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Genetic characterization of avian-origin H3N2 canine influenza viruses isolated from Guangdong during 2006–2012

Heng Wang · Kun Jia · Wenbao Qi · Minze Zhang · Long Sun · Huanbin Liang · Guohao Du · Likai Tan · Zhenwen Shao · Jiahui Ye · Lingshuang Sun · Zhenpeng Cao · Ye Chen · Pei Zhou · Shuo Su · Shoujun Li

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Abstract Canine influenza virus (CIV) is an emerging pathogen that causes severe and acute respiratory disease in dogs. In 2006, the H3N2 canine influenza virus was first identified in dogs from Guangdong province in China. Up to now, nine CIVs have been isolated from different populations in Guangdong. The nine isolates were grouped together with the canine H3N2 viruses isolated from dogs and felines in Korea, when the eight phylogenetic trees constructed were compared. These findings emphasize the importance of CIV surveillance in this region for understanding the genesis of this virus, and it is important to remain aware of the potential of H3N2 CIV to be transmitted from dogs to the human population.

Keywords Canine influenza virus · Pet dogs · Farmed dogs · Feral dogs

Introduction

Influenza A viruses infect a wide range of birds and mammalian hosts, and vary in their degree of host adaptation [1–3]. It is of global concern to be aware of the emergence of novel influenza virus subtypes and their interspecies transmission to prevent influenza epidemics and pandemics. Direct transfer of an essentially unaltered virus from one species to another is a fundamental mechanism in interspecies transmission of influenza viruses [4, 5]. In 2004, a laboratory-confirmed case of equine-origin H3N8 influenza A virus was reported in a dog [6]. Since the isolation of H3N8 canine influenza virus from racing greyhounds in 2004, the virus has been reported to cause influenza outbreaks among pet dogs in the USA [3]. The transmission of H3N8 equine influenza viruses to dogs has been reported in Australia and the United Kingdom. In 2007, the Republic of Korea reported another canine infection, in a pet dog, that was caused by an H3N2 canine influenza virus (CIV) of avian origin. Under experimental conditions, dogs are infected via nasal inoculation or contact (respiratory fluid exchange) [7]. In China, the first CIV strain isolated was an H3N2 subtype influenza virus of avian origin that was isolated from a pet dog in Guangdong province in 2006. Most outbreaks of canine influenza have been associated with pet dogs that attend veterinary clinics in southern China, and all these isolates were H3N2 of avian origin [4, 6]. During the last decade, reports of influenza A virus infection in dogs have drawn considerable attention from veterinary practitioners, virologists, and epidemiologists. The emergence of H3N2 CIV infection of avian origin in China may have resulted from ecological changes in China. Socioeconomic circumstances have been changing over the last 20 years in this country. More and more dogs are kept as companions and also raised for food, particularly in densely populated areas [4]. Recently, avian-origin CIV infections in pet and farmed dogs had been reported worldwide [6, 8–15].

Besides the evidence of H3 subtype influenza infections in dogs, other subtypes that may pose a public health

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Heng Wang, Kun Jia, and Wenbao Qi contributed equally to this work.

H. Wang · K. Jia · W. Qi · M. Zhang · L. Sun · H. Liang · G. Du · L. Tan · Z. Shao · J. Ye · L. Sun · Z. Cao · Y. Chen · P. Zhou · S. Su · S. Li (✉)
College of Veterinary Medicine, South China Agricultural University, Guangzhou, Guangdong 510642, China
e-mail: shoujunli@scau.edu.cn

S. Su
e-mail: ssh5658485@163.com

hazard have also been found in domestic dogs. For example, during the H1N1/2009 influenza virus pandemic in humans toward the end of 2009, dogs in the USA and China were reported to be clinically infected with pandemic H1N1/2009 influenza virus following close contact with infected humans.

We carried out molecular biology and epidemiological characterization of nine avian-origin H3N2 CIV strains which were isolated from pets, farmed and feral dogs in Guangdong, China from 2006 to 2012.Gene sequences of the nine virus strains were used to determine mutation frequencies associated with replication of the virus in canine hosts.

Materials and methods

Samples

We reported four cases of H3N2 canine influenza in southern China, which were identified in sick pet dogs from May 2006 to October 2007. The dogs showed similar signs of sneezing, copious nasal discharge, coughing and low fever on presentation to the clinics. The patients were treated with ribavirin, and they recovered from the disease. In order to monitor the prevalence of the avian-origin H3N2 CIV in different dog populations, six large-scale dog-rearing farms were selected in six different cities in Guangdong province. The six farms were chosen on the basis of their population size and geographic location, and included four farms in Guangdong province that had the highest productivity in terms of dog rearing. A total of 360 nasal swabs were collected from farmed dogs, and 100 nasal swabs were collected from free-roaming dogs. In addition, 500 nasal samples were collected from 50 different veterinary hospitals located in the same cities. All animal research was conducted under the guidance of the Centers for Disease Control and Prevention (CDC)'s Institutional Animal Care and Use Committee in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The study was approved by the Guangdong Province Animal Disease Control Center.

Virus isolation

Embryonated SPF chicken eggs (9–10 days old) were inoculated with nasal swab samples for virus isolation. The allantoic fluid was harvested after incubating for 72 h at 37 °C. The virus was identified with a one-step reverse transcriptase polymerase chain reaction (RT-PCR) using specific primers [16]. A hemagglutination test was performed to determine the presence of virus.

RNA extraction and RT-PCR

Genomic viral RNA was extracted from infected allantoic fluid using TRIzol reagents (Invitrogen, USA as per the manufacturer's manual). The RT-PCR was carried out using a Uni12 primer (AGCAAAGCAGG) as described previously [17]. The eight viral gene segments (NA, HA, NS, NP, PA, PB1, PB2, and M) were amplified by PCR using primers specific for each [17]. The PCR products were purified with the Agarose Gel DNA Purification Kit (TaKaRa, Dalian), and cloned subsequently into a pMD18-T vector, followed by sequencing (TaKaRa, Dalian).

Sequence analysis

Reference sequences were obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Comparisons of nucleotide and deduced amino acid sequences were made using DNASTAR software. The phylogenetic trees were generated with MEGA 5.0 software using the neighbor-joining method. Bootstrap values were calculated based on 1,000 replicates of the alignment.

Viruses

Nine H3N2 canine influenza viruses were isolated from different dog populations in Guangdong during 2006–2012 and designated: A/canine/Guangdong/01/2006(H3N2), A/canine/Guangdong/02/2006(H3N2), A/canine/Guangdong/01/2007(H3N2), A/canine/Guangdong/02/2007(H3N2), A/canine/Guangdong/1/2011(H3N2), A/canine/Guangdong/2/2011(H3N2), A/canine/Guangdong/3/2011(H3N2), A/canine/Guangdong/4/2011(H3N2), and A/canine/Guangdong/5/2011(H3N2).

Results

Analysis of similarity of nucleotide sequences

The viral genomes from nine isolates were amplified, sequenced and submitted to GenBank, except for the PB1 gene of A/canine/Guangdong/4/2011(H3N2), which has not yet been sequenced. The GenBank accession numbers for the nine viruses genes are from: GU433345 to GU433376, JX195341 to JX195347, JX195348 to JX195355, JX195356 to JX195363, JX414229 (M gene of A/canine/Guangdong/4/2011), JX414230 (NA gene of A/canine/Guangdong/4/2011), JX414239 to JX414243, and JX414244 to JX414251. Their sequence similarity was determined by comparisons with sequences available in GenBank. Each of the canine H3N2 influenza isolates originated from avian influenza virus (AIV). There was a high degree of genetic similarity (>97 %) in eight genes among the nine Guangdong canine

isolates. They were also highly homologous (97.2–99.3 %) with H3N2 CIVs circulating in Korea and southern China.

Amino acid analysis

The deduced amino acid sequences of our isolates obtained in 2011 were aligned and compared with those of other avian-origin H3N2 viruses [including the A/canine/Guangdong/01/2006(H3N2), A/canine/Guangdong/02/2006(H3N2), A/canine/Guangdong/01/2007(H3N2) and A/canine/Guangdong/02/2007(H3N2), which were obtained by this laboratory from 2006 to 2007]. The H3N2 influenza viruses isolated from avian species and 2006–2007 canine isolates of this study contained the PEKQTR/G motif at the HA cleavage site. A single amino acid substitution (lysine to arginine) occurred at the −4 position of all sequences isolated since 2009, resulting in a PERQTR/G motif. The nine avian-origin H3N2 CIV isolates were compared with the most similar strain, A/canine/Jiangsu/02/2010 (H3N2). None of the mutations was found to occur in proposed antigenic sites, receptor-binding sites, and potential glycosylation sites, although mutations were found in other sites. The results revealed that few changes had occurred in CIVs after several years of spread.

Five H3N2 CIVs isolated after 2011 contained an insertion of two amino acids (positions 74 and 75) in the NA protein. A substitution at one of the five amino acids (positions 26, 27, 30, 31, and 34) within the transmembrane domain of M2 in all five isolates has been shown to confer resistance to amantadine. As with H3N2 avian influenza viruses, our nine canine isolates have 627E in the PB2 protein, while human influenza viruses have K at this position.

Phylogenetic analysis

Phylogenetic analysis was carried out by analyzing and comparing the sequences to those of other influenza viruses

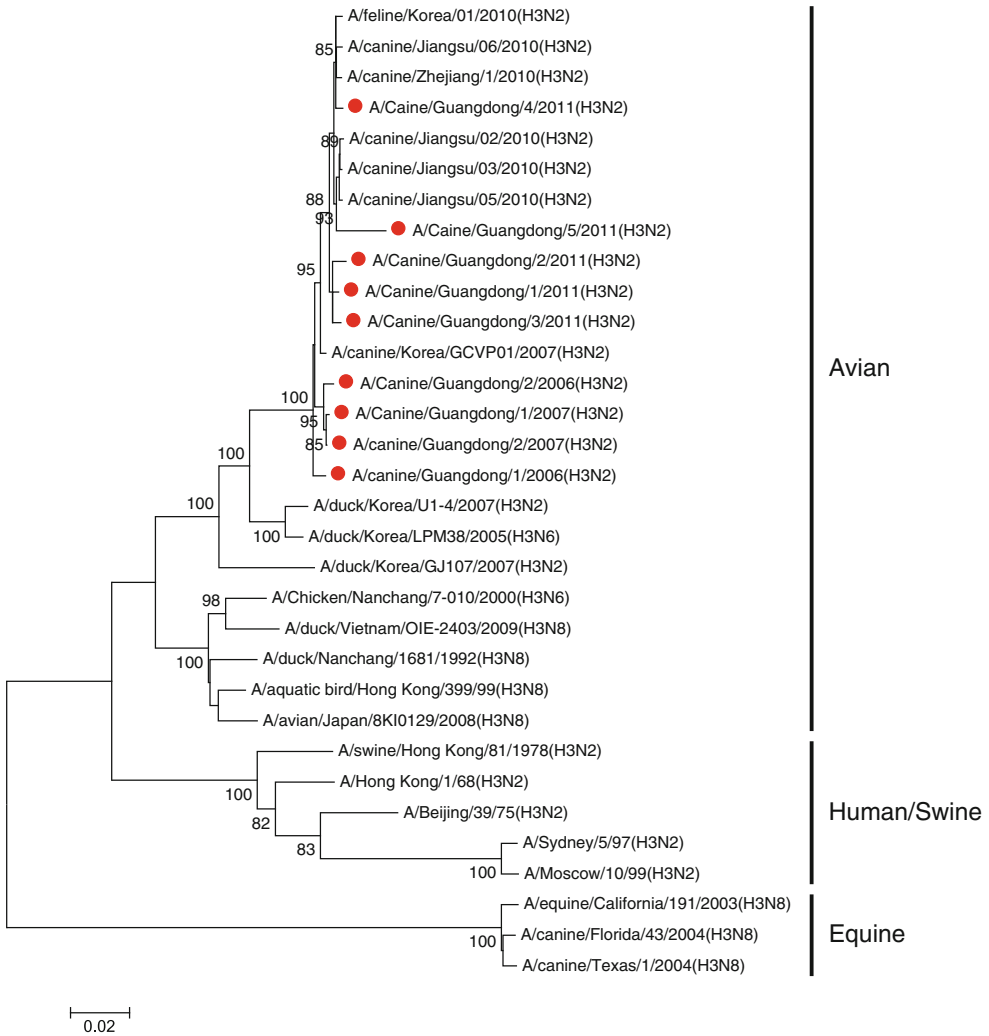

Fig. 1 Phylogenetic trees for eight H3N2 influenza A virus genes. The analysis was based on the nucleotide sequences in open reading frames of the HA gene. Trees were generated with the MEGA program (version 5.0), using neighbor-joining analysis

obtained from the GenBank database. A neighbor-joining nucleic acid tree was generated which divided the gene segments into three subgroups correlating with avian lineages, human and swine lineages, and equine lineages (Figs. 1, 2). When the eight phylogenetic trees constructed were compared, the nine viruses isolated between 2006 and 2012 in Guangdong province were grouped together with the canine H3N2 viruses isolated from dogs in Korea and China. In addition, all eight genes of the nine viruses were most closely related to the Jiangsu and Korea canine isolates (SUP.1–SUP.6). Phylogenetic analysis showed that the five recent isolates were most similar to the feline isolate from Korea, whereas the four earlier isolates were most similar to the canine isolates from Korea. This difference indicated that the most recently isolated CIVs and the CIV isolates of 2006–2007 in Guangdong might have been the result of separate introductions from Korea. The five recent isolates and the four earlier isolates formed a sub-clade that was separated from the clades of avian H3 influenza viruses. The internal genes of the CIVs were most closely related to the A/canine/Korea/GCVP01/2007 (H3N2) strain, and they all clustered closely with different Chinese avian influenza viruses.

Discussion

We carried out molecular and epidemiological characterization of nine H3N2 CIV strains of avian origin which were isolated from pet, farmed and feral dogs in

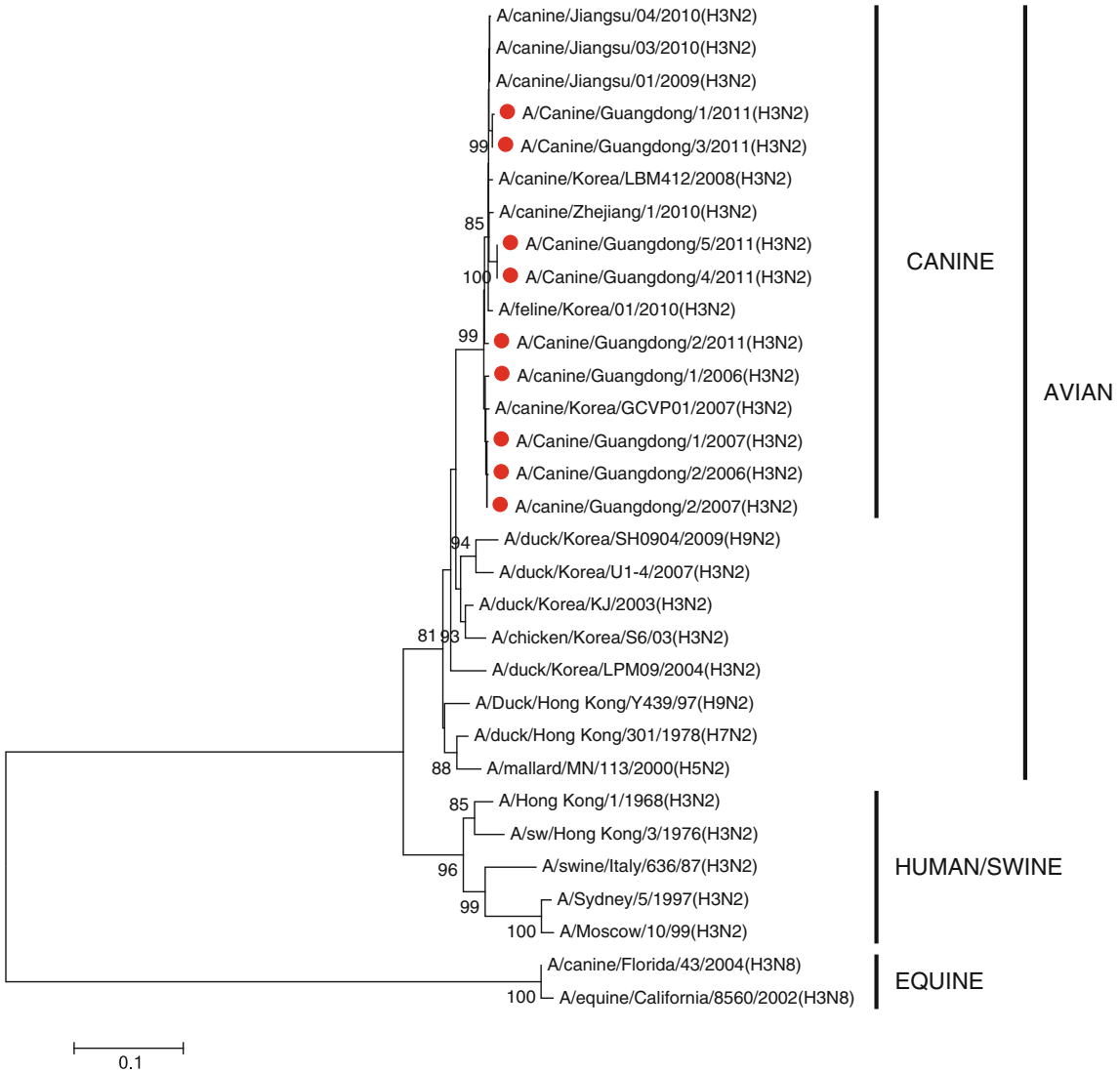

Fig. 2 Phylogenetic trees for eight H3N2 influenza A virus genes. The analysis was based on the nucleotide sequences in open reading frames of the NA gene. Trees were generated with the MEGA program (version 5.0), using neighbor-joining analysis

Guangdong, China from 2006 to 2012. In comparison with the strains that we isolated in 2006 and 2007, several mutations were identified in five new isolates. These variations may have facilitated adaption of the avian influenza virus to mammals. When and how these mutated CIVs obtained the ability to infect dogs remains to be discovered. It is known that H3N2 causes severe and acute respiratory disease in dogs, manifested by fever, cough, rhinorrhea, sneezing, anorexia, depression, and dyspnea. However, it is still unclear whether all CIV infections have clinical manifestations. Hence, whether the new CIV is capable of causing an epidemic among dogs in China is unknown, although it is highly possible. In southern China, there are many large-scale dog farms in poor rural conditions. The close interaction of feral and pet dogs with farmed dogs and the surrounding wildlife provide frequent opportunities for within- and cross-species viral transmission. As a companion animal, the dog is kept in close contact with humans, although the H3N2 CIV is genetically and antigenically different from viruses currently circulating in humans. Whether CIVs have the ability of transmitting to humans remains to be determined; we should nevertheless be aware of the potential for cross-species transmission to occur. Therefore, further in-depth study is required because the H3N2 CIV has become established in different dog populations and poses a potential threat to public health.

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Conflict of interest None.

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