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Characterization of an H4N2 influenza virus from Quails with a multibasic motif in the hemagglutinin cleavage site

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ABSTRACT

The cleavage motif in the hemagglutinin (HA) protein of highly pathogenic H5 and H7 subtypes of avian influenza viruses is characterized by a peptide insertion or a multibasic cleavage site (MBCS). Here, we isolated an H4N2 virus from quails (Quail/CA12) with two additional arginines in the HA cleavage site, PEK**RR**TR/G, forming an MBCS-like motif. Quail/CA12 is a reassortant virus with the HA and neuraminidase (NA) gene most similar to a duck-isolated H4N2 virus, PD/CA06 with a monobasic HA cleavage site. Quail/CA12 required exogenous trypsin for efficient growth in culture and caused no clinical illness in infected chickens. Quail/CA12 had high binding preference for α2,6-linked sialic acids and showed higher replication and transmission ability in chickens and quails than PD/CA06. Although the H4N2 virus remained low pathogenic, these data suggests that the acquisition of MBCS in the field is not restricted to H5 or H7 subtypes.

Introduction

Avian influenza viruses (AIVs) are classified by the World Organization of Animal Health (OIE) (OIE, 2012) into two pathogenicity groups: low-pathogenic avian influenza (LPAI) viruses cause mild or asymptomatic infections in chickens, and highly pathogenic avian influenza (HPAI) viruses cause severe morbidity and mortality. Of the 16 influenza virus subtypes isolated from avian species to date, only some strains of H5 and H7 are classified as HPAI viruses.

The virulence of HPAI viruses has been largely attributed to the presence of additional, often basic amino acids at the hemagglutinin (HA) protein cleavage site. During virus replication, the precursor HA protein (HA_0) is cleaved by proteases into functional subunits HA_1 and HA_2 to allow for fusion with host membranes. LPAI strains possess a monobasic XXR/G cleavage motif and are cleaved by tissue-restricted proteases while, HPAI strains have inserted peptides or at least 4 basic amino acids RX (K/R)R/G (known as multibasic cleavage site) at the cleavage site which enables cleavage by ubiquitous furin-like proteases (Garten and Klenk, 2008; Rott et al., 1995), leading to increased tissue tropism and multiorgan involvement in infected hosts (Horimoto and Kawaoka, 1994; Stieneke-Grober et al., 1992; Steinhauer, 1999). In laboratory, these HPAI viruses do not require exogenous trypsin for *in vitro* growth. Importantly, HPAI strains are believed to emerge when LPAI strains are introduced into domestic poultry and acquire additional amino acids in the HA cleavage site during virus replication.

While MBCS is a prerequisite to a highly pathogenic phenotype, it is not the only contributing factor. Non-H5/H7 subtypes (including H4) influenza viruses can support a MBCS but enhanced pathogenicity also depended on the internal gene constellation (Veits et al., 2012; Munster et al., 2010; Gohrbandt et al., 2011; Schrauwen et al., 2011) However, with the exception of a canine H3N2 virus isolated from in 2009 in China (Sun et al., 2013), non-H5/H7 subtypes with MBCS-like motifs are not isolated in the field.

In August 2012, we isolated an H4N2 virus from a farm that reported a 1.6% mortality in its quail flock. Sequence analyses revealed that A/Quail/California/D113023808/2012 (Quail/CA12) contained a multibasic amino acid motif in the cleavage site of

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HA and was most closely related to a duck-origin H4N2 virus, A/Pekin Duck/California/P30/2006 (PD/CA06), isolated in 2006, with a typical monobasic cleavage motif. As Quail/CA12 contained an atypical cleavage site, we studied its biological features and potential risk to poultry species and humans.

Results

Phylogenetic analyses

BLAST analysis of the HA and NA sequence of Quail/CA12 showed that it had the highest nucleotide identity to the HA (94.4%) and NA (93.9%) of an H4N2 virus isolated from a Pekin duck in California in 2006. However, the Pekin duck H4N2 virus lacked the insertion at the MBCS in the HA. As expected, phylogenetic analyses confirmed that although the HA and NA of Quail/CA12 was still within the same phylogenetic subgroup as PD/CA06, but both viruses formed distinct monophyletic clades (bootstrap value of 100 on the HA and NA branch node) (Fig. 1). The PD/CA06 H4 was grouped with other H4 genes from viruses isolated from the western United States at around the same time, but the Quail/CA12 H4 appeared to be distinct. The N2 gene was less divergent between the two viruses and formed a subgroup that was distinct from other North American N2 genes. Notably, the top 50 BLAST hits for the quail and Pekin duck NA gene in GenBank yielded strains isolated in the 1970s and 1980s, indicating a gap in surveillance data. The constellation of internal genes, however, was different between the viruses (Supplemental Fig. 1). The PD/CA06 contained internal genes that had greatest similarity to those of avian viruses that did not seem to circulate after 2009, whereas Quail/CA12 had internal genes from contemporary avian viruses. This suggests that Quail/CA12 acquired its' HA and NA from
PD/CA06-like virus, but underwent reassortment and acquired the internal genes from currently circulating avian viruses.

The HA of Quail/CA12 shares approximately 95% amino acid sequence identity with PD/CA06. The most notable difference was the acquisition of dibasic arginine (–RR-) residues at positions 345 and 346 (H3 numbering used for all amino acid residues, as per alignment in Fig. 2), giving rise to the cleavage motif PEK**RR**TR/G. This motif arose from a threonine (T) to R substitution at position 345 and a –R insertion at position 346. The insertion did not arise during egg passage, as the sequence of the original swab collected for diagnostic workup had the same atypical cleavage site. A search of all publicly available H4 sequences available at GenBank and the Influenza Research Database ($N=301$ non-identical sequences) showed that this motif was unique to Quail/CA12. Both viruses retained the Q and S at positions 226 and 228, respectively, indicating that they retained the avian-like receptor-binding preference (Yamada et al., 2006). Five potential glycosylation motifs—22NYT, 38NGT, 182NLT, 314NVS and 502 NGS— were conserved, but Quail/CA12 may have an additional glycosylation sequon because of the lysine (K) to T substitution at position 149 (Fig. 2). By mapping the mutations onto the H3 protein crystal structure (Supplemental Fig. 2), we noted that the changes A206G, K213V, and K210N, sit on the 190-helix, which forms part of the receptor binding domain (Yamada et al., 2006; Ha et al., 2003).

In NA, Quail/CA12 shared 94% amino acid identity with PD/CA06 with 12 amino acid differences. Seventeen-amino-acid residues were deleted from position 54 to 70. The stalk deletion was a motif that commonly arises during the adaptation of influenza viruses from aquatic birds to terrestrial poultry (Cilloni et al., 2010; Hossain et al., 2008).

Infectivity, pathogenicity, and transmission in quails and chickens

To determine if Quail/CA12 meets OIE's specification for a highly pathogenic avian influenza virus, we performed the intravenous pathogenicity test (IVPI) in chickens according to OIE's specification (OIE, 2012). Quail/CA12 did not cause overt clinical signs in infected chickens and thus, scored 0, suggesting that despite the presence of MBCS, Quail/CA12 is a low-pathogenic avian influenza virus.

To determine the extent of infectivity and pathogenicity of both viruses in quails, we infected quails and examined the birds for clinical symptoms and viral shedding. Apart from mild depression, none of the quails in either group displayed any overt signs of clinical illness. All six quails infected with Quail/CA12 shed virus for at least 5 days, whereas only three quails in the PD/CA06 group shed the virus (Fig. 3A) for the same amount of time. All shedding occurred via the oropharyngeal route. In the transmission experiment, all four contacts quails infected with Quail/CA12 shed the virus for at least 3 days (Fig. 3B). None of the contact quails in the PD/CA06 group became infected. All directly infected quails (4/4) in the Quail/CA12 group seroconverted, but only 2 of 4 quails in the PD/CA06 group seroconverted (Fig. 3E). None of the contact quails in either group seroconverted.

To assess the severity of infection, infected quails were sacrificed and quail tissues were collected on day 5 post-infection. In the Quail/CA12–infected birds, virus was recovered from the nasal turbinate, esophagus, and brains, while virus was recovered from the nasal turbinate of only PD/CA06-infected bird (Table 1). The brain was also strongly positive for the viral antigen in both quails for the Quail/CA12 group and 1 quail in the PD/CA06 group, although virus was not isolated from the latter (Supplementary Fig. 3).

Because quails have been implicated in facilitating the adaptation of duck viruses to infect chickens, we tested whether Quail/CA12 were more infectious or transmissible than PD/CA06 in chickens in a similar experimental set-up. All chickens in the Quail/CA12–infected group shed virus for longer compared to the PD/CA06 group (Fig. 3C). All chickens shed virus via the oropharyngeal route, except for one chicken in the PD/CA06 group that shed virus via the cloaca on days 5 and 7. One contact chicken in the Quail/CA12–infected group shed virus for 3 days (Fig. 3D), but none of the contact chickens in the PD/CA06 group shed virus. In the infected chickens, Quail/CA12–viruses were recovered from the nasal turbinate and esophagus, while PD/CA06 was only recovered from the trachea of infected birds.

In the Quail/CA12–infected group, all the directly inoculated chickens and 1 contact chicken seroconverted while in the PD/CA06 group, only 3 of 4 directly inoculated chickens but no contact chickens, seroconverted.

In vitro characterization of quail/CA12 and PD/CA06

To determine whether the presence of the MBCS enabled trypsin-independent replication, we compared plaque formation, HA proteolysis, and growth of Quail/CA12 and PD/CA06 in the presence and absence of trypsin. In plaque assay, neither viruses formed plaques in the absence of trypsin (data not shown). Similarly, the precursor HA (HA_0) was cleaved to its subunits HA_1 and HA_2 only in the presence of trypsin (Fig. 4A) for both viruses. In the presence of trypsin, both viruses grew to high titers in the quail cell line, QT-6 but only PD/CA06 replicated efficiently in DEF cells (Fig. 4B) after 72 h. These data suggests that despite the MBCS-like motif, Quail/CA12 still depended on trypsin for efficient growth. No significant differences were observed in the replication kinetics between the two viruses in MDCK, QT-6 and NHBE cells. The chicken fibroblast cell line, DF-1 did not support

efficient replication of both viruses when incubated at 37 °C (Supplemental Fig. 4).

Since some of the amino acid differences between Quail/CA12 and PD/CA06 were observed around the receptor-binding domain, we then tested the sialic-acid binding preferences of Quail/CA12 and PD/CA06 in a solid-phase sialoglycopolymer direct binding assays (Gambaryan et al., 1997). The 2009 pandemic strain A/California/04/2009 (H1N1) (CA/09), which had α2,6-SLN binding preference, was used as a point of comparison in this assay. PD/CA06 showed a higher binding preference for α2,3-SL (Fig. 4C), whereas Quail/CA12 had a higher binding preference for α2,6-SLN, indicating that Quail/CA12 has shown stronger binding specificity toward human like receptor.

We then tested the acid-stability of HA for the two viruses in the hemolysis assay. The Quail/CA12 HA was activated at a relatively high pH of 6.0, whereas the PD/CA06 HA was activated at a pH of 5.2 (Fig. 4D). This suggests that the HA of Quail/CA12 is less acid stable than the HA of PD/CA06.

Since the length of the NA stalk affects viral biological function and virulence (Castrucci and Kawaoka, 1993), we compared the levels of NA activity of Quail/CA12 and PD/CA06. When 2′-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid (MU-NANA) was used as a substrate, there were no differences in the NA activity of the two viruses (data not shown). We then compared the NAs for their abilities to cleave larger membrane-bound substrates in the virus elution assay, using guinea pig RBCs (which contains more α2,6-linked sialic acids) and chicken RBCs (which contains more α2,3-linked sialic acids). PD/CA06 was eluted out of bound guinea pig RBCs within 3 h, whereas some virions remained bound to chicken RBCs even at the end of the 12 h assay period. In contrast, Quail/CA12 was not eluted out of either type of RBCs (Fig. 4E). This result is consistent with previous findings that a short stalk does not affect NA's catalytic ability but negatively affects its ability to access the membrane-bound substrate (Castrucci and Kawaoka, 1993; Mitnaul et al., 2000).

In summary, Quail/CA12 HA has stronger mammalian-like receptor binding preference, is less acid-stable, and its NA does not cleave membrane-bound substrate.

Pathogenicity and transmission in ferrets

Since Quail/CA12 showed mammalian-receptor binding preference, we infected ferrets to assess its infectivity and transmissibility. Quail/CA12 was able to infect the directly inoculated ferrets, which shed virus for up to 5 and 7 days (Fig. 5). Apart from a temperature spike on the day after infection in one of the infected ferrets, no other clinical signs were observed (data not shown). Both ferrets seroconverted by day 21 p.i. None of the contact ferrets shed virus or seroconverted.

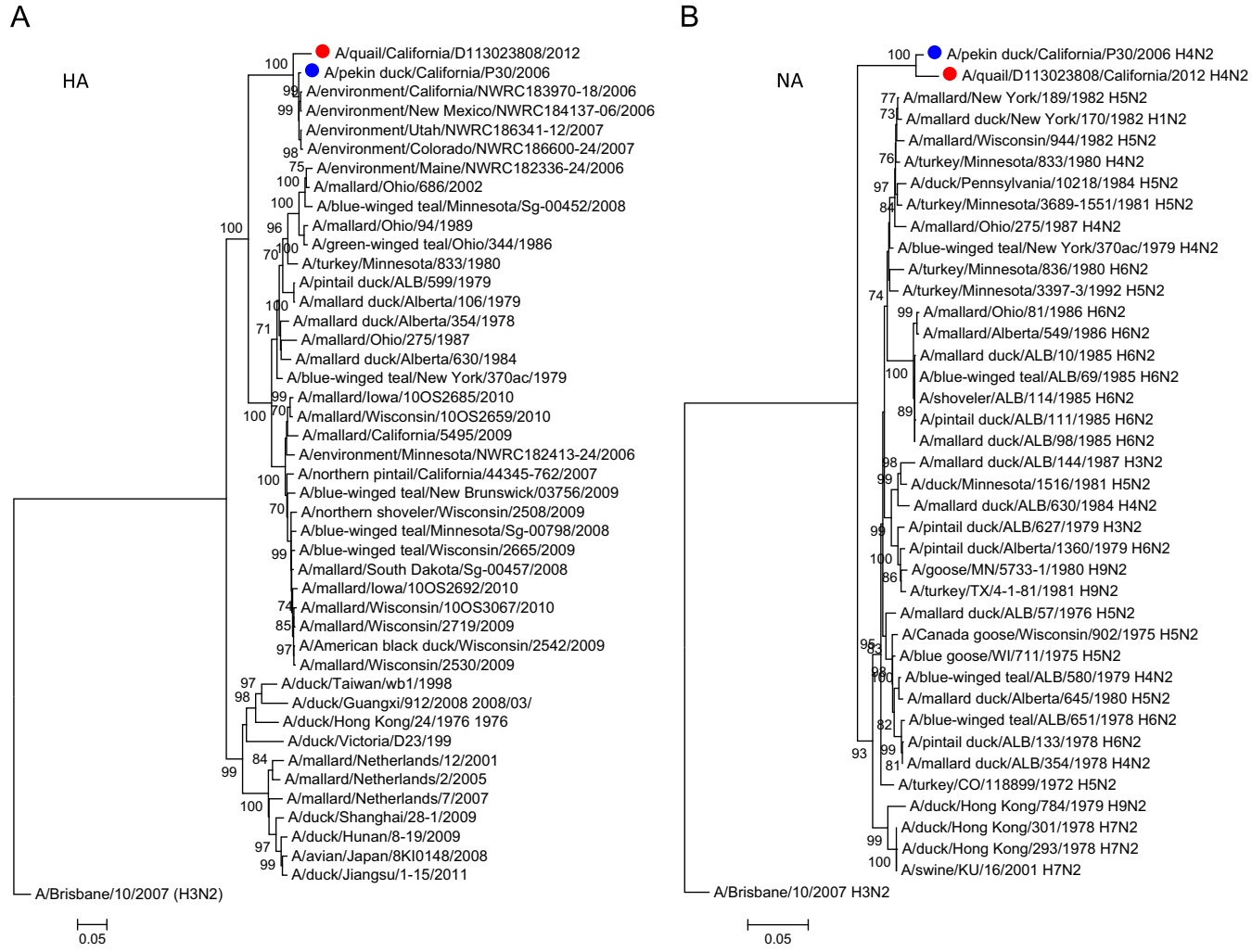

Fig. 1. Origin of A/Quail/California/D113023808/2012 (H4N2) (Quail/CA12). The phylogenetic relationship of the (A) HA and (B) NA genes of Quail/CA12. The tree was constructed by using the neighborhood-joining method with a bootstrap value of 1000. The scale bar indicates the number of nucleotide substitutions.

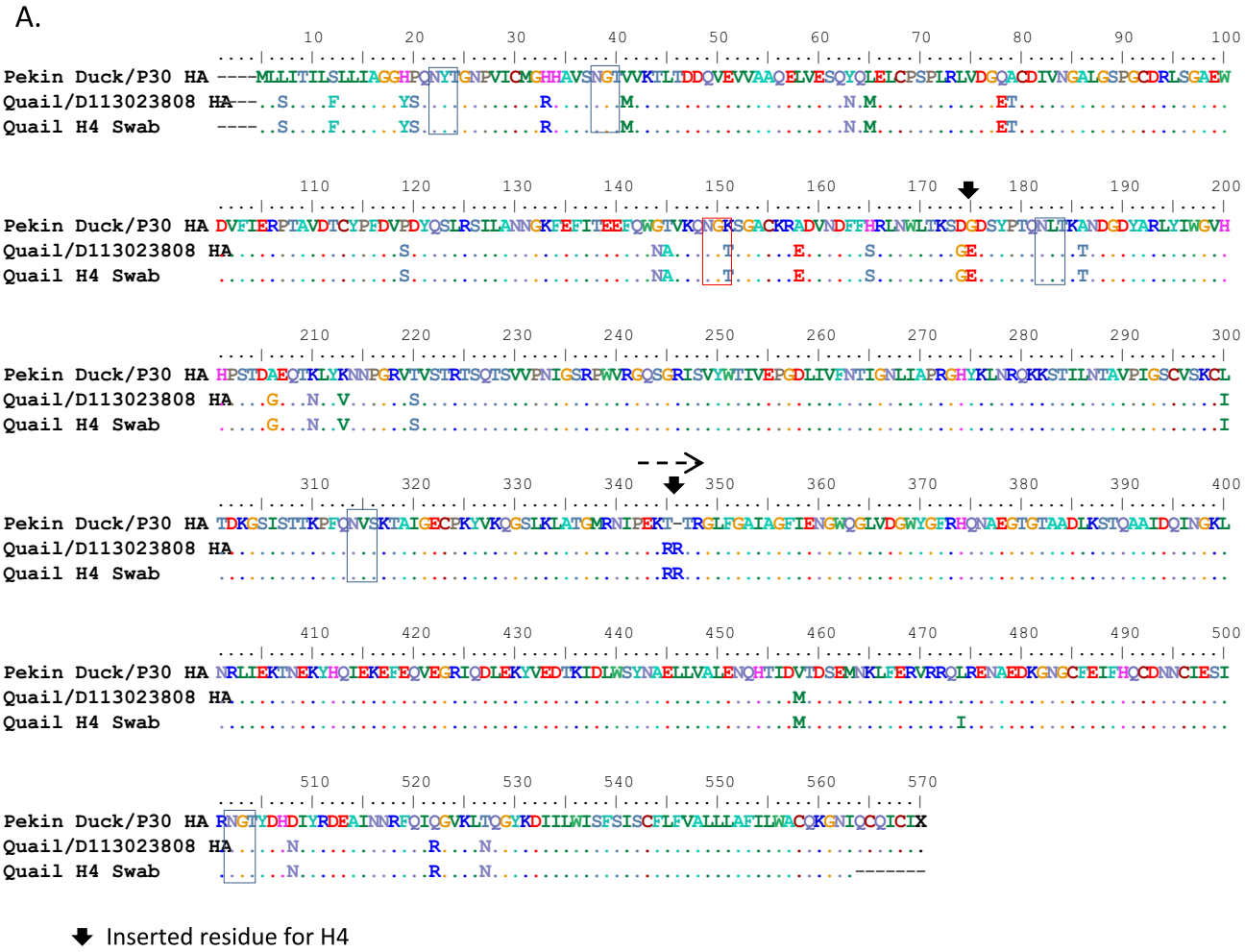

Fig. 2. Sequence comparison of the HA gene of Quail/CA12, PD/CA06, and the original field swab of Quail/CA12. Amino acids are aligned and numbered according to H3 numbering. Conserved putative glycosylation sequons are boxed in blue, and the sequon that was unique to a strain is boxed in red. The black arrow indicates an additional residue for the H4 sequences, and the red arrow indicates the cleavage site.

Discussion

Quails have been postulated to be an alternate "mixing vessel" to swine and several groups have adapted duck-origin viruses in quail to assess their role in ecology of influenza viruses (Cilloni et al., 2010; Hossain et al., 2008; Giannecchini et al., 2010; Yamada et al., 2012; Sorrell and Perez, 2007). In these studies, adapted virus often showed a change in receptor-binding preference and a reduction in NA stalk length, consistent with what we observed in Quail/CA12. The higher binding preference for α2,6-linked sialic acids in the HA of Quail/CA12 likely altered its ability to infect duck-origin cells, and enhanced its ability to infect chickens (Hossain et al., 2008; Ghendon et al., 1984; Gambaryan et al., 2002). Other molecular changes such as the K151T mutation (which results in the introduction of a putative glycosylation sequon in the 130-loop) (Matrosovich et al., 1999; Baigent and McCauley, 2001), and the DG156/157GE and A158E on the top and the edge of the globular head, respectively (Giannecchini et al., 2010; Yamada et al., 2012) were also observed in laboratory -adaptation experiments and could have occurred to compensate for the reduced NA activity due to a shorter stalk (Matrosovich et al., 1999; Baigent and McCauley, 2001; Banks et al., 2001). These molecular changes support the idea that Quail/CA12 evolved from a duck-origin virus and had adapted in the terrestrial avian species. In addition, some of these changes also resulted in antigenic differences. Of the sera collected from experimentally infected animals, we found that most were able to differentiate between the two viruses by HI assay (Supplemental Table 1).

Another notable finding is that Quail/CA12 was recovered from the brain tissues of quails, a feature not typically observed with LPAI infection of avian species. Quail/CA12 infected quails did not show classic neurological symptoms such as torticollis, paralysis and locomotor dysfunction typically observed in HPAI infection in gallinaceous poultry (reviewed in (Frana and Brown, 2014)). Indeed, broader tissue distribution beyond the oropulmonary or gastrointestinal sites is associated with the presence of MBCS in the HA cleavage site (Horimoto and Kawaoka, 1994; Stieneke-Grober et al., 1992; Steinhauer, 1999). One notable exception however, is with the H7N9 virus currently circulating in China that has caused significant mortality in humans. Experimental animal infection with H7N9 showed that this virus replicated in the brains of ferrets, macaques and in quails, but yet not chickens (Watanabe et al., 2013; Zhu et al., 2013), despite the lack of a multibasic cleavage site. Since the brains of PD/CA06-infected quails were also positive for viral antigen, this suggests that neurotropic infections occur more readily in quails with certain LPAI viruses. This aspect of the LPAI pathobiology merits further investigation.

In H5 viruses, enhanced cleavability in viruses with four basic residues at the cleavage site was only observed when accompanied by a loss of glycosylation sites (Kawaoka and Webster, 1989). In Quail/CA12, there are two potential glycosylation sites encoded

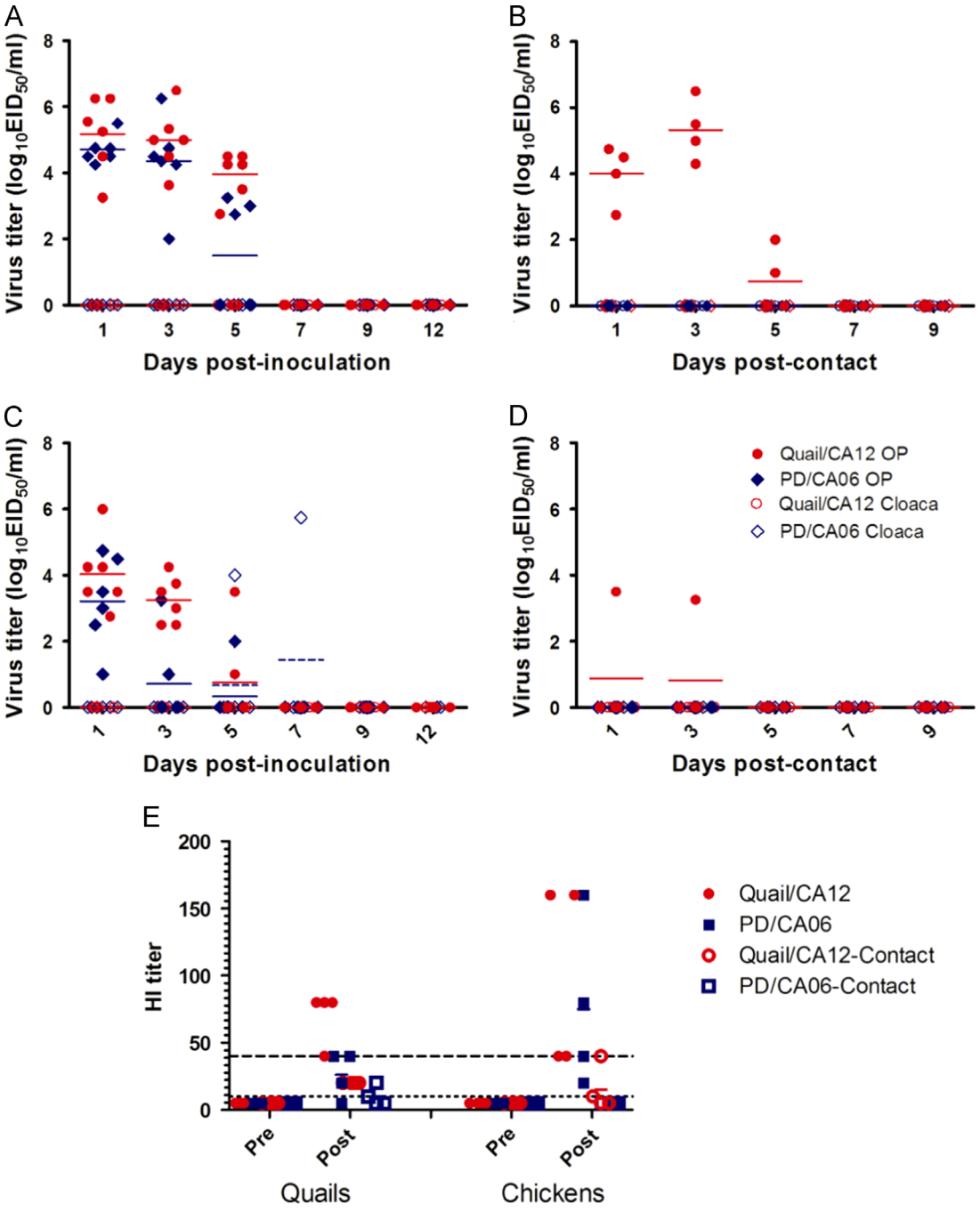

Fig. 3. Infectivity and transmissibility of Quail/CA12 and PD/CA06 in quails and chickens. Viral titers of oral and cloacal swabs collected at different time points post-infection in (A) direct-inoculated quails, (B) contact quails, (C) direct-inoculated chickens, and (D) contact chickens. Panel (E) shows the serum hemagglutination inhibition (HI) titers pre-infection and at day 21 post-inoculation. The lower dashed line indicates the starting sera dilution (1:10), while the higher dashed line indicates the reciprocal titer of 1:40, the minimum-titer required to qualify for seroconversion.

by the sequences 22NYT25 or 38NGT40 that lie near the cleavage site that may block enhanced HA cleavage (Kawaoka and Webster, 1989; Kawaoka and Webster, 1988; Garcia et al., 1996; Walker and Kawaoka, 1993). However, at present, only a single nucleotide substitution (C to A/G) in Quail/CA12 that changes threonine to arginine or lysine is needed to convert the current motif to a five-basic amino acid stretch, which can then be cleaved by furin-like proteases, despite the presence of a glycosylation site (Kawaoka and Webster, 1989). Continued evolution of Quail/CA12 in poultry may lead to acquisition of a highly cleavable HA phenotype or increased pathogenicity as has been experimentally shown in an LPAI H5N2 virus isolated in Taiwan (Cheng et al., 2010). Quail/CA12 was more infectious and transmissible in chickens than PD/CA06, already posing a significant threat to the poultry industry. Although historically non-H5/H7 subtype viruses had not been associated with highly pathogenic phenotypes, modern day poultry farming practices with high animal densities could lead to more opportunities for adaptation of low pathogenic avian viruses into highly pathogenic viruses. The six years interval between the isolation of PD/CA06 and the emergence of Quail/CA12 poses the question of where this lineage of virus had been circulating, and supports the need for a rigorous avian influenza surveillance system. Given the importance of the poultry industry in the US and the associated populations-at-risk, increased vigilance in not

Table 1
Virus titers in tissues of quails and chickens infected with Quail/CA12 and PD/CA06.[a]

Tissue	Virus titer (log EID_{50}/g)			
	Quails		Chickens	
	Quail/CA12	PD/CA06	Quail/CA12	PD/CA06
Nasal turbinate	5.09, 6.93	–, 2.41	–, 2.06	–,–
Lungs	–,–	–,–	–,–	–,–
Trachea	–,–	–,–	–,–	–,–
Liver	–,–	–,–	–,–	–,–
Heart	–,–	–,–	–,–	–,–
Kidney	–,–	–,–	–,–	–,–
Duodenum	–,–	–,–	–,–	–,–
Jejunum	–,–	–,–	–,–	–,–
Colon	–,–	–,–	–,–	–,–
Cecum	–,–	–,–	–,–	–,–
Esophagus	1.41, 3.46	–,–	–, 3.51	–,–
Brain	3.46, 4.25	–,–	–,–	–,–
Blood (log EID_{50}/ml)	–,–	–,–	–,–	–,–

[a] Birds were infected intranasally with 10^6 EID_{50} in a 0.5-mL volume. Two birds per group were euthanized on day 5 post-infection. Individual titers (log EID_{50}/g tissue, except as indicated) are shown, and a dash indicates that no virus was isolated.

only detecting, but in further characterizing influenza viruses in addition to H5 and H7 subtypes from avian populations is advised to curtail potential future threats.

Materials and methods

Sequencing and phylogenetic analyses

Viral RNA was extracted using the RNeasy Viral RNA extraction kit (Qiagen, CA) and reverse-transcribed using Superscript Reverse Transcriptase II (Invitrogen, CA). Each segment was amplified with gene-specific primers using Phusion High Fidelity Polymerase (New England Biolabs, MA) and sequenced by The Sanger method. For phylogenetic analysis, at least 10 strains closest to Quail/CA12 and PD/CA06 identified using the BLAST program (Altschul et al., 1997) and selected reference strains were retrieved from GenBank and aligned using Bioedit version 7.1.11 (Hall, 1999). A phylogenetic tree was generated using Mega 5.1 (Tamura et al., 2011), using the neighbor-joining method with the 2-parameter model. The bootstrapping was set at 1000 iteration, with the threshold set at 70%. For the internal genes, A/Equine/Praque/1/1957 (H7N7) was used to root the tree.

Viruses and cells

Madin–Darby canine kidney (MDCK) cells were grown in minimal essential medium (MEM) supplemented with 10% fetal calf serum, 100U penicillin/streptomycin, 2 μM L-glutamine, and vitamins (Gibco, NY). The quail-derived fibroblast cell line QT6 was grown in Ham's F12K medium (Life Technologies, NY) supplemented with 5% fetal calf serum, 100U penicillin/streptomycin, and 10% tryptose phosphate broth. Chicken-origin DF-1 cells were grown in Dulbecco's MEM that was similarly supplemented. Primary duck embryo fibroblast (DEF) cells were grown in supplemented, antibiotic-free MEM. All cells were grown at 37 °C in a 5% CO_2 environment.

Normalized human bronchial epithelial (NHBE) cells from a healthy male were purchased from Lonza (Walkersville, MD), plated in 0.33 cm^2 Transwell inserts (Corning, NY) and allowed to differentiate in an air–liquid interface environment of 95% air and 5% CO_2.

Passage 1A/Quail/California/D113023808/2012 (Quail/CA12) was received from the University of California, Davis- and egg passage 2A/Pekin Duck/California/P30/2006 (PD/CA06) was received from the University of Minnesota. Both viruses were propagated for an additional passage in embryonated chicken eggs.

Growth kinetics Experiments

MDCK, QT6, and DF-1 cells were infected at a multiplicity of infection (MOI) of 0.01 in duplicate wells in 6-well plates. NHBE cells were infected in triplicate transwell inserts. Cells were washed once before infection and virus was allowed to adsorb for 1 h. After adsorption, cells were washed and incubated with infection media in the presence or absence of exogenous trypsin-TPCK. For growth kinetics experiments, 0.5 μg/mL of trypsin was used for QT-6 and DEF cells and 0.1 μg/mL of trypsin was used for DF-1 cells. At various time points post-infection (p.i.), an aliquot of the culture supernatant was removed and virus titers were determined by the Reed–Muench method (Reed and Muench, 1938).

Western blotting

MDCK cells were infected with Quail/CA12 or PD/CA06 at an MOI of 10 in the absence of trypsin in duplicate wells in a 6-well plate. At 24 h p.i., 2 μg/mL of trypsin-TPCK was added to 1 well and incubated for 30 min. Cells were washed with phosphate-buffered saline (PBS) and then incubated 300 μL of cold RIPA buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, Sigma) for 30 min on ice. Cell lysates were resolved on 4–12% SDS-PAGE gels, transferred onto a polyvinylidene difluoride membrane, and immunoblotted using homologous chicken antisera collected from the animal experiments.

Receptor specificity assay

Virus receptor specificity was determined as previously described (Matrosovich and Gambaryan, 2012). Three biotinylated sialylglycopolymers were used: 3′-sialyllactose (3′SL, Neu5Acα2-3Galβ1-4Glc), 6′-sialyllactose (6′SL, Neu5Acα2-6Galβ1-4Glc), and 6-sialyl-N-acetyllactosamine (6′–SLN, Neu5Acα2-6Galβ1-4GlcNAc) (Sigma). 96-well fetuin-coated (1 μg/well) plates were washed, blocked with PBS containing 1% bovine serum albumin (BSA), and incubated overnight with 32 HA units of influenza viruses at 4 °C. Plates were washed, 100 μL of biotinylated glycans were added and incubated at 4 °C for 2 h. The plates were washed and incubated with 100 μL of horse-radish peroxidase–conjugated streptavidin at 4 °C for 1 h. After a final wash, 50 μL of the tetramethylbenzidine substrate was added and incubated for 10 min at room temperature. The reaction was stopped with 50 μL of 50 mM hydrochloric acid, and absorbance was measured at 450 nm.

Virus elution assay

A 2-fold serially diluted virus stock starting from an HA titer of 1:128 in a volume of 50 μL was incubated with 50 μL of either 0.5% chicken or 1% guinea pig erythrocytes at 4 °C for 1 h in a microtiter plate. The plate was then incubated at 37 °C, and the reduction in HA titer was recorded periodically for up to 12 h. The assay was performed with calcium saline (6.8 mM $CaCl_2$–154 mM NaCl in 20 mM borate buffer, pH 7.2) as a diluent.

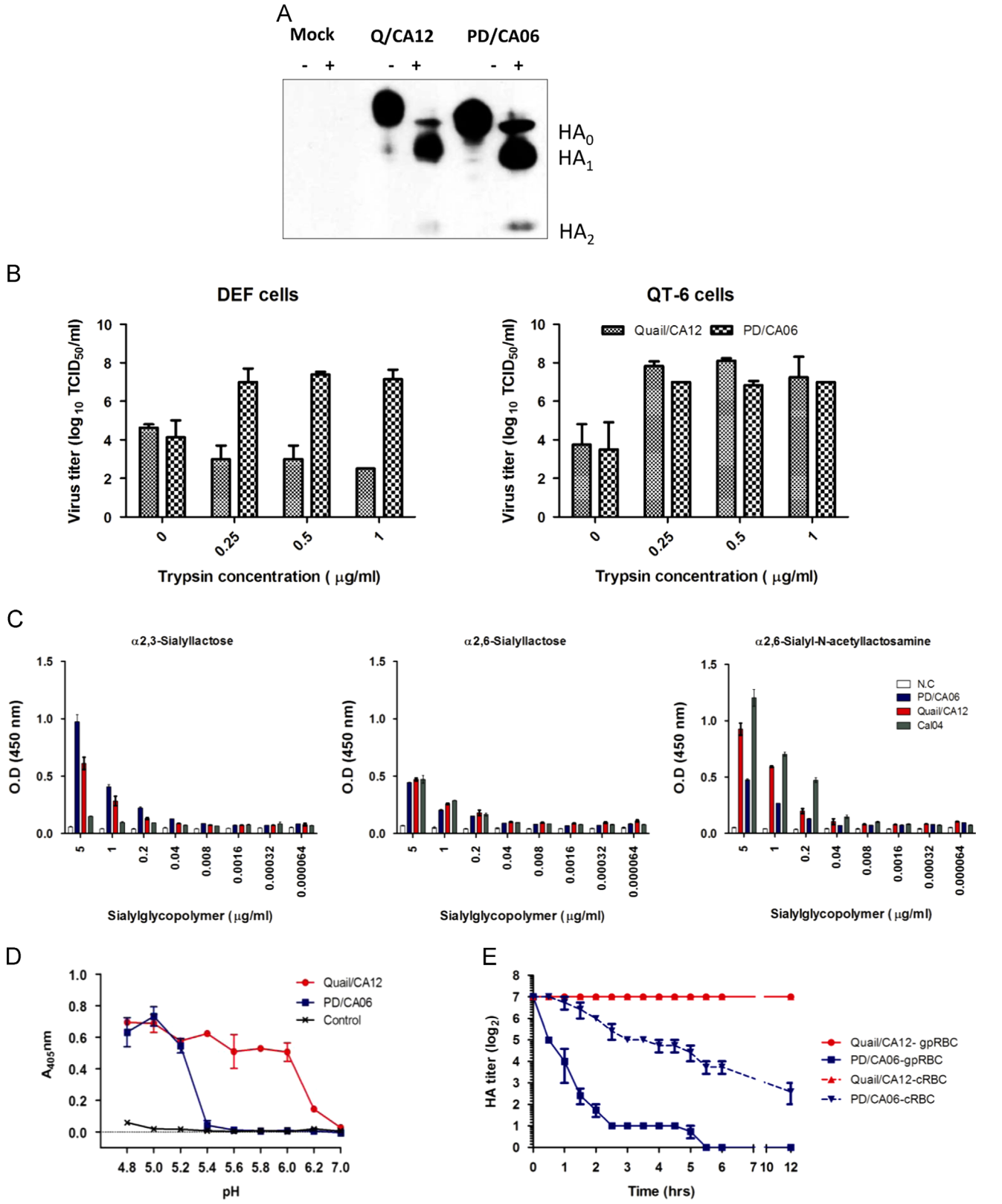

Fig. 4. Biological features of the HA and NA of Quail/CA12 and PD/06. (A) Proteolytic cleavage of HA in the presence (+) and absence (−) of trypsin. (B) Replication in duck embryonic fibroblasts (DEF) (B) and quail-origin QT-6 cells (C) across a range of trypsin concentrations after 72 h. (C) Receptor-binding specificity (A) to α2,3- and α2,6-linked sialic acids was determined in a solid-phase binding assay. N.C denotes negative control. (D) Stability of the HA protein across a range of pH values was assessed by determining the pH at which virus fusion was triggered to cause hemolysis of RBCs. (E) NA activity was determined by a virus elution assay. GpRBC and cRBC indicate hemagglutination performed with guinea pig and chicken RBC, respectively.

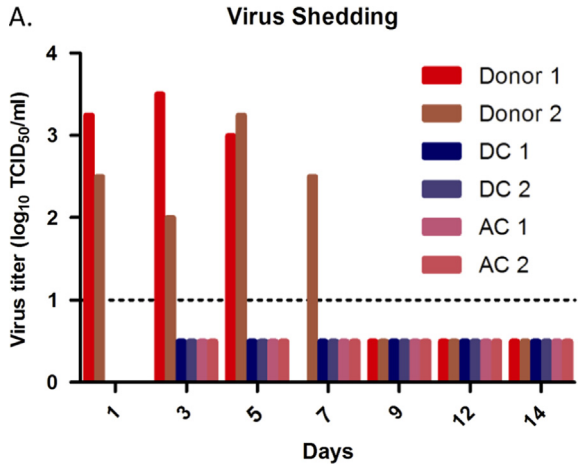

Fig. 5. Infectivity and transmissibility of Quail/CA12 and PD/CA06 in ferrets. (A)Viral titers of nasal washes collected at different time points post-infection in direct-inoculated ferrets (donors), direct-contact ferrets (DC), and airborne-contact ferrets (AC).

Hemolysis assay

Standardized virus stocks (64 HA unit in 50 μL) were diluted in 1% chicken red blood cells (RBCs) at a ratio of 1:4 and incubated on ice for 1 h to allow the binding of virus to cells. Pelleted cells were resuspended in 100 μL of pH-adjusted PBS buffer and incubated at 37 °C for 1 h to allow activation of the HA protein and fusion. Cells were pelleted, and the amount of fusion-induced cell lysis was determined as a function of hemoglobin released in the supernatant by measuring absorbance at 405 nm.

Animal experiments

All animal experiments were carried out in accordance Guide for the Care and Use of Laboratory Animals and were approved by the St. Jude Children's Research Hospital Institutional Animal Care and Use Committee.

Pathogenicity and transmission experiments in quails and chickens

Six-week-old Japanese quails (B&D Game Farm, OK) and ten-week old SPF White Leghorn chickens (CRL Avian Products and Services, MA) were infected with either Quail/CA12 or PD/CA06 ($N=6$ per group) at the dose of 10^6 EID_{50} by the oropharyngeal and ocular route. Tracheal and cloacal swabs were collected on alternate days up to 12-days p.i. Two directly infected quails were euthanized on day 5 and the following tissues were collected for subsequent virus titration, or stored in 10% formalin for immunohistochemical analysis: nasal turbinate, lungs, trachea, liver, heart, kidney, duodenum, jejunum, colon, cecum, esophagus, brain and blood (serum) (serum was not stored in formalin). For immunohistochemistry analysis however, only tissues that were positive or likely to be positive for virus (nasal turbinate, lungs, trachea, esophagus and brains) were processed. For the transmission experiment, 4 quails were placed into contact with the infected quails at day 1 p.i. Sera were collected at days 0 and 21 p.i. from all birds for the hemagglutination inhibition (HI) assay.

Infectivity and transmission experiments in ferrets

Three-month-old influenza-seronegative ferrets were purchased (Triple F Farms, PA) for the infectivity and transmission experiments. Two ferrets were infected intranasally with 1 mL of 10^6 $TCID_{50}$/mL Quail/CA12 under isoflurane sedation. At day 1 p.i., naïve ferrets were placed into the cage of the infected ferrets (total $N=2$) as the direct contact, whereas another two naive ferrets was placed into an adjacent cage as the airborne contact. Nasal washes were collected on alternate days for two weeks and titrated on MDCK cells. Sera were collected at day 0 and day 21 p.i. for the HI assay.

Statistical analyses

Data collected were graphed using GraphPad Prism version 5.03. Data from growth kinetic experiments were statistically analyzed by the analysis of variance with the Bonferroni correction, with $P<0.05$ being considered statistically significant.

Sequence deposition

The full genome of Quail/CA12 has been submitted to Genbank and designated the accession numbers KF986859-KF986866.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.07.048.

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