Genetic evidence for avian influenza H5N1 viral transmission along the Black Sea–Mediterranean Flyway

Sen Zhou,† Huaiyu Tian,† Xiaoxu Wu, Bo Xu, Jing Yang, Karen Kie Yan Chan, Shanqian Huang, Lu Dong, John Brownstein and Bing Xu

1Ministry of Education Key Laboratory for Earth System Modelling, Center for Earth System Science, Tsinghua University, Beijing, PR China
2State Key Laboratory of Remote Sensing Science, College of Global Change and Earth System Science, Beijing Normal University, Beijing, PR China
3Ministry of Education Key Laboratory for Biodiversity and Ecological Engineering, Beijing Normal University, Beijing, PR China
4Boston Children’s Hospital, Harvard Medical School, Boston, MA, USA
5Department of Geography, University of Utah, Salt Lake City, UT, USA

The current epidemic of highly pathogenic avian influenza H5N1 virus is considered to pose a significant threat to the health of wild and domestic avian species, and even to human beings. The Black Sea–Mediterranean Flyway is one of the most important epidemic areas of H5N1. However, the epidemic along this flyway has not been fully explored. To better understand the role of hosts in the spread and evolution of H5N1 virus along the flyway, a phylogeographic study was conducted using haemagglutinin (HA) gene sequences obtained during 2005–2013. To infer phylodynamic spread in time and space, we used a flexible Bayesian statistical framework and modelled viral spatial diffusion as a continuous-time Markov-chain process along time-measured genealogies. Our results revealed that H5N1 virus isolated from wild birds showed an increase in genetic variation of HA gene from 2005–2007. The mean genetic distance of viruses isolated from poultry reached its peak in 2010, and dropped in 2011, increasing again in 2012–2013. The reconstruction of virus circulation revealed a different viral-migration network of H5N1 virus by different hosts. Western Russia constituted a link in viral migration from Russia to Europe and Africa. Cross-species transmission of H5N1 viruses predominated in the migration network of the Black Sea–Mediterranean Flyway. This might be due to the migration of birds across long distances and interaction between local poultry and migratory birds. Additionally, the short-distance spread of H5N1 viruses among poultry followed local transportation networks. Such findings will aid in developing effective disease control and prevention strategies.

INTRODUCTION

The global spread of highly pathogenic avian influenza (HPAI), H5N1, is considered a major health threat to wild birds, poultry, animals and humans. The remarkable genetic diversity and extensive genetic reassortment characteristics of H5N1 viruses have led to their widespread distribution (Guan et al., 2009; Vijaykrishna et al., 2008). H5N1 viruses were grouped into different clades based on phylogenetic characterization and sequence homology of the HA gene (WHO, 2008). These clades of H5N1 virus have different geographic characteristics (Tian et al., 2015a). Among all H5N1 clades, clade 2.2 viruses were characterized by their diverse geographical distribution (Li et al., 2014) across the most affected regions, including Asia (Kang et al., 2011), Europe and Africa (Alexander, 2007; Brown, 2010).

The initial death of close to 6000 birds at Qinghai Lake in 2005 (Kou et al., 2009) was due to H5N1 virus which had
spread north to Russia, and to European and African countries (Hulse-Post et al., 2005; Kilpatrick et al., 2006). Since then, H5N1 HPAI clade 2.2 virus has become the dominant virus on the flyway, raising serious public health concerns. Even now, chickens and ducks in Egypt still suffer from disease due to clade 2.2.1.1 viruses.

Clade 2.2 remains the dominant lineage on the Black Sea–Mediterranean Flyway and has raised a serious public health concern. Currently, there is limited knowledge about the circulation patterns of H5N1 viruses among different hosts along the Black Sea–Mediterranean Flyway. It is therefore necessary to understand the geographical dispersion of these viruses in order to identify regions that play an important part in their migration and evolution. We studied the distribution, migration and evolution of H5N1 viruses carried by poultry and wild birds along the Black Sea–Mediterranean Flyway from a phylogeographical perspective.

RESULTS

Evolution of H5N1 viruses along the Black Sea–Mediterranean Flyway

The genetic distance reflected the virus divergence (Fig. 1a, b). H5N1 HPAI viruses were detected among poultry on the Black Sea–Mediterranean Flyway in late 2005, with almost 20 sequences in Russia, Europe and Turkey obtained from viruses in poultry during that year. Since 2005, as the severity of the epidemic increased, a significant growth in the number of sequences was noted as was an increase in the genetic distances between these sequences (Fig. 1a). The mean genetic distance reached its peak in 2010, and plummeted in 2011, gradually increasing again in 2012–2013. Most importantly, the viruses detected within this period were only isolated in North Africa after 2009. Similar to the evolution of viruses isolated in poultry, the evolutionary pattern of H5N1 isolated in wild birds showed a rapid increase from 2005 onwards, peaking in 2007 (Fig. 1b). After 2007, few viruses were detected in wild birds.

We constructed a temporally structured maximum-clade-credibility phylogenetic tree. The tree was colour coded according to the six geographical regions where viruses were sampled (Fig. 1c, d). Viruses isolated from wild birds belonged to two main clades, clade 2.2 and clade 2.2.1, which showed a temporal and spatial pattern in the phylogenetic tree (Fig. 1d). Since the first H5N1 HPAI virus was isolated in 2005 in the Central Russian region, these viruses have spread westwards and southwards. They have been detected along most of the Black Sea–Mediterranean Flyway, infecting both wild birds and poultry (Fig. 1d). The viruses appear to have quickly evolved from clade 2.2 to clade 2.2.1 upon arrival in Turkey and other African countries in late 2005 and in 2006 (Fig. 1d). As a new sub-clade emerged, existing clade 2.2 viruses were not replaced but rather coexisted with subclade 2.2.1. Clade 2.2 and clade 2.2.1 viruses were the predominant clades circulating in the study area in the following 2 years. After 2008, few viruses were detected in the northern part of the study region (Fig. 1d). However, viruses in North Africa continued to evolve and a new sublineage emerged, namely, clade 2.2.1.1. The dominant clades became clades 2.2.1 and 2.2.1.1. Interestingly, unlike clades 2.2 and 2.2.1 that showed a wide distribution, clade 2.2.1.1 viruses only occurred in North Africa (Fig. 1d).

In general, viruses belonging to clades 2.2 and 2.2.1 were predominant among both wild birds and poultry along the Black Sea–Mediterranean Flyway in 2005 to 2007/2008, according to the phylogenetic trees (Fig. 1d). In 2007, clade 2.2.1.1 viruses were detected in poultry. After 2007, most viruses, particularly those infecting poultry, belonged to clades 2.2.1 and 2.2.1.1. Clade 2.2.1.1 viruses were locally persistent in North Africa.

Spatial dispersion of H5N1 viruses along the Black Sea–Mediterranean Flyway

We reconstructed the migration network and spatial-dispersion patterns of H5N1 viruses along the Black Sea–Mediterranean Flyway (Fig. 2). In general, three major migration pathways of H5N1 viruses were detected (Fig. 2). The predominant pathway of the gene flow was inter-regional virus transmission among wild birds and poultry. The observable transmission routes of viruses from wild birds to poultry were from Western Russia to Europe and West Africa, which are strongly supported by a bayes factor (BF>10), and from West Africa to North Africa, which are decisively supported (BF>100). Western Russia constitutes a link in viral migration from Russia to Europe and African countries. In addition, viruses circulating among poultry were transmitted from Europe to wild birds in Western Russia, North Africa and West Africa. Another decisively supported pathway was virus transmission among wild birds and poultry in a specific region, such as Central Russia, Western Russia and Europe; the latter pathway represents the gene flow within poultry and wild birds. These include transmission pathways for poultry between neighbouring regions of Europe and Western Russia, Europe and Turkey and from West Africa to Central Russia; the pathway for wild birds was from Western Russia to West Africa.

DISCUSSION

There have been recent peaks of H5N1 outbreaks in African countries (Monne et al., 2015). Contrary to global patterns, there have been continued human fatalities from the H5N1 viruses in Egypt (World Health Organization, 2015). Influenza viruses exhibit rapid evolutionary dynamics, a high mutation rate and rapid replication. This hinders the development of effective prevention and control measures, which are urgently needed. Our study study investigated the transmission and evolution of H5N1 viruses along the Black Sea–Mediterranean Flyway. The findings indicate the
following: (1) The evolutionary dynamics of H5N1 viruses differ among wild birds and poultry. (2) The interaction of wild birds and poultry contributes to the predominant viral migration along this flyway. (3) Virus circulation in African countries is locally persistent and responsible for virus evolution and genetic variation.

Cross-species virus transmission networks

The main pattern found was the cross-species transmission of H5N1 viruses between wild birds and poultry. This is due to the migration of birds, the poultry trade and contact between wild birds and poultry. Evidence has shown that during the endemic in the winter of 2005–2006, the high density of birds along the 0°C isotherm contributed to the spread and geographical distribution of outbreaks (Reperant et al., 2010). Wild birds can carry virus from one place to another, and thus influence viral evolution. This is consistent with the geographical distribution and evolution of H5N1 viruses in the study area.

Evidence supports the proposition that contact among free-grazing poultry (Wang et al., 2013), backyard poultry (Jiang et al., 2014) and wild birds (Gilbert et al., 2006) has contributed to the spread of H5N1 influenza virus. In African countries and Turkey, it is common practice to keep multispecies poultry in a free-range system. Poultry may therefore become infected from close contact with wild birds, and spread virus further to other wild birds and other poultry species (Akanbi & Taiwo, 2014; Wang et al., 2014). In this way, the interactions between wild bird and poultry contributed to the diverse transmission patterns observed. Furthermore, European countries are the global top producers and exporters of poultry products. Poultry import and export may contribute to the regional transmission pattern of viruses from poultry to wild birds and among poultry.

Poultry and local persistence of viruses

The re-emergence and evolution of H5N1 viruses due to the persistence of local viruses is a major concern (Russell et al., 2008). The huge number of poultry and resident bird species, which serve as reservoirs of the viruses, contribute to the local persistence of H5N1 viruses (Hill et al., 2012; Lewis et al., 2015). Since 2008, when clade 2.2.1.1 was first detected in poultry along the flyway, it has developed as a regional strain. Clade 2.2.1.1 virus are endemic and were only detected in North Africa, allowing co-circulation of multiple lineages. In fact, clade 2.2.1 coexisted with clade 2.2.1.1 viruses in this region. Although viruses in poultry were isolated in North Africa after 2009, the genetic distance increased in 2010, then decreased significantly, before increasing slightly again. Local persistence of the viruses may result in the variation of genetic distance. Local practices of small-scale backyard breeding systems and the preference for purchasing freshly slaughtered poultry from poultry markets have placed North Africa at further risk (De Benedictis et al., 2007; Zhang et al., 2014). These practices have led to frequent contacts amongst birds, poultry and humans, in addition to providing ideal conditions for inter- and intra-species transmission (Abdelwhab et al., 2010; Li et al., 2004). Increased efforts should be made toward the prevention and control of viruses in these high-risk areas to effectively minimize the next epidemic. The selection of suitable vaccine candidates may, however, prove difficult, as local virus persistence could cause co-circulation of multiple lineages (Tian et al., 2015b). Such problems have already been encountered by Abdelwhab and colleagues, who reported the failure of commercially available H5 poultry vaccines (Abdelwhab et al., 2010).

Roles of different regions in the virus network

Studies have shown that H5N1 viruses were introduced from Asian countries by migratory birds (Olsen et al., 2006), then spread to Western Russia and continued westwards and southwards. Russia (Central Russia and Western Russia) needs to be vigilant to prevent and control the outbreak of H5N1 virus infection in case of another wave of avian influenza epidemic along the Black Sea–Mediterranean Flyway. Wild birds along the 0°C isotherm are important monitoring targets. European countries are both migration and breeding sites for wild birds (Gaidet et al., 2008; Reperant et al., 2010). In conjunction with its extensive poultry industry, Europe also needs to be vigilant and carry out active surveillance to monitor potential outbreaks. Large-scale poultry production, poultry-feeding systems (e.g. backyard feeding systems) and live poultry markets increase contact between wild birds and poultry, and among poultry species. Increased control measures and strict animal welfare policies need to be implemented in the poultry industry, particularly in Turkey and African countries, to reduce the risk of future epidemics.

Limitations

There are some limitations in this research. The sequence data and outbreak cases represent active monitoring data (Peterson, 2006; Peterson & Williams, 2008; Wilking et al., 2009). Extensive transmission of the viruses and high prevalence were detected, but if an outbreak occurred but remained undetected it would still be recorded in this monitoring system (Wilking et al., 2009). Virus migration within each region was not investigated and future studies should focus on both spatial and temporal migration of H5N1 viruses. Subsequent studies should also analyse the interaction between poultry and human activities to tackle the issue of human H5N1 infections. Finally, further in-depth analysis of the migration network along the Black Sea–Mediterranean Flyway is required.

METHODS

Virus sequence data. The haemagglutinin (HA) genes of H5N1 viruses were used to study the migration pattern of these viruses. To
analyse the period 2005–2013, 881 HA sequences of at least 1500 nucleotides were obtained from the GenBank database affiliated to the National Center for Biotechnology Information (NCBI) (Table S1, available in the online Supplementary Material). Hosts were classified into wild birds and poultry groups according to species.

We divided the Black Sea–Mediterranean Flyway into six regions: Central Russia (including the central part of Russia, and Kazakhstan), Western Russia, Europe, Turkey, West Africa (including Nigeria and its neighbouring countries) and North Africa (including Egypt and its neighbouring countries). As shown in Fig. 1, Central Russia, West Africa and North Africa are three discrete regions in the study area. Europe, Western Russia and Turkey, in the middle section of the study area, have been divided into three regions following natural boundaries (such as Caucasus Mountains, Black Sea, Caspian Sea, Aegean Sea and Mediterranean Sea, Carpathians). These boundaries could influence human activity and bird migration, consequently affecting virus evolution and transmission.

Identical sequences were removed from the database. In total, 768 virus sequences hosted by poultry and 113 virus sequences hosted by wild birds were selected. The sequence datasets of viruses isolated from wild birds and poultry were grouped into six regions.

**Phylogenetic analysis.** We employed MAFFT (multiple alignment using fast Fourier transform) to align the sequences (Katoh et al., 2002). The pairwise genetic distance was calculated using MEGA version 5.2 software (Fig. 1a, b). Bayesian phylogeographic trees were generated via BEAST. The trees were colour coded according to the six geographical regions where viruses were sampled and also to the host species.

**Fig. 1.** The evolution and genetic diversity of H5N1 viruses along the Black Sea–Mediterranean Flyway. (a) Genetic distance of viruses isolated in poultry. (b) Genetic distance of viruses isolated in wild birds. (c) Study area along the Black Sea–Mediterranean Flyway. The black points denote the locations of H5N1 outbreaks (http://empres-i.fao.org/eipws3g/). (d) The phylogenetic tree of H5N1 HA gene sequences. The analysis was based on coding nucleotides 1–1701 of HA genes. The tree was midpoint rooted. Viral strains are represented by coloured circles to indicate their locations. The blue area is Central Russia, including the central part of Russia, and Kazakhstan. The dark green area is Western Russia. The orange area is Western Russia. The red area is Turkey. The light purple area is North Africa, including Egypt and its neighbouring countries. The cyan area is West Africa, including Nigeria and its neighbouring countries. The black points denote viruses isolated from wild birds. No viruses were isolated from wild birds in Turkey.

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**Table S1.**

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(Fig. 1d). Virus strains were classified into clades according to the unified nomenclature system (WHO, 2008).

A Markov chain model was constructed to estimate the rates of viral migration across regions. The general patterns of H5N1 virus circulation were reconstructed. The model was restricted to the six previously identified regions. In this analysis, we applied a constant-size coalescent tree prior to the phylogeny and a HKY85 nucleotide substitution model (Lemey et al., 2009; Rabaa et al., 2010). The MCMC chain was run for 50 million iterations, with sub-sampling every 10,000 iterations. The most relevant migration pathways between locations were identified by applying the Bayesian stochastic search variable selection (BSSVS) approach. BSSVS was employed to reduce the number of parameters to those with significantly non-zero transition rates (Lemey et al., 2009). This explores and efficiently reduces the state space by employing a binary indicator (I). A Bayes factor (BF) test can be applied to assess the significance of individual rates. The BF is a function of I and is deemed statistically significant when I>0.5 and the BF>6. Supported state transitions when the mean I was <0.5 were indicated with a dashed line. In this analysis, BF>100 indicates statistically decisive support, 30<BF≤100 indicates very strong support, 10<BF≤30 indicates strong support.

**ACKNOWLEDGEMENTS**

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