Infection, Genetics and Evolution 41 (2016) 279–288

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

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

Research paper

A point mutation in the polymerase protein PB2 allows a reassortant H9N2 influenza isolate of wild-bird origin to replicate in human cells

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ARTICLE INFO

Article history:
Received 11 February 2016
Received in revised form 8 April 2016
Accepted 10 April 2016
Available online 14 April 2016

Keywords:
Influenza
H9N2
PB2
Polymorphisms
Viral polymerase

ABSTRACT

H9N2 influenza A viruses are on the list of potentially pandemic subtypes. Therefore, it is important to understand how genomic reassortment and genetic polymorphisms affect phenotypes of H9N2 viruses circulating in the wild bird reservoir. A comparative genetic analysis of North American H9N2 isolates of wild bird origin identified a naturally occurring reassortant virus containing gene segments derived from both North American and Eurasian lineage ancestors. The PB2 segment of this virus encodes 10 amino acid changes that distinguish it from other H9 strains circulating in North America. G590S, one of the 10 amino acid substitutions observed, was present in ~12% of H9 viruses worldwide. This mutation combined with R591 has been reported as a marker of pathogenicity for human pandemic 2009 H1N1 viruses. Screening by polymerase reporter assay of all the natural polymorphisms at these two positions identified G590/K591 and S590/K591 as the most active, with the highest polymerase activity recorded for the SK polymorphism. Rescued viruses containing these two polymorphic combinations replicated more efficiently in MDCK cells and they were the only ones tested that were capable of establishing productive infection in NHBE cells. A global analysis of all PB2 sequences identified the K591 signature in six viral HA/NA subtypes isolated from several hosts in seven geographic locations. Interestingly, introducing the K591 mutation into the PB2 of a human-adapted H3N2 virus did not affect its polymerase activity. Our findings demonstrate that a single point mutation in the PB2 of a low pathogenic H9N2 isolate could have a significant effect on viral phenotype and increase its propensity to infect mammals. However, this effect is not universal, warranting caution in interpreting point mutations without considering protein sequence context.

1. Introduction

Influenza A viruses (IAVs) have caused several pandemics and continue to pose significant threats to human public health (Horimoto and Kawaoka, 2005; Fineberg, 2014). The natural reservoirs of IAVs are wild birds, in which IAVs establish mostly asymptomatic infections in the gastrointestinal tract (Boyce et al., 2009). Occasionally, IAVs infect domestic birds or cross the species barrier to infect mammals including humans, with manifestations that may vary from mild to fatal respiratory illness (Runstadler et al., 2013). Surveillance of IAVs in wild birds has increased substantially following the spillover of the H5N1 subtype to humans resulting in mortalities. Generally, the goals of influenza surveillance in birds and mammalian species are twofold: to increase understanding of the origin of novel epidemic strains and to identify those strains before they become a risk for human health. In the past 10 years, data gathered from surveillance efforts worldwide have aided in identifying the evolutionary origins of zoonotic subtypes, including pandemic 2009 H1N1, H7N9 and H10N8 viruses (Smith et al., 2009; Lam et al., 2013; Wu et al., 2013; Qi et al., 2014). Yet, little work has explored the value of avian surveillance in *a priori* identification of genomic segments derived from wild bird viruses that could potentially pose threat to humans.

Searching for influenza strains carrying unusual genetic features or markers of mammalian adaptation is a common practice for identifying potentially pandemic viruses. We sought to test the value of this approach by examining and assessing a group of wild bird-origin H9N2 isolates, a subtype of current world health concern due to it's endemic status in Chinese poultry markets and propensity to sporadically infect humans (Fusaro et al., 2011; Zhou et al., 2012; Monne et al., 2013). Some H9N2 strains circulating in Asian poultry have acquired HA receptor-binding site mutations that enable them to bind more

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http://dx.doi.org/10.1016/j.meegid.2016.04.011
1567-1348/

efficiently to human α-2,6 sialylated glycan receptors(Matrosovich et al., 2001; Li et al., 2014). Other H9N2 strains isolated from different avian species were transmissible to direct contacts in the ferret model (Wan et al., 2008). In addition, an engineered recombinant virus carrying the surface proteins of an avian H9N2 and the internal segments of a human H3N2 virus was able to transmit efficiently following adaptation in the ferret model *via* respiratory droplets (Sorrell et al., 2009). These experimental results coupled with sporadic human infections placed H9N2 viruses among the list of potentially pandemic subtypes (Peiris et al., 1999; Lin et al., 2000; Alexander et al., 2009). Furthermore, a recent global analysis of the ecosystem interactions between H9 viruses showed frequent two-way transmission between wild and domestic birds and highlighted a role for wild birds in the long-distance dispersal of these viruses between different geographic locations (Ramey et al., 2015; Bahl et al., 2016). Therefore, understanding correlations between genetic and phenotypic diversity among H9 viruses circulating in wild birds is an important component of pandemic preparedness efforts and warrants a closer examination of strains identified by current surveillance efforts.

Little is known about how frequently reassortment and genetic polymorphisms alter viral phenotype and drive the evolution of low pathogenic H9 viruses in their wild bird reservoirs, despite the fundamental role in diversifying influenza virus strains (Dugan et al., 2008; Lu et al., 2014). Surveillance efforts in North America have so far yielded 36 fully sequenced H9N2 genomes, which is a relatively small number compared to other subtypes such as H3 and H4. Six of these fully sequenced H9N2 viruses were isolated in Alaska, an important breeding grounds that attracts millions of migratory birds each year, a scenario that facilitates the mixing of IAV from American and Eurasian lineages of virus and generates interhemisperhic reassortants (Ramey et al., 2010; Winker and Gibson, 2010; Pearce et al., 2011). We identified a wild Alaskan H9N2 isolate (A/northern shoveler/Interior Alaska/8BM3470/2008, henceforth AK08) with a large number of polymorphisms distinguishing it from other H9N2 viruses circulating in North American wild birds. It also showed evidence of multiple reassortment events involving both surface and internal proteins, worthy of further genotypic and phenotypic characterization.

The viral polymerase complex, which is encoded by the PB2, PB1, PA and NP segments, plays an important role in the adaptation of IAVs to mammalian hosts (Boivin et al., 2010), and served as the focal point of this investigation. E627K and D701N are among the most well characterized PB2 adaptive mutations, and are widely considered as important molecular markers for pathogenicity (Subbarao et al., 1993; Hatta et al., 2001; Li et al., 2005; de Jong et al., 2006; Gao et al., 2009; Russell et al., 2012). However, some highly pathogenic H5N1 and almost all pandemic 2009 H1N1 [A(H1N1)pdm09] viruses did not possess either of these two PB2 markers; and introducing them into genetically engineered viruses did not impact the overall viral phenotype (Garten et al., 2009; Herfst et al., 2010; Jagger et al., 2010) suggesting additional unexplored complexity in the PB2 segment. In this study, we investigated the evolutionary history of the AK08 virus and examined the effects of reassortment and PB2 polymorphisms on the *in vitro* polymerase activity and viral phenotype. Our findings suggest that mutations in the PB2 segment of this wild-bird origin H9N2 isolate strongly impacts viral phenotype. This work demonstrates the potential value of targeted investigation of unusual low-pathogenic isolates discovered through routine surveillance, the complexity of interpreting viral phenotype, and proposes improved methods for the high-throughput analysis of surveillance isolates from the wild bird reservoir that may be informative for understanding viral pathogenesis.

2. Methods

2.1. Phylogenetic analysis and data mining

All sequences of Influenza A H9 virus from avian hosts collected globally were downloaded from the Influenza Research Database (IRD; www.fludb.org) on October 30th, 2014, aligned using MUSCLE and any incomplete and low-quality sequences were removed. Using PAUP, neighbor-joining trees were generated using the HKY85 substitution model and taxa that formed outgroups were randomly downsampled. The final number of sequences included in each dataset included: PB2 (n = 359), PB1 (n = 358), PA (n = 309), H9 (n = 295), NP (n = 321), N2 (n = 249), M (n = 260), NS (n = 270). Bayesian phylogenetic trees for each of the 8 segments of the AK08 virus were constructed using BEAST v1.8.0 (Drummond and Rambaut, 2007), with the time of most recent common ancestor (TMRCA) inferred. At least 3 independent Bayesian Markov Chain Monte Carlo (MCMC) chains of 50 million generations each were run and sampled, under the Bayesian skyline coalescent model and the lognormal relaxed time clock, to produce 10,000 trees. The quality of BEAST runs was checked in Tracer (http://beast.bio.ed.ac.uk/Tracer). After removing about 10–30% burn-in from each chain, we combined trees, and constructed maximum clade credibility trees.

PB2 sequences were downloaded from the IRD on November 30, 2014. All subtypes, hosts and countries were considered. Sequences were filtered such that only those that were 759 amino acids long (full consensus length) were analyzed, comprising 23,917 out of 23,975 downloaded. Custom scripts were written in the Python programming language to identify the geographic distribution of the Q591K mutation, and are archived on Zenodo (DOI: 10.5281/zenodo.33746).

2.2. Host ecology & distribution

We used wild bird-origin viruses that shared phylogenetic ancestry with the AK08 virus to identify host species relevant to the transmission and circulation of the virus. The plausibility of transmission between host species based on their seasonal distribution in North America was assessed using bird-banding data. We obtained all available recovery/recapture data of banded birds (1951–2015) for the relevant wild bird species from the U.S. Geological Survey Bird Banding Laboratory (3 January 2015). The spatial overlap between species was quantified for each state/province in North America using QGIS 2.0 (Open Source Geospatial Foundation Project: http://qgis.osgeo.org).

2.3. Cells and viruses

Human embryonic kidney (293T) cells (ATCC CRL-3216) and Madin-Darby canine kidney (MDCK) cells (ATCC CCL-34) were maintained in DMEM containing 10% FBS and penicillin/streptomycin at a final concentration of 50 IU/ml penicillin and 50 μg/ml streptomycin. Normal human bronchial/tracheal epithelial (NHBE) cells (Lonza) were supplemented with the BEGM BulletKit for optimal growth according to the manufacturer instructions. AK08 and A/northern shoveler/Interior Alaska/10BM16764R0/2010 (henceforth AK10) are 2 H9N2 viruses isolated at Minto Flats, a wetland system in the boreal forest region of Interior Alaska that supports high densities of breeding and migrating waterfowl. Full-genome sequencing of these 2 viruses was carried out by the J. Craig Venter Institute. Reverse genetics techniques were used to rescue: i) wild-type AK08, ii) AK08 virus carrying the PB2 segment of the AK10 virus and iii) four other mutants (SR, GR, SK and GK) of the AK08 virus containing single or double amino acid substitutions at positions 590 and 591 of the PB2 gene. A/Victoria/361/2011(H3N2) virus was obtained from BEI Resources (www.beiresources.org).

2.4. Plasmid constructs and mutagenesis

All PCR amplifications were conducted using Phusion High_Fidelity DNA polymerase (NEB) according to the manufacturer's instructions. Gaussia luciferase (Gluc) was used as a marker for our minigenome polymerase assays. Gluc was amplified by PCR from a pGLuc-Basic vector (NEB) template using primers carrying the 3′ and 5′ UTR sequences of the NA segment and BsmBI restriction sites (Table S1). The PCR product

was digested with BsmBI, and cloned in the minus-sense orientation into the pHH21 vector (obtained from Yoshihiro Kawaoka, University of Wisconsin-Madison), which encodes the human RNA polymerase I promoter and the mouse RNA polymerase I terminator, flanked by BsmBI sites (Neumann et al., 1999). The resulting recombinant plasmid (pHH21-Gluc) was verified by sequencing and tested for its ability to express luciferase using a set of pCAGGS plasmids encoding the polymerase complex genes of the A/WSN/1/33 virus (also obtained from Yoshihiro Kawaoka). In order to investigate the effect of point mutations on the *in vitro* polymerase activity of the AK08 virus in mammalian cells, we constructed a set of mammalian expression vectors encoding the polymerase complex genes. Viral RNA was extracted from AK08, AK10 and Victoria/2011 viruses using QIAmp Viral RNA Mini kit (Qiagen). The PB2, PB1, PA and NP ORFs of AK08 and AK10 viruses were amplified from viral cDNA using primers carrying restriction enzyme recognition sequences at their 5′ ends (Table S1) inserted into the multiple cloning site of a pCI mammalian expression vector (Promega) using standard molecular cloning techniques (Sambrook and Russell, 2001). For the Victoria/2011 wild type and mutant polymerase segments, the Circular Polymerase Extension Cloning (CPEC) assembly method was used (Quan and Tian, 2009). Briefly, plasmid backbone and ORFs were PCR-amplified with overlapping 40-mer primers. The plasmid backbone and ORF fragments were DpnI-digested and then gel purified and assembled in a secondary PCR reaction, with the overhanging regions priming the extension of its neighboring parts. Assembly products were purified and then chemically transformed into DH5α cells. The identity of all resulting recombinant plasmids was verified by restriction digestion and Sanger sequencing (Genewiz). Maxipreps of all plasmid DNAs were obtained using the Plasmid Maxi Kit (Omega Bio-Tek). To rescue wild-type and mutant viruses by reverse genetics, PCR-amplified full genomic segments were digested with LguI restriction enzyme (Thermo Scientific) and cloned into the ambisense pDZ vector (obtained from Adolfo Garcia-Sastre, Icahn School of Medicine at Mount Sinai) (Martinez-Sobrido and Garcia-Sastre, 2010). The PB2, PB1, PA, HA and NA segments were amplified using combinations of universal and internal primers, while the NP, M and NS segments were amplified using a series of segment-specific primers (Table S1). The resulting 8-plasmid system was subjected to sequencing to verify that no spurious mutations were introduced during PCR. All site-directed mutagenesis was carried out by the overlap extension PCR method (Sambrook and Russell, 2001).

2.5. Gaussia luciferase polymerase assay

The *in vitro* polymerase activity was assessed using the minigenome gaussia luciferase assay system as previously described (Zhu et al., 2011). Briefly, the luciferase marker plasmid pHH21-Gluc and the four plasmids encoding the polymerase complex genes were co-transfected (0.5 μg each) into duplicate wells of confluent 293T cell monolayers grown in 12-well plates using Lipofectamine (Lifetechnologies) according to the manufacturer's instructions. Negative control wells were included in each experiment, where the pHH21-Gluc vector was transfected by itself without any polymerase segments, and these background values were subtracted. Forty-eight hours post-transfection, supernatants were collected and the luciferase signal was detected using a BioLux Gaussia Luciferase Assay Kit (New England Biolabs) on a Varioskan plate reader.

2.6. Virus rescue

Wild type and mutant viruses carrying point mutations in the PB2 gene were rescued from the 8-plasmid reverse genetics system as previously described (Martinez-Sobrido and Garcia-Sastre, 2010). Briefly, 8 plasmids encoding the full genomic segments of the desired viruses were co-transfected (1 μg each) into 293T cells. Sixteen hours post-transfection, transfected cells were scraped off in PBS and inoculated into the allantoic sacs of 10-day old embryonated chicken eggs. The allantoic fluids were harvested 72 h post-inoculation, tested by HA assay, aliquoted and stored at −80 °C for downstream experiments. The research being reported in this manuscript is based on work that was completed prior to the U.S. government Gain-of-Function deliberative process and research funding pause on selected Gain-of-Function research involving Influenza, MERS, and SARS viruses.

2.7. Viral growth kinetics

Twelve-well plates were seeded with MDCK or NHBE cells and allowed to grow until confluent monolayers were obtained. On the day of the experiment, one monolayer from each cell type was trypsinized in 0.25% Trypsin–EDTA and counted in a hemocytometer. Titrated viral stocks were diluted and used to infect cells in duplicate at a multiplicity of infection (MOI) of 0.1. Briefly, viruses were allowed to adsorb for 60 min, then the virus inoculum were aspirated and cells were washed once with sterile PBS. Supernatants were collected at 12, 24, 48 and 72 h post-infection and titrated by plaque assay on fresh MDCK monolayers.

3. Results

3.1. AK08 is a reassortant virus with intercontinental origins

Protein sequence alignments identified the AK08 virus with 10 PB2 amino acid signatures that were detected at relatively low frequency compared to the majority of H9N2 viruses (Table 1). We examined the role of reassortment in the evolutionary history of the AK08 virus. Using BEAST phylogenetic reconstruction (Figs. S1–S8), we observed that 4 segments (PB2, NP, NA and M) of 4 viruses: AK08, A/white fronted goose/Korea/20 36/2007(H9N2), A/snow goose/Montana/466771-4/2006(H5N2) and A/snow goose Quebec/1416/2006(H9N2) always clustered together in the North American lineage, suggesting that they shared common ancestry (Fig. 1C). Frequent reassortment of these genes among viruses circulating in North America is typical of the wild bird reservoir (Dugan et al., 2008; Bahl et al., 2013). Phylogenetic reconstruction of the PB2 gene segment from a representative dataset revealed three co-circulating lineages (indicated as I, II, and III in Fig. 1A). Isolate AK08 shared ancestry with other lineage III isolates (Fig. 1C). Among all isolates (globally distributed), the PB2 mutations found in the AK08 virus were rare. However, ~90% of lineage III have a S590 mutation. This lineage is primarily found in AIV viruses circulating in North American wild birds. In contrast, the S590 mutation was observed in ~4% of isolates belonging to lineage I and II. The remaining nine mutations were found in 10% or fewer isolates in Lineage III. Examining the tree topologies suggested multiple reassortment events that involved recent ancestors of the AK08, A/snow goose/Montana/466,771 4/2008(H5N2) and A/snow goose/Quebec/1416/2006(H9N2) viruses, possibly resulting in acquisition of the AK08 PB2, NP, NA and M segments from an H5N2 virus.

The PB1 and PA segments appear to be typical of North American isolates, with ancestors circulating in the dabbling ducks of the Pacific Northwest region since early the 1950s (Figs. S2 and S3). The NS segment belongs to the allele B group, which is commonly in avian viruses (Marc, 2014). Clustering of viruses isolated from China & Korea within this North American clade provided evidence of at least 3 recent incursions of North American H9 viruses into Eurasia. The AK08 and A/white fronted goose/Korea/20-36/2007 viruses were sister taxa on the PB2, HA, NP, and M trees implicating this lineage in North America-to-Eurasia viral flow *circa* 2004. The same pattern of interhemispheric viral flow was evident for A/egret/Hunan/1/2012 virus (Wang et al., 2012), which clustered with the HA and NA segments of the AK08 virus. Additionally, an earlier interhemispheric movement of virus originating from North America in 2002 was detected for the A/wild bird/Korea/8G39/2005(H9N2) virus.

Table 1
Numberings of the 10 amino acid positions (top row) where polymorphisms of the AK08 PB2 were detected. Under each position, the various polymorphisms (left column) and their global frequencies % (right column) across all H9N2 strains are shown. The asterix (*) denotes polymorphisms tested in this study. Amino acid residues present in the AK08 PB2 are shown in bold.

106		107		147*		274		390		473		508		575		590*		679	
T	92.5	S	98.3	I	77.3	A	99.6	D	99.6	M	95.6	R	84.2	M	87.8	G	86.9	P	98.6
A	**6.8**	N	1.3	V	15.8	**T**	**0.2**	**N**	**0.2**	V	2.2	**Q**	**9.1**	L	6	**S**	**12.2**	Q	0.9
S	0.5	**D**	**0.3**	**T**	**3.8**	V	0.2	E	0.1	**I**	**1.2**	L	4.7	**V**	**5.5**	C	0.8	**L**	**0.3**
K	0.1	I	0.1	M	3.1			H	0.1	T	0.7	K	1.1	I	0.7	R	0.1	R	0.1
N	0.1									L	0.2	M	0.9					S	0.1
										Q	0.1								

3.2. Polymorphisms at positions 590 and 591 of PB2 modulate H9N2 polymerase activity

The AK08 HA (accession # CY079646) was typical of low pathogenic strains, with no polybasic insertion at the cleavage site. No HA mutations, such as Q226L, that were reported to enhance the binding of H9 viruses to human α-2,6 sialylated glycans were detected (Wan and Perez, 2007), nor were mutations present, such as E627K and D701N, commonly reported in the literature to be associated with mammalian adaptation (Gabriel et al., 2013). However, among the 10 unique PB2 substitutions of AK08 virus, we observed a G590S substitution, which is present in about 12% of H9N2 isolates. This G590S substitution constitutes half of the SR polymorphism (S590/R591) signature reported as a marker of pathogenicity for A(H1N1)pdm09 viruses. Thus, in order to examine whether PB2 segment acquisition *via* reassortment had any impact on AK08 viral polymerase function, we generated a reporter construct encoding the gaussia luciferase gene (in the negative sense orientation and flanked by viral UTRs) and a 4-plasmid system expressing the polymerase protein complex of the reassortant AK08 virus. Additionally, we constructed another contemporary H9N2 viral polymerase (strain AK10) that did not show any genetic evidence of reassortment and had a PB2 segment that matched the most common North American H9N2 isolates. The AK08 viral polymerase was 1.5 fold more active than that of the AK10 virus (Fig. 2A). This suggested that the acquisition of the PB2 segment from an H5N2 virus, as inferred by the BEAST tree (Fig. 1C), had minimal effects on AK08 viral polymerase function.

In an effort to understand how minor genetic changes could exert substantial phenotypic effects, we investigated the effect of individual AK08 PB2 polymorphisms on polymerase activity. We tested both natural polymorphisms and additional mutations that could arise by simple genetic drift and with functional significance based on prior studies. Out of the 10 amino acid substitutions found in AK08 PB2, only 2 positions have been previously linked to polymerase phenotypic changes: 147 and 591. In contrast to the majority of H9N2 viruses, which have isoleucine (I) at position 147, the AK08 virus has threonine (T). Other polymorphisms reported in nature at position 147 are valine (V) and methionine (M). We made four mutants of the AK10 PB2: I147T, I147V, I147M and I147L. None of them exhibited a marked increase in polymerase activity when compared to the wild type I147 (Fig. 2B).

We then investigated the effects of amino acid polymorphisms at PB2 positions 590 and 591 on polymerase activity. AK08 PB2 had a serine residue at position 590, which is found in about 12% of H9 viral isolates worldwide (Table 1). Global sequence analysis identified the following polymorphisms in PB2 at positions 590 and 591: GQ (most frequently detected signature, also found in AK10), SQ (found in the AK08 virus), CQ, GK, GL, RQ, SK and SL. In addition to the aforementioned natural polymorphisms (Fig. 2C and D), we also engineered 2 additional mutants (GR and SR) to dissect the effects of a basic residue at position 591, which has been reported before to enhance polymerase activity of an H5N1 virus (Yamada et al., 2010). The 4 PB2 mutants having an arginine (R) at position 591 (AK08-S590/Q591R, AK08-S590G/Q591R, AK10-G590/Q591R and AK10-G590S/Q591R) exhibited a 3-fold increase in polymerase activity regardless of whether the amino acid residue at position 590 was an S or G (Fig. 2C and D). When lysine (K) was introduced to position 591, we noticed a 4-fold increase with a glycine (G) at position 590 (AK08-S590G/Q591K and AK10-G590/Q591K) and about 5- to 6-fold increase with a serine (S) at position 590 (AK08-S590/Q591K and AK10-G590S/Q591K). The remaining natural polymorphisms tested had no noticeable effects on polymerase function. The 590S/591K signature, which displayed the highest levels of polymerase activity, has been previously recorded in nature in 13 Asian isolates (Table S2).

The PB2 mutation E627K is a widely reported marker for mammalian adaptation (Subbarao et al., 1993). Furthermore, a synergistic effect has been observed for the PB2 mutations D253N and Q591K (Mok

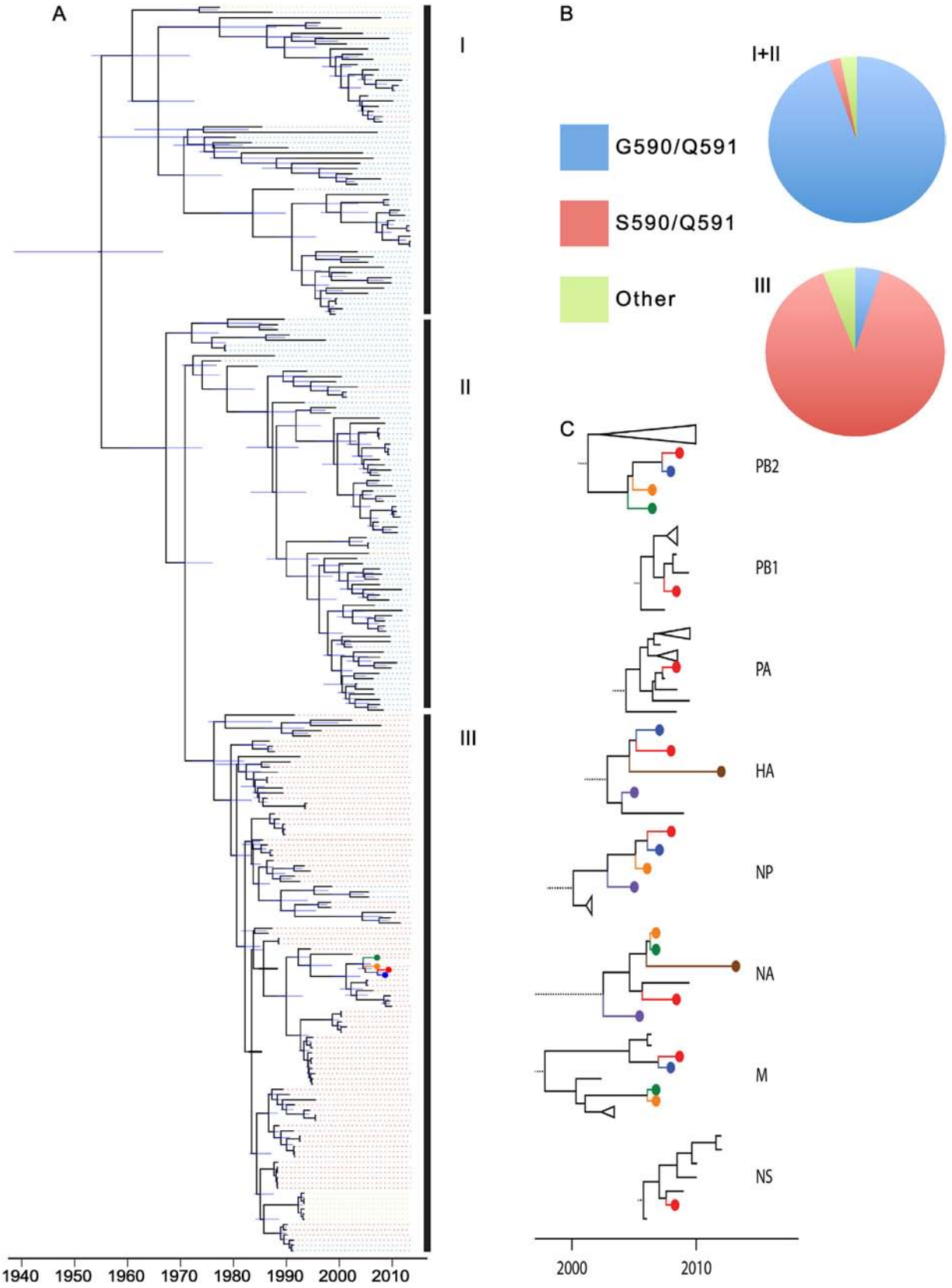

Fig. 1. Phylogenetic reconstruction showing the reassortment history of AK08. (A) PB2 gene genealogy; three monophyletic lineages are circulating globally. AK08 is found in Lineage III. (B) Pie charts showing the proportion of isolates in Lineage I and II (top) and Lineage III (bottom) with the S590 mutation. (C) Putative reassortment history of the AK08 viral gene segments. The horizontal axis represents the time scale in years. For simplicity, some sister clades were collapsed. The AK08 virus is highlighted for each of the 8 segments (red branch ending with a closed circle marker). Viruses sharing ancestry and that were considered relevant to the evolutionary history of the AK08 virus across multiple segments are color-coded as follows: ● A/white-fronted goose/Korea/20-36/2007(H9N2), ● A/snow goose/Montana/466771-4/2006(H5N2), ● A/snow goose/Quebec/17416/2006(H9N2), ● A/egret/Hunan/1/2012(H9N2), ● A/wild bird/Korea/8G39/2005(H9N2). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

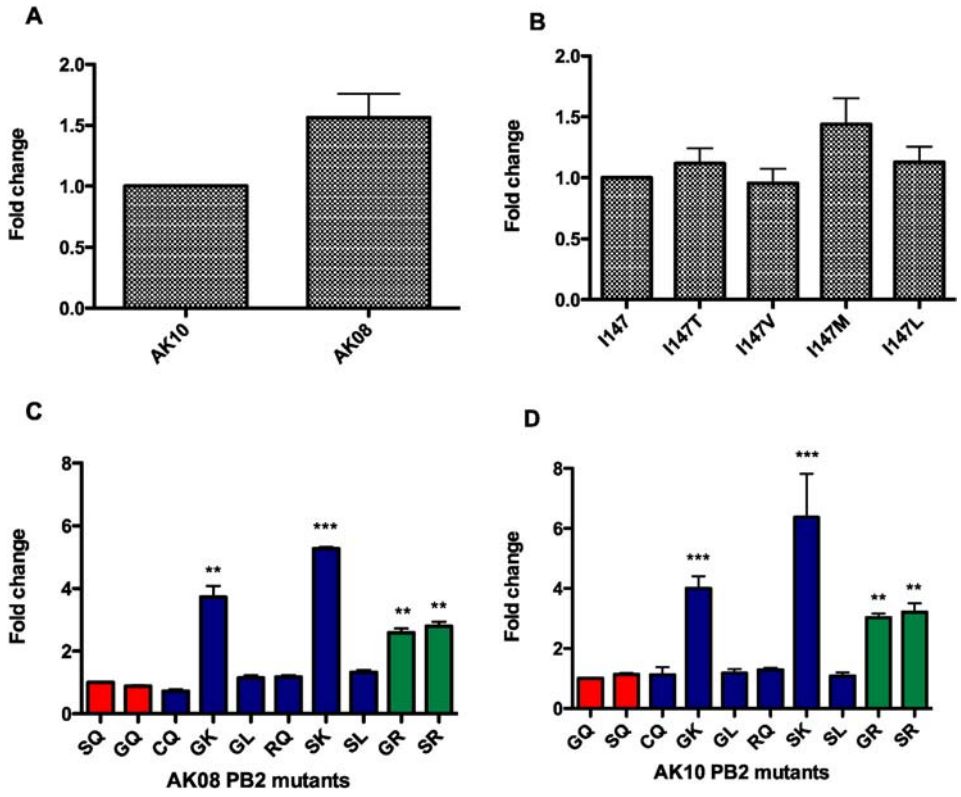

Fig. 2. Effects of PB2 reassortment and polymorphisms on AK08 viral polymerase activity. Polymerase activity was measured using a luciferase minigenome assay. Results are displayed as fold change to the parental PB2 in arbitrary light units (after subtracting the negative control background values). Error bars represent standard error of the mean (SEM) of at least 2 independent experiments. The Mann–Whitney U-test was used for comparisons. Statistically significant differences between the mutant and wild type PB2s are denoted by asterisks. (A) The polymerase activity of the AK08 virus as compared to that of the AK10 virus. (B) The effects of PB2 polymorphisms at position 147 on AK10 viral polymerase activity. (C) and (D) The effects on PB2 polymorphisms at positions 590/591 on polymerase activity of the AK08 and AK10 viruses, respectively. Red bars represent the 590/591 amino acid signatures found in the AK08 and AK10 viruses, blue bars represent all 590/591 polymorphisms found in nature in H9N2 viruses circulating globally, and finally the green bars represent 590/591 polymorphisms that did not exist in nature but were tested in our study. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

et al., 2011). Therefore, we examined the effects of combining either E627K or D253N mutations with some of the PB2 polymorphisms tested above. Introducing E627K or D253N mutations increased the polymerase activity of wild type AK08 and AK10 PB2 by about 4–6 fold, but did not offer any synergistic effect to the SK and GK mutants (Fig. 3), suggesting that the Q591K or Q591R polymorphism by itself is sufficient for enhancing polymerase function.

3.3. Q591K PB2 polymorphism enhances AK08 virus replication kinetics in MDCK and NHBE cells

To investigate the effects of PB2 point mutations on the intact viral phenotype, we generated 6 H9N2 viruses by reverse genetics. In addition to the wild type AK08, we also rescued an AK08 virus carrying the whole PB2 segment of its AK10 counterpart and four other AK08 mutants carrying single or double amino acid substitutions at positions 590 and 591 that had resulted in the highest levels of polymerase activity from the minigenome luciferase assays: SR, GR, GK and SK (Fig. 2C and D). These viruses were used to infect MDCK and NHBE cells at MOI of 0.1, after which viral titers were monitored over a period of 72 h. All 6 viruses grew in MDCK cells, but the GK and SK mutants replicated to significantly higher titers than the wild type virus, particularly at 48 ($p = 0.0408$ and 0.0294 for GK and SK, respectively) and 72 ($p = 0.0294$) h post-infection (Fig. 4A). Interestingly, the replication kinetics of the GK and SK mutants in NHBE cells followed similar patterns, where they exhibited significantly higher titers that reached early peaks at 12 h post-infection ($p = 0.0408$) and were detectable until 48 h post-infection. For the other 4 viruses, replication in NHBE cells was transient and titers were only detectable at 12 h post-infection (Fig. 4B).

3.4. Global distribution of the 591 K PB2 polymorphism

To interrogate the global distribution of the 591 K polymorphism, we mined the IRD for all PB2 sequences collected between 2008 and 2014 inclusive. We found that 591 K was present in PB2 genes from viruses isolated from swine, human and avian hosts, which belonged to 6 different viral subtypes (H1N1, H1N2, H3N2, H7N9, H5N2 and H9N2), and were distributed across 7 countries (Fig. 5). In other words, the 591 K polymorphism has a global distribution and is not associated with a particular host, subtype or geographic location.

3.5. AK08 PB2 segment carrying the Q591K polymorphism does not enhance the polymerase activity of a human H3N2 virus

Given the fact that the PB2 Q591K mutation is widely distributed across several IAV subtypes isolated from different hosts and geographies (Fig. 5), and the recent findings that demonstrated H9N2 viruses circulating in wild birds as frequent donors of internal gene segments(Lam et al., 2013; Wu et al., 2013; Qi et al., 2014), we investigated the effect of Q591K on the polymerase activity of a human adapted H3 virus using experimental systems that mimicked both reassortment and genetic drift. In a polymerase minigenome assay, we replaced the PB2 of A/Victoria/361/2011(H3N2) virus with AK08 and AK10 PB2 plasmids carrying the Q591K mutation, and observed a reduction in activity when compared to the wild type (Fig. 6). We then introduced the Q591K point mutation alone into the PB2 of A/Victoria/H3N2 virus by site-directed mutagenesis and observed no change in polymerase activity, which could be due to the fact that this PB2 segment already

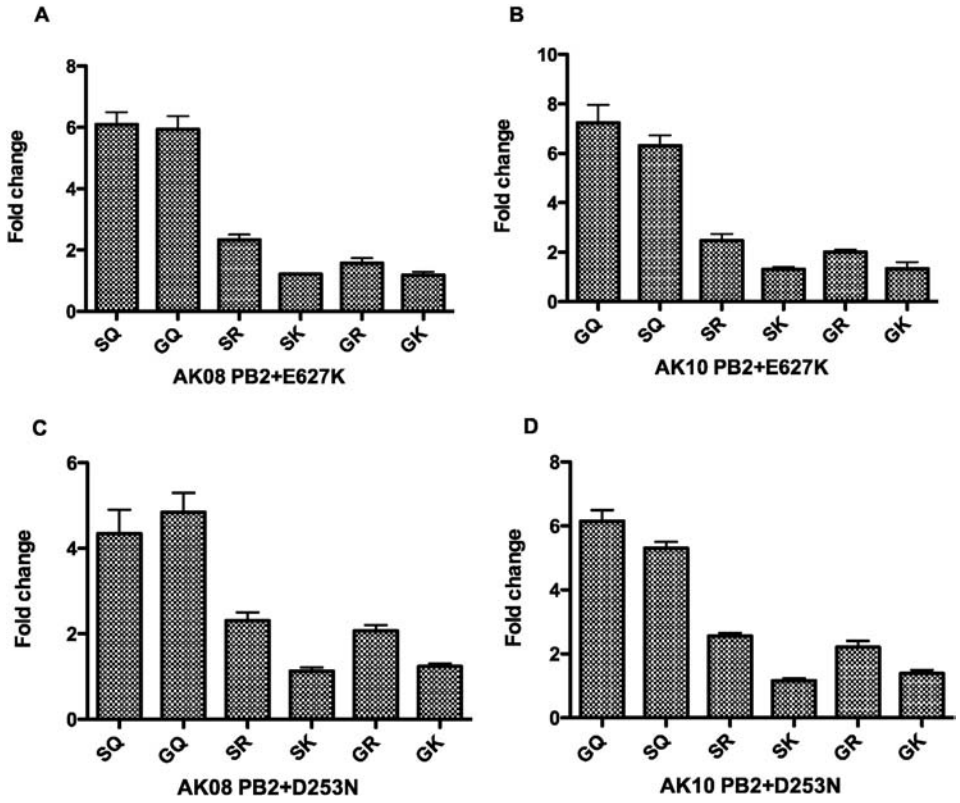

Fig. 3. Effects of combining E627K and D253N mutations to a panel of H9 PB2 polymorphisms. In order to test whether E627K or D253N mutation offer any synergistic effects to a panel of PB2. Proteins carrying polymorphisms at positions 590 and 591, these mutations were introduced into the same plasmids by site-directed mutagenesis. Results represent fold change in arbitrary light units as compared to E627K- and D253N-free plasmids. Error bars represent SEM of 2 independent experiments.

carries an E627K change (Fig. 6). In order to dissect the effect of Q591K on an E627K-free A/Victoria/H3N2, we created 2 PB2 mutants: VicPB2K627E (carrying neither K591 nor K627) and VicPB2Q591K/K627E (carrying K591 only). As expected, the VicPB2K627E mutant exhibited about 70% reduction in polymerase activity when compared to the wild type A/Victoria/H3N2 polymerase complex (Fig. 6). However, the VicPB2Q591K/K627E mutants displayed only a minimal reduction in polymerase activity, suggesting that the Q591K could compensate for the lack of K627 within the A/Victoria/H3N2 viral genomic constellation.

4. Discussion

Since the emergence of highly pathogenic H5N1 in 1997, the intensified influenza surveillance efforts have generated thousands of whole genome sequences. Accumulated knowledge about genotype–phenotype relationships has highlighted certain sequence features as markers for pathogenicity, which have become a fundamental component of risk assessment whenever a new virus of public health significance is encountered. Here we report that a single amino acid substitution (Q591K) in the PB2 segment significantly enhanced polymerase activity and conferred the ability of an H9N2 isolate identified through AIV surveillance of wild birds, to replicate in human cells. H9N2 strains derived from wild birds were shown to be donors of internal gene segments to some IAV subtypes that have recently emerged in humans (Lam et al., 2013; Wu et al., 2013). Interestingly, the AK08 PB2 segment carrying Q591K mutation did not enhance the polymerase activity when combined with the PB1, PA and NP segments of a human-adapted H3N2 virus (Fig. 6), indicating that molecular markers of PB2 pathogenicity are context-dependent, though not ruling out effects on other circulating human strains.

Despite being the natural reservoirs, very little work has been done to investigate the effects of genetic diversity generated in wild bird hosts *a priori* to avian influenza outbreaks in mammalian or agricultural hosts (Dugan et al., 2008; Boyce et al., 2009; Krauss and Webster, 2010; Runstadler et al., 2013). We investigated the evolutionary history of a low-pathogenic H9N2 isolate (A/northern shoveler/Interior Alaska/8BM3470/2008) identified through wild bird surveillance. This isolate was markedly different from contemporary H9N2 strains circulating in North America, as evidenced by the reassortment origins of its PB2, NP, NA and M segments (Fig. 1). Our findings indicate that the broad-scale sympatry of waterfowl hosts in North America (Fig. S9) is conducive to a complex exchange of viruses between a diversity of species and consequently increases chances for genetic reassortment between diverse virus strains. Moreover, we detected multiple incursions of Alaskan viruses hosted by wild birds into East Asia, demonstrating mixing of hemispheric virus pools due to wild bird-mediated dispersal. While our study provides evidence of North America-to-Asia transport of viruses, recent studies have detected whole genome H9N2 virus of East Asian origin in Alaska, supporting bidirectional virus flow (Ramey et al., 2015).

The BEAST phylogenetic trees suggest, but do not rule out other scenarios, that the AK08 virus most likely acquired its PB2 segment from an H5N2 virus, which made it stand out from other North American H9N2 strains. Highly pathogenic forms of H5N2 have been reported in healthy wild waterfowl (Gaidet et al., 2008); they also caused extensive outbreaks in domestic poultry (Donatelli et al., 2001; Lee et al., 2005, 2014; Zhao et al., 2012), have been transmitted to pigs (Lee et al., 2009), and left serological evidence of human exposure (Ogata et al., 2008; Yamazaki et al., 2009; Okoye et al., 2013; Wu et al., 2014). In addition, an avian H2N1 (A/green-winged teal/Ohio/175/1986) virus, which exhibited low levels of polymerase activity in human cells, replicated better and had increased polymerase function in human cells after it had acquired a PA segment from a human H1N1 virus (Mehle et al., 2012) indicating that swapping polymerase segments could have strong effects on the viral phenotype. We investigated whether the newly

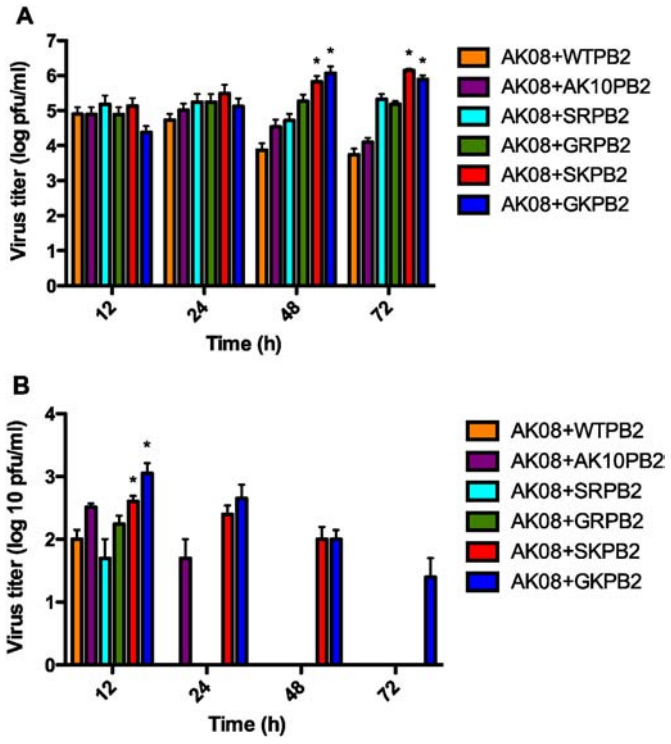

Fig. 4. Replication kinetics of wild type and mutant AK08 viruses in MDCK (A) and NHBE (B) cells. A total of 6 viruses were generated by reverse genetics, all based on the AK08 virus backbone. The only difference between the 6 viruses was the PB2 segment, which was either wild type or mutant PB2 carrying SR, GR, SK or GK polymorphisms at position 590 and 591. In one of the viruses, the whole PB2 segment was replaced with that of the AK10 virus. Cell monolayers in 12-well plates were infected at an MOI of 0.1 in duplicates. Virus progeny produced was monitored in the supernatants over a period of 72 h. Error bars represent SEM of 2 independent experiments. The Mann–Whitney U-test was used for comparisons. Statistically significant differences between the wild type and mutant viruses carrying G590/K591 and S590/K591PB2s are denoted by asterisks.

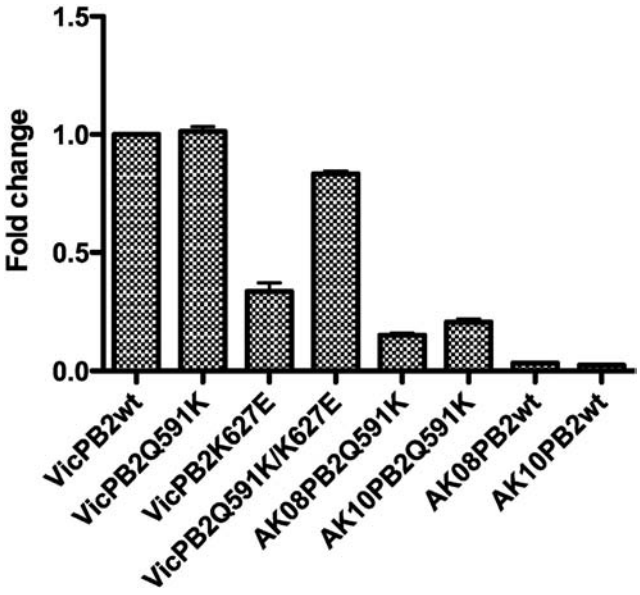

Fig. 6. Effect of Q591K on human H3N2 polymerase activity. To mimic a Q591K arising in human-adapted virus, this mutation was introduced into the wild type A/Victoria/H3N2 (VicPB2wt) by site-directed mutagenesis producing mutant VicPB2Q591K. To mimic a reassortment scenario of a human-adapted virus acquiring an AK08 segment carrying the Q591K polymorphism, the PB2 segment of A/Victoria/H3N2 virus was replaced with AK08 and AK10 viral PB2s with or without Q591K. In order to dissect the effect of Q591K on an E627K-free A/Victoria/H3N2, 2 more A/Victoria/H3N2 PB2 mutants were constructed: VicPB2K627E (carrying neither K591 nor K627) and VicPB2Q591K/K627E (carrying K591 only). Results are displayed as fold change to the wild type A/Victoria/H3N2 PB2. Error bars represent SEM of 2 independent experiments.

acquired PB2 substitutions impacted the polymerase function of the reassortant virus we isolated from avian surveillance. A minigenome reporter assay revealed that the polymerase complex of the AK08 virus was only 1.5 fold more active than that of an H9N2 isolate that was more closely related to North American strains (Fig. 2A), indicating that the PB2 acquisition through the reassortment event did not drastically affect polymerase function.

More than 86% of H9 PB2s have glycine (G) at position 590 and 93% of them have glutamine (Q) at position 591. G590S is present in only about 12% of H9 isolates and was among the 10 polymorphisms that the AK08 PB2 had likely inherited from its H5N2 precursor. In some H1N1 and H5N1 viruses, polymorphisms at positions 590 and 591,

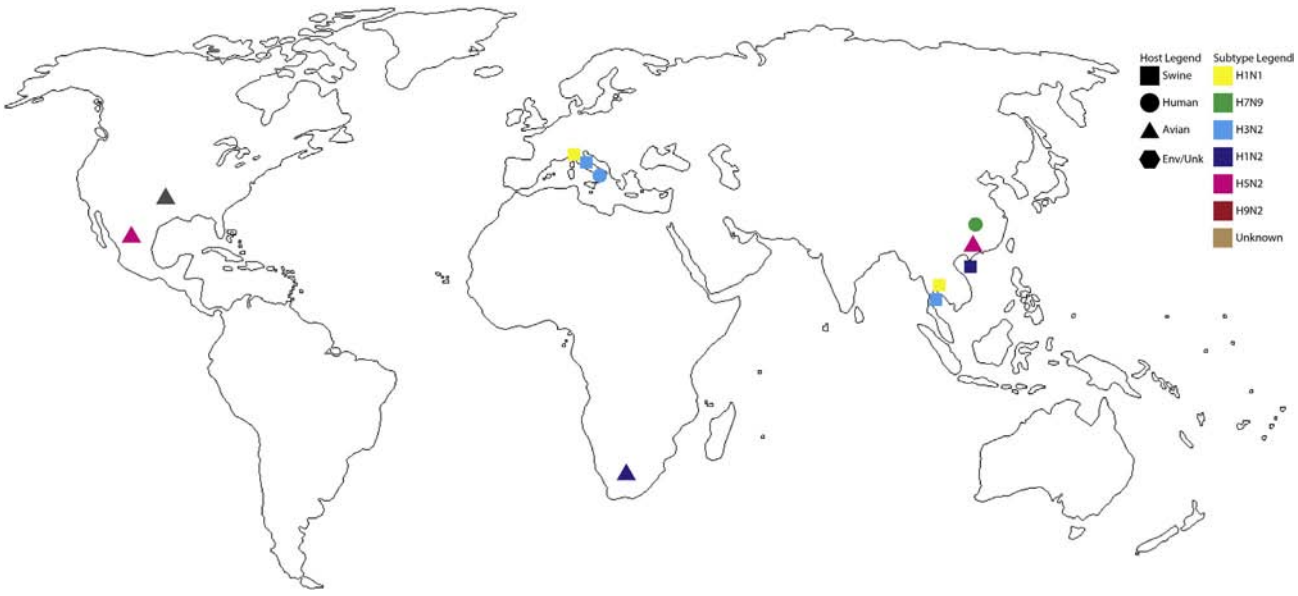

Fig. 5. Global distribution of the K591 polymorphism from 2008 to 2014. This analysis was based on publically available sequences acquired from the Influenza Research Database (www.fludb.org). Subtypes are color coded, while host types are indicated by different shape markers. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

designated as S590/R591 or "SR polymorphisms", have been described as important pathogenicity markers (Mehle and Doudna, 2009; Yamada et al., 2010). Although the Q591R polymorphism has not been detected yet in naturally circulating H9 isolates, it takes only a single nucleotide change to switch from glutamine (Q) to arginine (R), which could emerge *via* simple genetic drift, and go undetected by limited surveillance efforts. Our polymerase assay data suggest that certain polymorphisms at position 591 of H9 PB2 are major determinants of polymerase activity. An R at 591 regardless of the amino acid residue at 590 resulted in 3-fold increase in polymerase activity (Fig. 2C and D). The highest polymerase activity was recorded for PB2s that had S590 and K591 combined. A variant of the A/Quail/Hong Kong/G1/1997(H9N2) virus carrying synergistic D253N and Q591K mutations in PB2 was previously reported to have enhanced polymerase activity (2.7-fold higher than the wild type); it also replicated better in NHBE cells and was more pathogenic for mice (Mok et al., 2011). In our experiments, the Q591K mutant of AK08 virus was more active, but did not work in synergism with either D253N or E627K (Fig. 3). Furthermore, contrary to the above study, which observed no effect for the Q591R substitution, we demonstrated a 3-fold increase in polymerase activity. These differences highlight the need for a global assessment of polymerase enhancer polymorphisms, but demonstrate the strong phenotypic effect that even point mutations may have on circulating avian strains.

Natural H9 isolates carrying the 591 K PB2 polymorphism represent about 1% of the circulating isolates. In addition to the wild type AK08 virus, we rescued 5 additional viral mutants by reverse genetics. All 5 engineered viruses carried all segments from AK08 except for the PB2, which was replaced by mutated versions containing the 590/591 polymorphisms that displayed the highest polymerase activities (GR, SR, GK and SK) or a common H9 PB2 segment (from AK10). The 591 K PB2 viral mutants replicated the best in MDCK cells and were the only ones to show evidence of replication in NHBE cells (Fig. 4). The fact that PB2 591R resulted in an increase in polymerase activity (Fig. 2C), but was not correlated with an enhancement in virus replication kinetics indicates a complex process, where factors other than increased polymerase processivity could be also involved. Introducing a 591 K into the PB2 of an avian H5N1 virus (A/chicken/Indonesia/UT3091/05) was previously reported to enhance its growth properties in NHBE cells (Yamada et al., 2010). Our results not only confirm an earlier observation related to an MDCK-grown H9N2 virus (A/Quail/Hong Kong/G1/1997) carrying a combination of D253N/Q591K (Mok et al., 2011), but also suggest that a single amino acid substitution in PB2 is sufficient to alter the phenotype of a wild H9N2 isolate's growth in human cells without prior viral adaptation.

Surveillance efforts have focused on sampling different influenza hosts and sequencing the resultant viruses to monitor virus evolution and understand the source of frequent outbreaks and potential pandemic strains. In addition to laboratory characterization, initial risk assessment relies on sequence interpretation. Several PB2 polymorphisms have been reported in the literature to be associated with mammalian adaptation (Gabriel et al., 2013; Manz et al., 2013). One caveat with this approach is that these polymorphisms are studied in specific genetic contexts, confounded by viral subtype. For example, E627K is a mutation commonly associated with mammalian adaptation and enhanced replication of H5N1 viruses in mammalian cells. However, this polymorphism did not exert similar effects for pandemic 2009 H1N1 strains (Herfst et al., 2010; Jagger et al., 2010). Similarly, we report here that a Q591K PB2 polymorphism, which enhanced the polymerase function and replication kinetics of a wild H9N2 virus in human cells, had a context-dependent effect on the polymerase activity of a human-adapted A/Victoria/361/2011(H3N2) viral polymerase. Swapping the PB2 segment of the A/Victoria/H3N2 virus with AK08 and a common H9 (AK10) PB2 carrying the Q591K mutation resulted in a marked reduction in polymerase activity (Fig. 6). Engineering the same point mutation into the PB2 of the A/Victoria/H3N2 virus did not enhance polymerase function either because this particular PB2 segment had already acquired the E627K mammalian adaptation mutation (Fig. 6).

This study demonstrates the value of current avian surveillance protocols in identifying low pathogenic strains that can improve our understanding of the complex genotype–phenotype relationship and inform about the risk of circulating strains. Moreover, our findings clearly suggest that the Q591K PB2 molecular marker is widely distributed across several subtypes, hosts and geographic regions (Fig. 5), but yet did not exert universal enhancing effects within other polymerase genetic contexts. Work presented here highlights the need for a more comprehensive assessment of the phenotypic effects of polymerase markers across all known genetic constellations in order to allow for a more informed risk assessment and maximize the outcome of molecular surveillance.

Acknowledgment

We would like to acknowledge the NIH/NIAID funded Center of Excellence for Influenza Research and Surveillance (CEIRS Contract no.: HHSN272014000008C). The BEAST phylogenetic analysis was facilitated by an inter-center CEIRS training grant. We would like to thank Adolfo Garcia-Sastre and Nacho Mena, Icahn School of Medicine at Mount Sinai, for the pDZ plasmid gift and excellent technical support. We would like also to thank Yoshihiro Kawaoka, University of Wisconsin-Madison, for his generous gift of the A/WSN/1/33 polymerase plasmids. Any use of trade names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meegid.2016.04.011.

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