Research paper

Intercontinental spread of Asian-origin H7 avian influenza viruses by captive bird trade in 1990's

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A B S T R A C T

Wild bird migration and illegal trade of infected poultry, eggs, and poultry products have been associated with the spread of avian influenza viruses (AIV). During 1992–1996, H7N1 and H7N8 low pathogenic AIV (LPAIV) were identified from captive wild birds; such as Pekin robin (*Leiothrix lutea*), magpie robin (*Copsychus saularis*), flycatcher sp. (genus *Empidonax*), a species of softbill and parakeet, sun conure (*Aratinga solstitialis*), painted conure (*Pyrrhura picta*), fairy bluebird (*Irena puella*), and common iora (*Aegithina tiphia*), kept in aviaries or quarantine stations in England, The Netherlands, Singapore and the United States (U.S.). In this study, we sequenced these H7 viruses isolated from quarantine facilities and aviaries using next-generation sequencing and conducted a comparative phylogenetic analysis of complete genome sequences to elucidate spread patterns. The complete genome sequencing and phylogenetic analysis suggested that H7 viruses originated from a common source, even though they were obtained from birds in distant geographical regions. All H7N1 and H7N8 viruses were LPAIV, except a H7N1 highly pathogenic AIV (HPAIV), A/Pekin robin/California/30412/1994(H7N1) virus. Our results support the continued need for regulation of the captive wild bird trade to reduce the risk of introduction and dissemination of both LPAIV and HPAIV throughout the world.

1. Introduction

Whereas historically pathogen spillover events were likely to remain local, even undetected due to natural, cultural and geographic barriers, globalized trade of domestic and wild animals allows infectious diseases to spread along various globally connected networks in a matter of days (Smith et al., 2017). It has been reported that trade of infected birds, eggs, and poultry products have caused the spread of avian influenza viruses (AIV) (Vandegrift et al., 2010). For example, the detection of Asian-origin H5N1 highly pathogenic AIV (HPAIV) in eagles confiscated at Brussels Airport, Belgium, which were smuggled from Thailand, and in captive-caged birds held in quarantine in England imported from Taiwan during 2004–2005 and in smuggled cage birds originating from Indonesia detected at Vienna airport, Austria in 2013, represent this important dissemination route of AIV around the world (Capua and Alexander, 2007; Ronquist et al., 2012). The dissemination of low pathogenic AIV (LPAIV), mainly H3, H4 and H7 subtypes, via international captive bird trade during the 1970s–1990s, also highlights the risk for intercontinental spread of AIV through trade in such birds (Alexander, 1995; Banks et al., 2000; Panigrahy and Senne, 1997). In

particular, H7N1 LPAIV was identified from Passeriformes and Psittaciformes birds; such as sun conure (*Aratinga solstitialis*), painted conure (*Pyrrhura picta*), fairy bluebird (*Irena puella*), common iora (*Aegithina tiphia*), and a species of parakeet kept in aviaries or quarantine in England, the Netherlands and Singapore during 1994–1995 (Banks et al., 2000). In the United States (U.S.), H7N1 and H7N8 viruses were detected from exotic birds such as Pekin robin (*Leiothrix lutea*), magpie robin (*Copsychus saularis*), flycatcher sp. (genus *Empidonax*), and a species of softbill, intended for importation into the U.S. during 1992–1996 (Panigrahy and Senne, 1997). The phylogeographic origin, epidemiologic linkages and reassortment of gene segments of these viruses are unknown.

The deduced amino acid profile at the HA cleavage site for the H7N1 and H7N8 viruses identified from Passeriformes and Psittaciformes birds were consistent with that of LPAIV and found to be non-pathogenic for chickens, except for the A/Pekin robin/California/30412/1994(H7N1) (Pekin robin/94) virus. The presence of polybasic amino acids at the cleavage site of HA is characteristic of HPAIV of the H5 and H7 subtypes which causes systemic virus replication and high mortality in poultry. The Pekin robin/94 virus met the molecular

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Table 1
Viruses used in this study.

Virus	Found in	Origin of birds	HA cleavage site	In vivo pathogenicity (Reference)
A/softbill/California/33445-158/1992(H7N1)	Quarantine facility	Unknown	PETPKGR/G	Low (Panigrahy and Senne, 1997)
A/softbill/California/33445-136/1992(H7N1)	Quarantine facility	Unknown	PETPKGR/G	Low (Panigrahy and Senne, 1997)
A/magpie_robin/California/028709-5/1993(H7N8)	Quarantine facility	China	PEIPKER/G	Low (Panigrahy and Senne, 1997)
A/magpie_robin/California/028710-14/1993(H7N8)	Quarantine facility	China	PEIPKER/G	Low (Senne et al., 1996)
A/Pekin_robin/California/30412/1994(H7N1)	Quarantine facility	China	PEIPKRRR/G	Low (Senne et al., 1996), High (this study)
A/softbill/California/13907-21/1994(H7N1)	Quarantine facility	Indonesia	PEIPKGR/G	Low (Senne et al., 1996)
A/flycatcher/California/014875-1/1994(H7N1)	Quarantine facility	Indonesia	PEIPKGR/G	Low (Senne et al., 1996)
A/Avian/Canada/IM-1474/1994(H7N1)	Quarantine facility	Unknown	PEIPKGR/G	Low (this study)
A/sun conure/England/766/1994(H7N1)	Dead, Private aviary	Unknown	PEIPKGR/G	Low (Banks et al., 2000)
A/painted conure/England/1234/1994(H7N1)	Dead, Private aviary	Unknown	PETPKGR/G	Low (Banks et al., 2000)
A/parakeet/Netherlands/267497/1994(H7N1)	Dead, Private aviary	Unknown	PEIPKGR/G	Low (Banks et al., 2000)
A/fairy_bluebird/Singapore/F92/1994(H7N1)	Bird destined for export	Singapore	PEIPKGR/G	Low (Banks et al., 2000)
A/common_iora/Singapore/F89/95(H7N1)	Smuggled bird	Indonesia	PEIPKGR/G	Low (Banks et al., 2000)

criterion for classification as HPAIV with an amino acid sequence of PEIPKRRR/G, but was not highly lethal in in vivo chicken pathotyping test (Senne et al., 1996).

In this study, we sequenced H7N1 and H7N8 viruses identified from exotic birds during 1992–1994 using next-generation sequencing (NGS). Molecular epidemiological approaches based on genome sequences of these viruses were used to describe the phylogeographic origin and potential epidemiological linkages. In addition, we analyzed the cleavage site sequences of HA gene of Pekin robin/94 using deep-sequencing looking for evidence of mixed infections of LPAIV and HPAIV strains to explain the disparity of low pathogenicity phenotype and high pathogenicity genotype in the previous report. Also, we repeated the in vivo chicken pathogenicity test.

2. Materials and methods

2.1. Genome sequencing

Complete genomes of 11 H7N1 and 2 H7N8 viruses listed in Table 1 (seven isolates detected from exotic birds in quarantine facility in California during 1992–1994, five isolates from Animal and Plant Health Agency in Weybridge, United Kingdom submitted from England, The Netherlands and Singapore during 1994–1995, and an isolate detected from a bird in a quarantine facility in Canada during 1994) were sequenced using illunina MiSeq (Illumina) or Ion Torrent Personal Genome Machine (Life Technologies) as previously described (Lee et al., 2017a). The de novo and directed assembly of genome sequences were performed using Geneious 9.1.2 (http://www.geneious.com) and GALAXY software as previously described (Dimitrov et al., 2017). Assembled sequence data were analyzed to find subpopulation using Geneious v8.1.2 program as previously described (Lee et al., 2017b). Nucleotide sequences have been deposited in GenBank under accession no.MH574746-69 and MH558685-92.

2.2. Phylogenetic analysis

On the basis of BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) searches of H7N1 and H7N8 viruses, reference sequences for phylogenetic analysis were retrieved from GenBank. AIV sequences that originated from samples collected before 1995 were downloaded from the National Center for Biotechnology Information Influenza Virus Resource database in June 2018 and added to sequence alignments. Sequences were aligned using Multiple Alignment with Fast Fourier Transformation (MAFFT) in Geneious v8.1.2 program and trimmed to remove nucleotides that were outside the coding region. Relationships were inferred from maximum-likelihood (ML) phylogenies for each gene segment with RAxML (Stamatakis, 2014) using the GTR nucleotide substitution model and visualized with MEGA 7 software (sequences per segment: PB2 = 172; PB1 = 179; PA = 192; HA = 88;

NP = 146; N1 = 97; N7 = 71; N8 = 81; M = 239; and NS = 175). Bootstrap support values were generated using 1000 rapid bootstrap replicates and shown next to the branches (> 70). Based on ML phylogenetic analyses of all eight genes, we classified the H7 viruses into different genotypes according to tree topology, high bootstrap values (> 70%), and high levels of nucleotide identity (> 95–97%).

The Bayesian relaxed clock phylogenetic analysis of the HA gene was conducted using BEAST v1.8.3 (Drummond and Rambaut, 2007). The temporal signal estimated by TempEst software (available online: http://tree.bio.ed.ac.uk/software/tempest/) exhibited a positive correlation between genetic divergence and sampling time and appeared to be suitable for phylogenetic molecular clock analysis in BEAST (Correlation coefficient: 0.9249, R squared: 0.8555). We used an uncorrelated lognormal distribution relaxed clock method, the HKY nucleotide substitution model and the GMRF Bayesian skyride coalescent prior (Minin et al., 2008). A Markov Chain Monte Carlo (MCMC) method to sample trees and evolutionary parameters was run for 5.0×10^7 generations. At least three independent chains were combined to ensure adequate sampling of the posterior distribution of trees. Convergence of the each run and the sufficient effective sample size (> 200) were confirmed using Tracer version 1.6 (http://tree.bio.ed.ac.uk/software/tracer/). BEAST output was analyzed with 10% burn-in. A maximum clade credibility (MCC) tree was generated for each data set using TreeAnnotator in BEAST (http://beast.bio.ed.ac.uk/TreeAnnotator/). FigTree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/) was used for visualization of MCC tree and the time to the most recent common ancestor (tMRCA) of each clade was estimated.

2.3. In vivo pathogenicity test

We pathotyped the Pekin robin/94 and A/Avian/Canada/IM-1474/1994(H7N1) viruses in chickens to confirm the pathogenicity phenotype. The Pekin robin/94 isolates were obtained from the National Veterinary Services Laboratories in Ames, Iowa. The isolates were received in allantoic fluid after a single passage in embryonating chicken eggs (ECE), and further propagated by one additional passage at the U.S. National Poultry Research Center (USNPRC) to make working stocks of the viruses. Working stocks were titrated in ECE using standard methods [25]. All virus titers were reported as mean embryo infectious dose (EID₅₀). The studies were performed in biosecurity level-3 enhanced (BSL-3E) facilities in accordance with procedures approved by the USNPRC Institutional Biosecurity Committee. The Pekin robin/94 virus was pathotyped by intravenous inoculation in eight 4-week-old White Plymouth Rock (WPR) chickens as previously described (OIE, 2018). Isolates that killed more than six chickens within 10 days were categorized as HPAIV. In addition, a group of eight chickens was intranasally (IN)-inoculated with $10^{6.8}$ EID₅₀ of infectious allantoic fluid to determine virulence by simulated natural exposure route. To evaluate the mean chicken infectious dose (CID50), birds were divided into
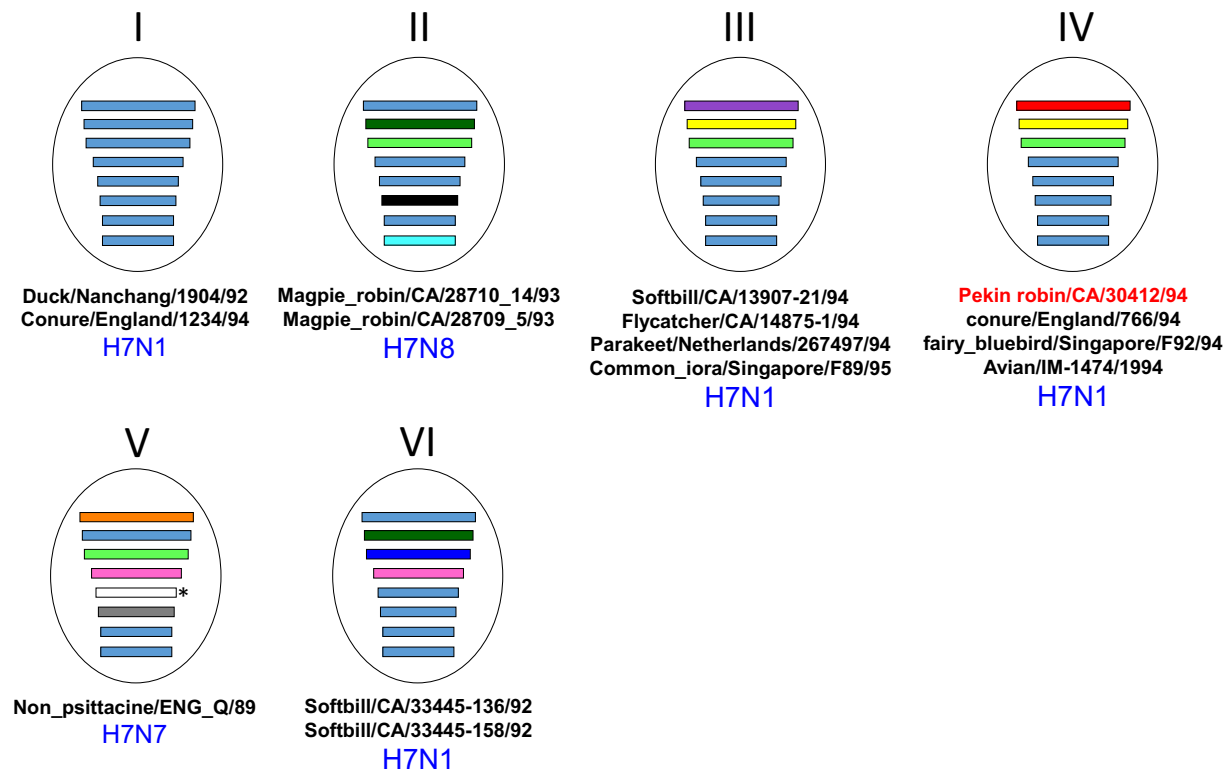
Fig. 1. Genome constellations of H7 viruses. Viruses are represented by ovals containing horizontal bars that represent the 8 influenza gene segments (from top to bottom: polymerase basic 2, polymerase basic 1, polymerase acidic, hemagglutinin, nucleoprotein, neuraminidase, matrix, and nonstructural) Each gene segment was colour coded according to the genetic clustering in ML phylogenetic trees. A highly pathogenic avian influenza virus, the Pekin robin/CA/30412/94, is colored in red. Asterisk indicates the gene belongs to North American lineage. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

groups of 8 birds, and each bird was IV-inoculated with 10^2, 10^4, or 10^6 EID_{50} in 0.1 ml of infectious allantoic fluid. The intravenous pathogenicity index (IVPI) of A/Avian/Canada/IM-1474/1994(H7N1) was performed at the Canadian Food Inspection Agency according to the standard procedures (OIE, 2018).

3. Results and discussion

3.1. Dissemination of reassortant H7 viruses via bird trade

A total of six distinct genotypes, genotype I-VI, were identified (Fig. 1), suggesting H7 viruses seem to have acquired their gene segments from different gene pools of Eurasian and North American LPAIV through reassortment (Fig. S2) and these genotypes may have co-circulated (Fig. 2 and S1). All genes of genotype III and IV viruses, except polymerase genes, shared high nucleotide identity (> 97.6%) and phylogenetically clustered at each gene segment with those of genotype I. Among all 8 segments, only the PB2 gene of genotype III did not share high nucleotide identity (88.5–89.1%) with Genotype IV. Based on the estimated tMRCA of HA gene (Fig. 2 and S1), H7 viruses sequenced as part of this study and an additional reference sequence from China likely diversified into genotype I-VI since 1984 March (95% Bayesian credible interval [BCI]: 1981 May – 1986 Aug, posterior probability: 0.99). The HA gene of genotype I-IV clustered together in Bayesian phylogenetic tree. The estimated tMRCA between genotype I-IV was 1989 February (95% BCI: 1986 Nov – 1990 Dec, posterior probability: 1.0). The HA gene of genotype V and VI clustered together and their tMRCA was estimated as 1986 August (95% BCI: 1984 May – 1988 Apr, posterior probability: 1.0). Between genotype V and VI, only HA, M and NS genes were clustered together (Fig. 1 and S2). The NP gene of A/non psittacine/England Q/1985/1989(H7N7) virus clustered with North American lineage LPAIV (Fig. S2). Interestingly, the PB2 gene of genotype IV appeared later in the Asian H9N2 LPAIV circulating in poultry. The PB2 gene of genotype IV phylogenetically clustered with H9N2 viruses identified in Middle East from 1999 and shared high nucleotide identity (97.4–97.7%) with the A/chicken/Middle East/ED-1/1999(H9N2) virus.

Our ML and Bayesian phylogenies of HA gene provided support for common ancestry between genotype I-IV viruses. The oldest virus in genotype I-IV cluster is A/duck/Nanchang/1904/1992(H7N1) showing the shortest branch length from the common ancestral node. Unfortunately, there are no full-length HA gene sequences that cluster with genotype V-VI available in the public databases; however, two H7N1 viruses identified from a quarantine station in California, A/softbill/California/33445-136/1992(H7N1) and A/softbill/CA/33445-158/1992(H7N1), share 99.1% nucleotide identity (110 of 111) with the 111 nt long partial HA gene of A/softbill/China/92(H7) virus (Banks et al., 1995). These data suggests that nine isolates identified from quarantine stations in California, Canada, and Singapore, and private aviaries in England and the Netherlands during 1994 most likely have been disseminated separately from a common origin possibly in Asia even though they were isolated from distant geographical regions. The isolations from quarantine stations in California and Singapore were from birds imported from China and Indonesia (Banks et al., 2000). The isolations in private aviaries in England and the Netherlands were from dead sun and painted conures, and parakeet, but the country of origin for these birds remains uncertain although the captive bird trade was theoretically banned at the time (Banks et al., 1995). During the period from 1981 to 2010, the live pet bird trades exported from China exceeded one million individual birds in contrast to approximately 80,000 individual birds imported to China during the same period (Li and Jiang, 2014). Parrots and passerines were the most
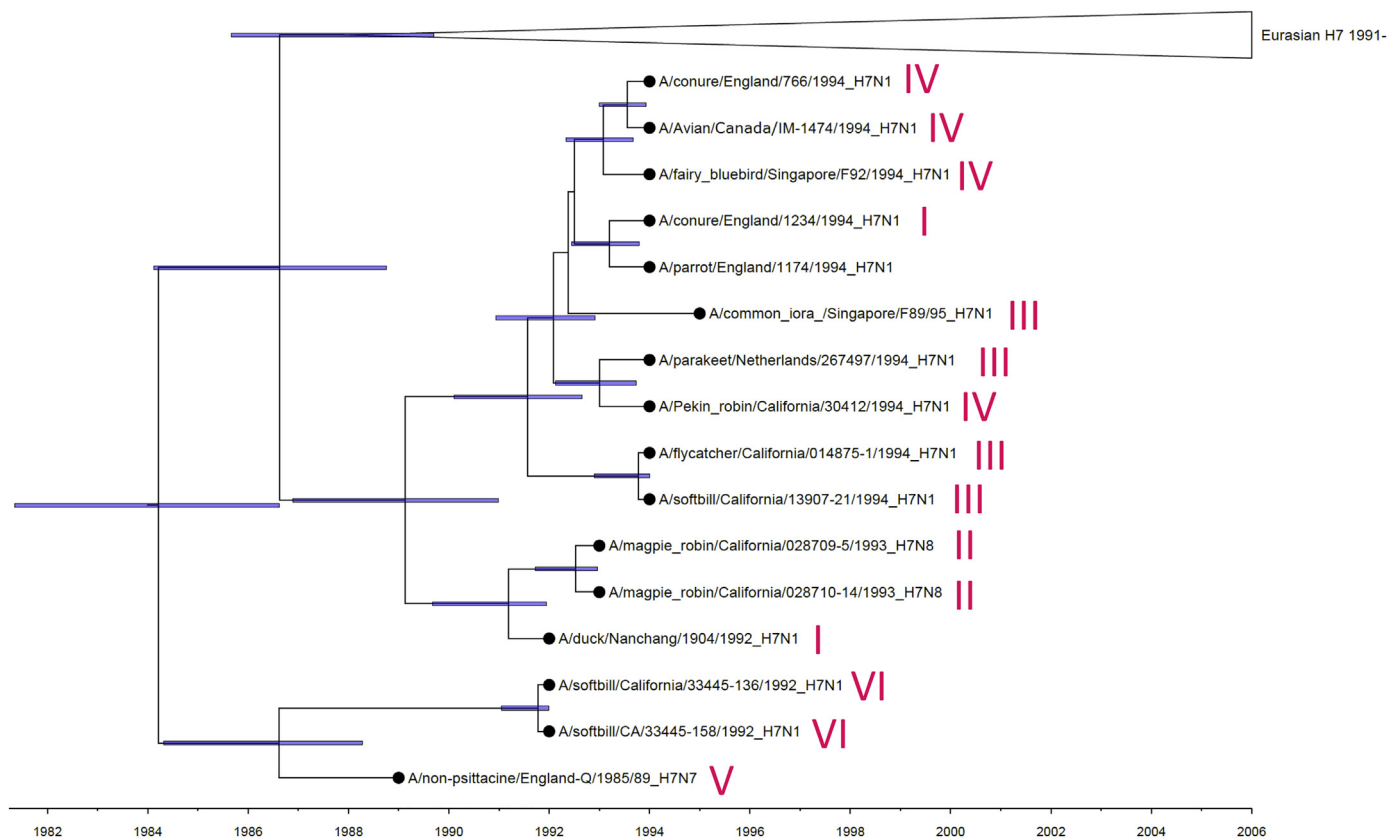
Fig. 2. Selected subtree of relaxed clock molecular phylogenetic tree for the hemagglutinin of H7 viruses. The phylogenetic relationships and temporal evolutionary history have been estimated by molecular clock analysis. The tree is scaled to the time (horizontal axis), with blue horizontal bars at nodes representing the 95% highest posterior density intervals for the node ages. Genotype of each virus is indicated next to taxa. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

common birds in trade: live bird exports from China were directed to Europe, Southeast Asia, Japan and the U.S., while exports from Africa and Southeast Asia were directed into China. Our phylogenetic analysis and bird trade data suggest that reassortant H7 viruses were co-circulating in captive bird populations in Southeast Asia and China, and dissemination of these viruses via export activities into multiple countries.

3.2. Pathogenicity of Pekin robin/94 virus

NGS confirmed that the deduced amino acid sequence for the H7 viruses isolated from captive birds during 1989–1995 bore two non-consecutive basic amino acids at the cleavage site (PEIPKER/G or PETPKGR/G [Table 1]), consistent with LPAIV, except for Pekin robin/ 94 virus. The Pekin robin/94 virus possessed multiple basic amino acids at the cleavage site (PEIPK<u>RRR</u>/G), likely representing mutation from an LPAIV motif through both non-synonymous substitution (single nucleotide polymorphism), and insertion of an additional basic amino acid. All viruses were LPAIV based on the HA cleavage site sequences and in vivo chicken testing in the present and previous studies (Banks et al., 2000; Panigrahy and Senne, 1997; Senne et al., 1996), except for Pekin robin/94 virus. In this study, the Pekin robin/94 virus was highly lethal in chicken pathotyping tests (Table 2). It produced high morbidity (8/8) and mortality (7/8) in IV-inoculated chickens. Mean death time (MDT) was 4.4 and 5.6 days in trial 1 and 2, respectively for chickens IN-inoculated with the $10^{6.0}$ EID_{50} dose. In the group inoculated with $10^{4.0}$ EID_{50} dose, all chickens showed clinical signs and six chickens died, with a MDT of 5.3 days. In $10^{2.0}$ EID_{50} virus dose inoculated group, seven chickens showed clinical signs and six chickens died, with a MDT of 6.7 days. The mean CID_{50} was $< 10^{2.0}$ EID_{50}

Table 2
Pathogenicity of the A/Pekin robin/CA/30412/1994(H7N1) virus in chickens.

Route	Dose (EID_{50})	Mortality	Morbidity	Mean death time (days)
Intravenous	$10^{6.8}$	7/8	8/8	4.4
	$10^{6.0}$	8/8	8/8	4.4
	$10^{4.0}$	6/8	8/8	5.3
	$10^{2.0}$	6/8	7/8	6.7
Intranasal	$10^{6.8}$	4/8	6/8	6.3

suggesting being highly adapted to the chicken host (Swayne and Slemons, 2008). The high pathogenicity of Pekin robin/94 virus produced in this study corresponds to the HA0 cleavage site sequences. The mean depth of coverage at the HA0 cleavage site was > 1000 which is sufficient to identify a variant with high confidence. None of the NGS read had the LPAIV motif at the HA0 cleavage site of Pekin robin/94 virus. It is unclear why the Pekin robin/94 virus had a low pathogenicity phenotype in the prior study while our study determined it was highly lethal for chickens and on NGS sequencing had an HA cleavage site of a high pathogenicity virus without a subpopulation of LPAIV. Historically, LPAIV mutation at the HA0 cleavage site to HPAIV has most consistently occurred in gallinaceous poultry with rare identification of HPAIV in non-gallinaceous species (Mathilde et al., 2017). However, with the Pekin Robin HPAIV, it remains unclear whether this HA0 mutation occurred during the infection cycle in this passerine species, or if the mutation occurred in gallinaceous poultry with exposure and transmission to the passerines prior to capture and/or during holding and trading.

4. Conclusion

During the last decade, migratory aquatic birds, especially in the orders Anseriformes and Charadriiformes, have been intensively monitored for influenza A viruses to better understand their role in the dissemination of A/goose/Guangdong/1/96 (H5N1) lineage H5 HPAIV over vast geographic distances (Machalaba et al., 2015). Our results highlight the risk of the live bird trade in other orders of birds which could be one of the possible routes for the introduction and dissemination of both LPAIV and HPAIV throughout the world. Many countries of origin for legal and illegal wildlife imports to the U.S. include "hotspots" of HPAIV, as well as other economically important livestock diseases (Smith et al., 2017). Based on the reported countries of origin from 2000 to 2013 for all declared US wildlife imports by shipment, China and Southeast Asia are primary regions of origin for US wildlife imports. Examples of identified live birds imported from these regions to the US included nearly 30 million live pheasants from China, and approximately 450,000 finch-like live pet birds from Taiwan, and over 85,000 kg of "edible-nest swiftlet" nests from Indonesia (Smith et al., 2017). In addition, LPAIV and HPAIV have been detected in intercepted illegal imported poultry and related products; for example, HPAIV (H5N1 clade 2.3.2.1c and H5N6 clade 2.3.4.4) and LPAIV (H9N2 and H1N2) were isolated from raw chicken and duck products carried by flight passengers from China and Taiwan to Japan during 2015 (Shibata et al., 2018). To minimize the risk of LPAIV and HPAIV introduction, strict testing, quarantine controls and monitoring for AIV should be continued for the importation of live poultry, and exotic and pet birds; fertile eggs and avian products before entering the country.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.04.028.

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