Evolution of H9N2 Influenza A Viruses in Quail From Southern China

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H9N2 influenza A viruses have become established and maintain long-term endemicity in terrestrial poultry in Asian countries. Occasionally these viruses transmit to other mammals including humans. Increasing epidemiological and laboratory findings suggest that quail may be an important host as they are susceptible to different subtypes of influenza viruses. To better understand the role of quail in influenza ecology and evolution, H9N2 viruses isolated from quail during 2000 to 2005 were antigenically and genetically characterized. Our results showed that only three subtypes of influenza viruses (H5, H6, H9) could be detected in quail in southern China. Furthermore, H9N2 viruses are prevalent year-round in southern China with higher isolation rates observed in the winter, and that those viruses mainly replicate asymptomatically in the respiratory tract of quail. Antigenic and genetic analysis revealed that both the G1-like (genotype A series) and Ck/Bei-like H9N2 lineages (genotype B series) were co-circulating in quail since 2000. Phylogenetic analyses demonstrated that most of the isolates tested were double or multiple reassortant variants, with four G1-like (A0-A3) and 16 Ck/Bei-like genotypes (B1-16) recognized. Non-reassortants of Ck/Bei-like viruses were not detected in quail. A novel genotype of G1-like virus, designated as genotype A3, had become predominant in quail since 2003, while multiple Ck/Bei-like genotypes were introduced to quail wherein they incorporated G1-like gene segments, but none of them became established in this host. Those Ck/Bei-like reassortants generated in quail have then been introduced to other poultry. These complex interactions form a two-way transmission system between quail and other types of poultry. The identification of HA and NP genes with high homology to Ty/WI/1/66 in some H9N2 viruses isolated from quail in 2001 suggested that those viruses had not evolved naturally. The present study provides evidence that H9N2 and H5N1 subtype viruses have also exchanged gene segments to generate currently circulating reassortants of both subtypes that have pandemic potential. Continuing influenza surveillance in poultry is critical to understanding the genesis and emergence of potentially pandemic strains in this region.

Introduction

Influenza A H9N2 viruses are present worldwide in poultry populations [1, 2]. In terrestrial poultry of southern China, two H9N2 virus lineages have become established since the mid-1990s. One virus lineage, represented by Ck/Bei/1/94 or Dk/HK/Y280/97, is mainly prevalent in chicken, while the other one, represented by Qa/HK/G1/97, is predominant in quail [3]. Recent studies suggested that quail may have participated in the genesis of the H5N1 virus (H5N1/97-like virus) responsible for the Hong Kong 'bird flu' incident [3] and are also susceptible to different subtypes of influenza viruses [4]. Therefore, quail may play an important role in the evolution and ecology of influenza A viruses.

Results

In the present study, H9N2 influenza viruses isolated from quail from 2000 to 2005 were genetically and antigenically characterized. Our findings showed that both the G1-like and Ck/Bei-like H9N2 influenza virus lineages co-circulate in quail. Novel Ck/Bei-like genotypes were introduced into quail and further reassorted with G1-like viruses endemic in quail. Those H9N2 reassortants with G1-like gene segments have then transmitted to other poultry forming a complex system of two-way transmission between quail and other types of poultry. Genetic analysis also provides evidence that H9N2 and H5N1 subtype viruses have a two-way exchange of gene segments to generate current genotypes of both subtypes that have pandemic potential.

Prevalence of H9N2 Influenza Viruses in Quai. Systematic surveillance of market quail from 2000 to 2005 resulted in 610 influenza isolates from 4,601 samples collected (total isolation rate, 13.3%). Three influenza subtypes were identified, H9N2 (n = 414), H6N1 (n=184) and H5N1 (n=12). H9N2 influenza virus in quail was prevalent year-round but an increased isolation rate was usually observed during the winter season (October to March). Three hundred and ninety-six of 414 (95.7%) of those H9N2 viruses were isolated from tracheal swabs, and only 18 influenza viruses were isolated from the cloacal samples. This information suggests that influenza A virus mainly replicates in the respiratory tract of quail in the field.

Phylogenetic Analysis of the Surface Genes. To understand the evolution and ecology of H9N2 viruses in quail, 73 representative viruses isolated during 2000 to 2005 were genetically characterized. Phylogenetic analysis of the H9 hemagglutinin (HA) gene revealed that 33 of those viruses belonged to the G1-like lineage, while the remaining 40 isolates were closely related to Ck/Bei-like viruses (Figure 1A). The G1-like viruses gave rise to a stable lineage reflecting their long-term endemicity and evolution in this host. Among those Ck/Bei-like viruses two subgroups were recognized in quail since 2000 (Figure 1A). Subgroup 1 consisted of 19 viruses, represented by Qa/ST/243/00, and subgroup 2 consisted of 21 viruses represented by Dk/HK/Y280/97 (Figure 1a). It is noteworthy that a single virus Ck/Heilongjiang/35/2000 (Ck/HLJ/35/00) is almost identical to (99.6% homology) to Ty/Wisconsin/1/1966 (Ty/WI/1/66) [5], an early H9N2 subtype reference strain from the North American lineage. Phylogenetic analysis of the neuraminidase (NA) gene revealed a similar evolutionary patterns as the HA gene tree, with the NA genes of all but one virus corresponding with the lineage of the HA

gene (Figure 1B). The exception was Qa/ST/4762/01 with a Ck/Bei-like HA had a G1-like NA gene, revealing reassortment between the two virus sublineages. These findings show that G1-like viruses have remained endemic in quail, and that Ck/Bei-like viruses were introduced into this species in 2000, wherein they have co-circulated with G1-like viruses.

Phylogenetic Analysis of the Internal Genes. Phylogenetic analysis of the six internal genes revealed that H9N2 viruses from quail in southern China have undergone extensive reassortment to generate multiple novel genotypes (data not shown). In the PB2 gene tree, representative H9N2 viruses clustered into three different lineages; 58 were G1-like, while 14 formed a group from an unknown avian source, likely derived from aquatic birds in the region. One virus, Qa/ST/1038/02, clustered with the H5N1/01-like viruses. Analysis of PB1 gene showed those H9N2 quail isolates formed three distinct lineages, including G1-like (n = 59), Ck/Bei-like (n = 9), and unknown avian (n = 5). Their PA genes also fall into three different groups, with most closely related to H5N1/01-like viruses. The NP gene of the H9N2 viruses separated into four groups: G1-like (n = 37), Ck/Bei-like (n = 14), H5N1/01-like (n = 16) and unknown avian (n = 6). It is interesting to note that the virus Ck/Shanghai/F/1998 (Ck/SH/F/98) contains an NP gene segment that was previously first detected in H5N1 virus in 2001 [6]. Furthermore, the NP gene of Ck/HLJ/35/06 is almost identical (99.9% homology) to Ty/WI/1/66. The M gene of 11 viruses grouped with Ck/Bei-like viruses and the remaining viruses were G1-like. The NS gene of 45 viruses was Ck/Bei-like, while that of the other 28 viruses were closely related to G1-like H9N2 viruses.

Genotyping. Viruses with a G1-like HA are designated as genotype A series and those with a Ck/Bei-like HA as genotype B series. Therefore, non-reassortant G1-like viruses are designated as A0, while reassortant G1-like viruses are designated sequentially as A1, A2, and so on, according to when the novel genotype was first identified. In the same manner, non-reassortant Ck/Bei-like viruses are designated as B0, and novel reassortants then numbered sequentially as B1, B2, and so on. Phylogenetic analysis revealed 20 different reassortant H9N2 genotypes isolated from 2000 to 2005 in quail. Four genotypes were G1-like (genotypes A0-A3), while 16 genotypes were Ck/Bei-like (genotypes B1-B16). In the G1-like lineage, non-reassortant G1-like virus (genotype A0) was detected from 2000 to 2002. Since 2002, H9N2 genotype A3 virus emerged and became predominant in quail and is the only G1-like virus detected in this host since 2003. For Ck/Bei-like viruses, a different compliment of reassortant H9N2 genotypes were detected in quail each year, but none of them became established in this host. These genotypes were all double or triple reassortants of Ck/Bei-like, G1-like, H5N1/01-like and unknown avian viruses, with the exception of genotypes B7, B10 and B15 which are four-way reassortants.

Discussion

Characterization of H9N2 influenza viruses isolated from quail from 6 years of influenza surveillance revealed that both G1-like and Ck/Bei-like viruses were co-circulating in this host in southern China since 2000. Genetic and antigenic studies demonstrated that a single H9N2 G1-like reassortant (genotype A3) had become established and predominant in this host since 2003, indicating these viruses were genetically stable and well adapted to quail. However, the Ck/Bei-like virus lineage (genotype B series) appeared very unstable, with new short-lived reassortants emerging each year, none of which had become established, indicating those viruses were not well adapted to this host. Our findings suggest that after introduced into quail, Ck/Bei-like viruses had further reassorted with G1-like viruses endemic in quail and subsequently transmitted to other poultry. These complex interactions formed a two-way transmission system between quail and other types of poultry. Through this system, quail severed as a "mixing vessel" to facilitate many reassortment events in the current influenza ecosystem. The present study also suggests that H5N1/01-like internal genes were first incorporated into Ck/Bei-like viruses in 2001, and then into G1-like viruses in 2002 (Fig. 2). However, the phylogenetic relationships of the NP genes suggests that a H9N2 virus, Ck/SH/F/98-like, may be a possible donor of H5N1/01-like internal genes. If this is the case then the gene flow between those subtypes may be in the reverse direction. Furthermore, the dominant G1-like virus found in quail (genotype A3), along with all Ck/Bei-like viruses isolated in this study since 2003, incorporate an H5N1/01-like PA gene that is also found in current H5N1 genotype Z viruses [7]. In this regard, quail may serve as a "mixing vessel" to facilitate reassortment events between H9N2, H5N1 and H6N1 viruses to facilitate the emergence of viruses with pandemic potential in this region.

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Figure 1. Phylogenetic relationships of the HA (A) and NA (B) genes of representative influenza A viruses isolated in Asia. Trees were generated by the neighbor-joining method in the PAUP* program (Bayesian analysis revealed similar relationships.) Numbers above and below branches indicate neighbor-joining bootstrap values and Bayesian posterior probabilities, respectively. Not all supports are shown because of space constraints. Analysis was based on nucleotides 129-1042 of the HA gene and 231-1297 of the NA gene. The HA and NA trees were rooted to Qa/Arkansas/29209-1/93 (H9N2) and Ck/Pennsylvania/8125/83 (H5N2), respectively. Genotypes characterized in this study were shown brackets. Scale bar, 0.01 substitutions per site. BJ and Bei, Beijing; Ck, chicken; Dk, duck; GD, Guangdong; Gf, Guinea fowl; GX, Guangxi; HLJ, Heilongjiang; HN, Henan; HK, Hong Kong; NC, Nanchang; Pg, pigeon; Ph, Pheasant; Qa, quail; SCk, silky chicken; SD, Shandong; SH, Shanghai; ST, Shantou; Ty, turkey; WDk, wild duck.