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Diffusion of influenza viruses among migratory birds with a focus on the Southwest United States

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ABSTRACT

The Southwest United States, including Arizona and New Mexico, has a diverse climate and is home to many different avian species. We sequenced the hemagglutinin (HA) gene of twenty influenza specimens for the years 2007–2009. This included four from Arizona, and sixteen from New Mexico. We analyzed the sequences and determined the following HA subtypes: H3, H4, H6, H8, and H11. For each subtype, we combined our virus sequences with those from a public database, and inferred phylogeographic models of influenza diffusion.

Statistical phylogeography indicated that overall evolutionary diffusion of avian influenza viruses is geographically structured ($p < 0.05$). In addition, we found that diffusion to the Southwest was often from nearby states including California.

For H3, H4 and H6, the intra-flyway gene flow rates were significantly ($p < 0.001$) higher than those of inter-flyway. Such rate difference was also observed in H8 and H11, yet, without statistical significance ($p = 0.132$, $p = 0.190$, respectively). Excluding any one flyway from the calculation generated similar results, suggesting that such barrier effect on gene flow rates is not exclusively produced by any single flyway.

We also calculated the Bayes factor test for the significant non-zero rates between states and identified significant routes both within and across flyways. Such inter-flyway spread of influenza was probably the result of birds from four flyways co-mingling on breeding grounds in northern regions or marshaling on staging areas post breeding in Canada or Alaska, before moving south each fall.

This study provides an initial analysis of evolutionary diffusion of avian influenza virus to and from the Southwest United States. However, more sequences from this region need to be generated to determine the role of host migration and other factors on influenza diffusion.

Abbreviations: AIV, avian influenza virus; IRD, influenza research database; NCBI, National Center for Biotechnology Information; RNA, ribonucleic acid; cDNA, complimentary deoxyribonucleic acid; PCR, polymerase chain reaction; USGS, United States Geological Survey; USDA, United States Department of Agriculture; KML, Keyhole Markup Language; AI, association index; PS, parsimony score; KL, Kullback–Leibler; MCMC, Markov chain Monte Carlo; MCC, maximum clade credibility; NIAID, National Institute of Allergy and Infectious Diseases; HA, hemagglutinin; NA, neuraminidase; BF, Bayes factor; BaTS, Bayesian tip-significance testing; LPAI, low pathogenic avian influenza; HPAI, highly pathogenic avian influenza; AK, Alaska; AB, Alberta; AZ, Arizona; BC, British Columbia; CA, California; DE, Delaware; GA, Georgia; IA, Iowa; IL, Illinois; LA, Louisiana; MB, Manitoba; MD, Maryland; MI, Michigan; MN, Minnesota; MO, Missouri; MS, Mississippi; NE, Nebraska; NB, New Brunswick; NL, Newfoundland; NJ, New Jersey; NM, New Mexico; NY, New York; ND, North Dakota; NS, Nova Scotia; OH, Ohio; ON, Ontario; OR, Oregon; PA, Pennsylvania; QC, Quebec; SK, Saskatchewan; SD, South Dakota; TN, Tennessee; TX, Texas; WA, Washington; WI, Wisconsin.

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1. Introduction

The Southwest United States, including Arizona and New Mexico, has a diverse climate (Lenart, 2008) and is home to many different avian species (Sharp, 2012). Arizona, in particular is part of the Pacific flyway which starts in Alaska and extends as far as South America (2013b). While there are four migratory flyways in the United States, the Pacific flyway is believed to pose the greatest risk of introduction of avian influenza (AIV) H5N1 virus due to mixing of birds from Asian countries (Hill et al., 2012b). Conversely, New Mexico is in the Central flyway that includes Colorado, Montana, Texas, Oklahoma, Wyoming, Kansas, North and South Dakota, and north into Canada (2013a). Despite the importance of the Southwest for avian migration, there remains uncertainty about its relationship to the diffusion of avian influenza viruses.

Phylogeography is a field that uses sequence data to model geographic diffusion and genetic diversity over time (Avise, 2000). RNA viruses including influenza are often studied because of their short genomes and rapid rate of nucleotide substitutions (Holmes, 2004). Here, geospatial data such as location of the infected host is used in the model to infer diffusion. While there have been many studies that have characterized influenza viruses in migratory birds in the United States (Bahl et al., 2013; Chen and Holmes, 2009; Cross et al., 2013; Dugan et al., 2008; Girard et al., 2012; Henaux et al., 2012; Hill et al., 2012b; Huang et al., 2014; Ip et al., 2008; Jackwood and Stallknecht, 2007; Koehler et al., 2008; Krauss et al., 2004; Lee et al., 2011; Lewis et al., 2013; Pearce et al., 2010; Ramey et al., 2011; Spackman et al., 2005; Suarez et al., 1999; Widjaja et al., 2004; Wille et al., 2011), none have focused on the Southwestern United States. Lam et al. (2012) studied the migration of influenza viruses among a variety of migratory birds in the United States. The authors obtained 100 samples in California, Washington, and Oregon from the United States Geological Survey (USGS) National Wildlife Health Center's archive, and analyzed with >1000 GenBank genome sequences (Lam et al., 2012). Results showed that diffusion of influenza viruses in the United States was isolated by geographic distance and shaped by flyways of the species (Lam et al., 2012). In another study, Girard et al. (2012) examined the phylogeography of influenza among California and Alaskan migratory birds in the Pacific flyway, and found that the geographic origin had the strongest association with virus phylogeny. A study by Hill et al. (2012b) focused on the relationship between migration strategy and avian influenza spread among mallards in the United States, in particular, the Pacific flyway. The authors found that diversity was greater in California, suggesting that wintering states, such as Arizona, might represent an important area for avian influenza reassortment (Hill et al., 2012b). A more recent study by Bahl et al. (2013) found that flyways play a less important role in the long-term persistence of North American avian influenza virus.

The purpose of this study is to understand the phylogeography of avian influenza among migratory birds in the Southwest United States. In addition to the Southwest, we included publically available sequences from North American isolates in order to obtain a complete picture of virus diffusion. We used the combined sequence data sets to identify the association between geography and virus evolution with a particular focus on the Pacific and Central flyways and the Southwest United States.

2. Materials and methods

2.1. Virus isolation and sequencing

We considered oropharyngeal/cloacal swab samples ($N = 227$) from the USDA National Wildlife Disease Program Avian Tissue Archive. All of the samples originated in Arizona (69/227) or New Mexico (158/227) from 2007 to 2011 and previously tested positive for avian influenza virus by real-time reverse-transcription polymerase chain reaction (rRT-PCR) for the matrix gene at their respective state laboratories. We stored the samples at −80 °C in the archive until use for this study. We inoculated each sample into 9–11 day old specific pathogen-free embryonated chicken eggs and incubated at 37 °C for 72 h. We conducted a second passage on all samples. We then harvested amniotic allantoic fluid from each egg and assayed for hemagglutination. We identified thirty-one hemagglutination positive virus isolation samples by rRT-PCR for the matrix gene and extracted RNA using the Ambion MagMAX™ AI/ND Viral RNA Isolation Kit (Life Technologies, Carlsbad, CA).

2.2. Amplification of hemagglutinin gene

We used Superscript II reverse transcriptase (Life Technologies, Carlsbad, CA) to synthesize cDNA from the RNA extracts using the manufacturer's protocol. We utilized the Uni12 primer (5′ – AGCAAAAGCAGG – 3′) designed by Hoffmann et al. (2001) in the reverse transcription reaction. We then included Hoffman's (Hoffmann et al., 2001) universal primers *Bm-HA-1* (5′ – TATTCGTCTCAGGGAGCAAAAGCAGGGG – 3′) and *Bm-NS-890R* (5′–ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT – 3′) to amplify the entire HA region of the influenza genome. We performed the reaction on a C1000 Touch™ thermal cycler (BioRad Carlsbad, CA) and included an initial denaturation phase of 4 min at 94 °C followed by 30 cycles of: denaturation at 94 °C for 20 s, annealing at 58 °C for 30 s, and extension at 72 °C for 7 min. This was followed by a final extension at 72 °C for 7 min. We ran a 1% agarose gel with GelRed™ stain (Biotium, Hayward, CA) for 45 min at 100 v and then used a Gel Doc™ XR + system (BioRad, Hercules, CA) for imaging. We excised bands on a transilluminator (VWR, Radnor, PA) and purified the DNA by using the E.Z.N.A.® Gel Extraction kit (Omega Bio-Tek, Inc., Norcross, GA) according to the manufacturer's protocol.

We used the CloneJET kit (Thermo Scientific, Waltham, MA) to perform blunting and ligation reactions according to the manufacturer's protocol. We transformed One Shot® TOP10 Chemically Competent *Escherichia coli* cells (Life Technologies, Carlsbad, CA) and incubated them with 250 µl of SOC media at 37 °C and 225 rpm for 1 h. We then plated 25 and 75 µl of the cell mixture on pre-warmed LB-amp plates and incubated them overnight at 37 °C.

We visually inspected the plates for the presence of colonies, added them to 3.5 ml of LB-amp (in 14 ml culture tubes), and incubated them overnight at 37 °C and 225 rpm. We screened colonies by performing PCR with the forward and reverse primers provided in the CloneJET kit. We ran the reaction on a C1000 Touch™ thermal cycler and included an initial denaturation phase of 3 min at 95 °C followed by 25 cycles of: denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 2 min. This was followed by a final extension at 72 °C for 2 min. We ran a 1% agarose gel with GelRed™ stain for 45 min at 100v and then used a Gel Doc™ XR + system (BioRad, Hercules, CA) for imaging. We extracted plasmids using a PureYield™ Plasmid Miniprep kit (Promega, Madison, WI) according to the manufacturer's protocol. We prepared a sequencing reaction using primers from the CloneJET kit and sent the plasmid template and primers to the sequencing lab at Arizona State University for Sanger sequencing.

We found reliable 5′ and 3′ reads to be about 800–850 bp. Since the hemagglutinin gene is approximately 1778 bp in length, we prepared a third sequencing reaction to capture the gap in the middle of the coding region. Here, we designed a primer on the 5′ sequence using Geneious (Biomatters, Auckland, NZ). We used Geneious to perform assembly and identify contigs and then concatenated the three sequences. We used the National Center