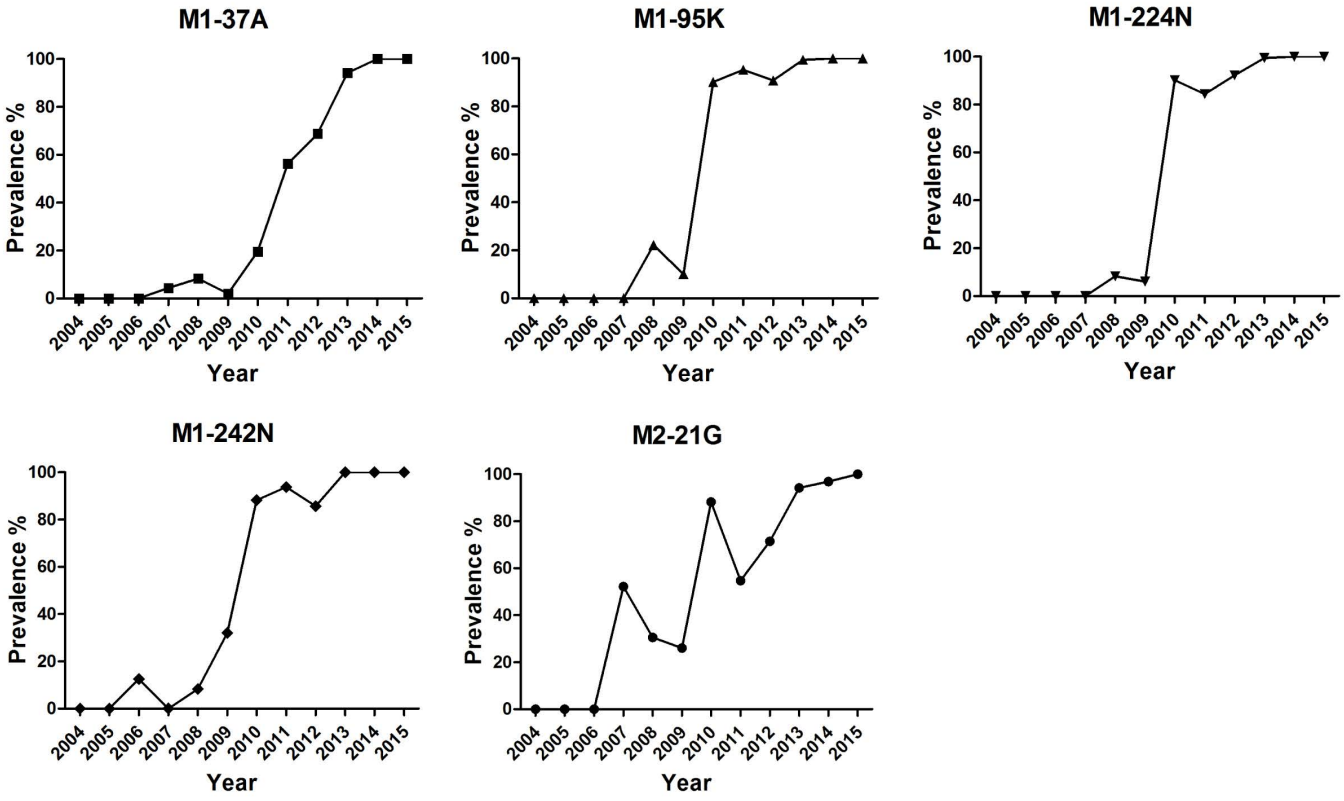
[a]The total number of the viruses either with G1-like M gene from major group, or with G1-like M gene from non-major group, or with BJ/94-like M gene is 427, 104 and 224 respectively.











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