

H9N2 influenza viruses from birds used in falconry

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Background H9N2 avian influenza viruses continue to spread in poultry and wild birds throughout Eurasia.

Objectives To characterize H9N2 influenza viruses from pheasants, quail, and white-bellied bustards (WBBs) used to train falcons in the United Arab Emirates (UAE).

Methods Four H9N2 viruses were isolated from pheasants, quail, and WBB used for falconry in the UAE, and antigenic, molecular, phylogenetic analysis, and *in vivo* characterization of H9N2 viruses were performed.

Results and conclusions The pheasant and WBB isolates were antigenically and molecularly clearly related and along with the quail isolates contained multiple “avian–human” substitutions. The release of smuggled H9N2-infected birds for falconry may contribute to the spread of these viruses to wild birds, domestic poultry, and humans.

Keywords Falconry, genetic mixing, H9N2 avian influenza viruses, Middle East, white-bellied bustard.

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Introduction

The avian H9N2 influenza virus that emerged in Asia in the 1990s—presumably from the aquatic bird reservoir—has become enzootic in domestic poultry in many Eurasian countries. These viruses have become ubiquitous in domestic chicken and quail (*Coturnix coturnix*), especially in Central Asia and the Middle East.¹ Although these viruses are classified as low pathogenic avian influenza viruses and alone cause limited disease in infected poultry, they have acquired many characteristics that make them a threat to veterinary and human public health. These characteristics include sporadic transmission to humans and swine,^{2–5} acquisition of receptor-binding characteristics associated with human infection (α -2,6 sialic acid binding),⁶ evolution of multiple stable lineages represented by prototype viruses A/Chicken/Beijing/1/94 (H9N2) and A/Quail/Hong Kong/G1/97 (H9N2),⁷ and high ability for reassortment.¹ Sequence analyses of the HP H5N1 isolated from the 1997 “bird flu” outbreak in Hong Kong have shown that the H5N1 circulating in quails were H9N2 double reassortants.⁸ Also in the H7N9 (2013) human outbreak in China, sequence analyses have shown that the H7N9 has six internal genes from avian origin H9N2 viruses.⁹ Under experimental

conditions, H9N2 viruses are not highly pathogenic in poultry, but can cause reduced egg production and, in conjunction with other diseases, leading to mortality.¹⁰ Therefore, vaccination programs have been implemented in many countries to control H9N2 infection.¹¹

Avian H9N2 influenza viruses are widespread in the Middle East¹ and have a spectrum of different gene constellations that are closely related to those of H9N2 influenza viruses from neighboring countries. The host species of origin of these “rainbow” H9N2 gene constellations are chickens, quail, pheasants, and falcons.¹ Quail and pheasants used for human consumption and for feeding and training of falcons are brought legally and illegally in small cages with white-bellied bustards (WBBs) into the Arabian Peninsula from Central Asia. The five subspecies of WBBs from Mauritania in western Africa to countries in eastern and southern Africa are not found in the Arabian Peninsula. These birds are smuggled into the UAE and sold for falconry.

To study a possible mechanism of genetic mixing and spread of the H9N2 influenza virus in wild birds, we isolated four H9N2 viruses from the Reeves's pheasant (*Syrnaticus reevesii*), common quail (*Coturnix c.coturnix*), and WBB (*Eupodotis senegalensis*) in the UAE and performed antigenic, molecular, phylogenetic analysis, and *in vivo* testing of the

isolates. The smuggling of wild birds for falconry into the UAE may help the spread and generation of H9N2 influenza viruses with diverse gene constellations into the Middle East.

Methods

For our study, H9N2 influenza viruses were isolated in chicken embryos^{2,3} from oropharyngeal swabs taken from diseased quail and pheasants that showed clinical signs such as clear ocular discharge, dyspnea, and anorexia. The WBB from which the virus was isolated had disease signs including conjunctivitis, respiratory signs, dyspnea, and anorexia before death occurred. Antigenic analysis by hemagglutination inhibition assays^{3,5} with post-infection ferret antisera to the reference strains of H9N2 influenza viruses and to the A/Quail/UAE/D1556/2011 (H9N2) revealed that the pheasant and WBB isolates were closely related to each other and very similar to H9N2 viruses isolated from Bangladesh and Pakistan but were distinct from the quail isolate (Table 1). The WBB isolate was antigenically related to the pheasant isolates.

To determine the pathogenic potential of the WBB and quail isolates, five specific pathogen-free white leghorn chickens were each inoculated with 10^6 EID₅₀ of each virus via all three routes (intranasal, intraocular, and intratracheal) at the same time and the birds were observed for 12 days. None of the birds showed clinical signs of the disease during the course of the study. Both viruses replicated to higher titers in the respiratory tract than in the intestinal tract. The WBB isolate replicated in all five birds to moderately high titers ($5.5\text{--}6.5 \log_{10}\text{EID}_{50}/\text{ml}$), whereas the quail isolate infected three of five birds and replicated to slightly lower titers ($\sim 5.5 \log_{10}\text{EID}_{50}/\text{ml}$).

Results and discussion

Phylogenetic analysis^{3,5,6} of the complete sequences of the four H9N2 viruses revealed that the *PB2*, *HA*, *NP*, *NA*, and *NS* genes belonged to the G1 lineage, but the *PB1*, *PA*, and *M* genes of all isolates were related to those of H7N3 viruses from Pakistan¹² (data not shown). All the eight genes of the pheasant and WBB isolates were most closely related to those of H9N2 influenza virus isolates from the Arabian Peninsula. In contrast, most genes in the quail isolate were closely related to those of H9N2 virus isolates from India and Pakistan (Figure 1).

Molecular analysis revealed that except for the quail isolate, the remaining H9N2 isolates carried the Q226L amino acid substitution in the receptor-binding site (RBS) of the HA, which is associated with α -2,6 (human) sialic acid binding⁶ (Table 2). All the UAE viruses had the H183 substitution in the RBS, which is also related to human virus-like receptor specificity.¹³ None of the UAE isolates had the polybasic cleavage site between the HA1 and HA2 of the precursor HA molecule, but the quail isolate had the Arg-Ser-Arg-Arg (R-S-R-R) motif. The pheasant and WBB HAs had seven potential glycosylation sites. The quail isolate had one additional glycosylation site (residue 206). In the internal genes, the UAE H9N2 viruses acquired several substitutions in positions previously described as avian-human signature positions,^{14–16} including E627K an important substitution in the PB2 gene and a key host range and virulence determinant of influenza viruses in mammals, in the quail isolate (Table 2). The M2 protein of the pheasant and WBB isolates carried the S31N substitution, which confers amantadine resistance.¹⁷ The pheasant and WBB isolates showed identical substitutions in protein composition described as avian-human signatures (Table 2). These substitutions were in the

Table 1. Antigenic analysis of H9N2 influenza viruses from UAE

H9N2 influenza viruses	Hemagglutination inhibition titre* with ferret antisera to:					
	G1/97	G9/97	PAK/2434	BD/600	D1556	DE/249
rg-A/Quail/Hong Kong/G1/97 - A/PR/8/34	320	<20	20	<20	<20	20
A/Chicken/Hong Kong/G9/97	<20	640	160	160	<20	160
A/Environment/Bangladesh/600/2008	<20	40	320	320	<20	<20
A/Quail/UAE/D1556/2011	<20	<20	<20	<20	80	<20
A/White-Bellied Bustard/UAE/D1520/2011	<20	<20	80	80	<20	<20
A/Pheasant/UAE/D1521/2011	<20	20	160	320	<20	<20
A/Pheasant/UAE/D1307.B/2011	<20	20	160	160	<20	<20
A/Shorebird/DE/249/2006	<20	<20	<20	<20	<20	80

*Hemagglutination inhibition tests were done with chicken red blood cells using receptor destroying enzyme treated post infection ferret sera according to the WHO manual.¹⁹ Homologous HI titers are indicated in bold.

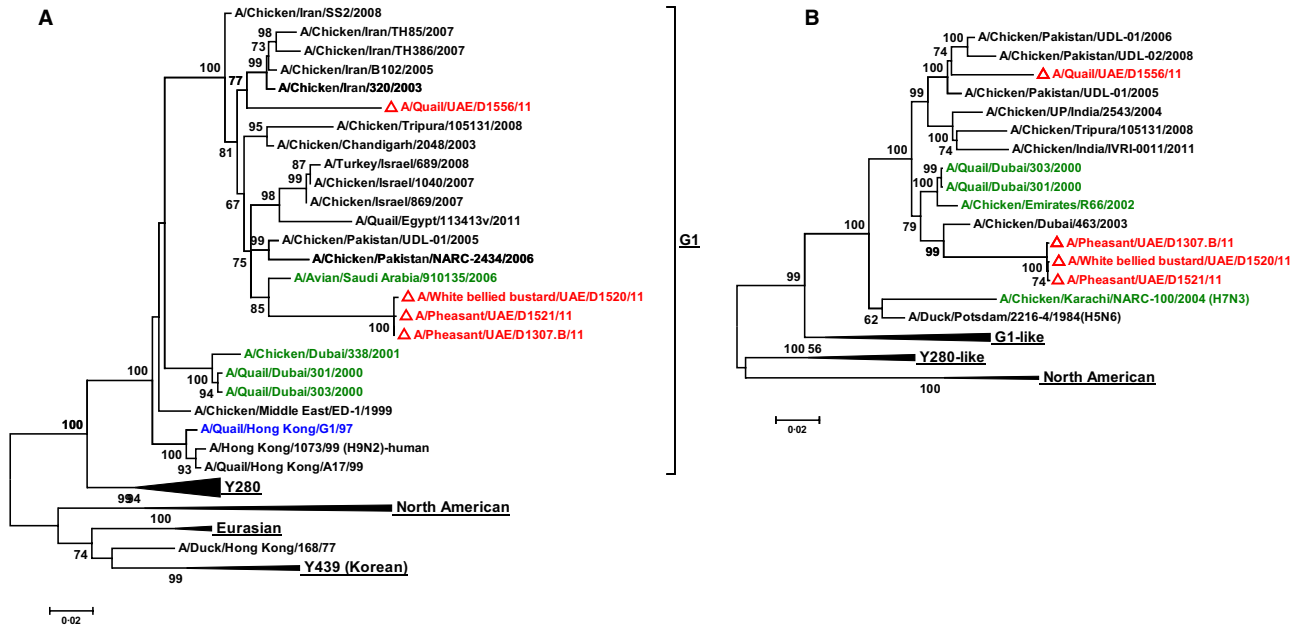


Figure 1. Phylogenetic relationship of HA (A) and PB2 (B) genes of H9N2 avian influenza viruses isolated from the UAE in 2011. The unrooted phylogenetic trees were generated by the maximum likelihood method using MEGA, version 5.1. The reliability of the trees was assessed by bootstrap analysis with 2000 replications. Numbers at the major node are bootstrap values (given as percentage); only bootstrap values >60% are shown. Viruses isolated from the UAE in this study are highlighted in red. Prototype virus of the major sublineage is shown in blue. Viruses isolated from the Middle East that are closely related to those isolated in the UAE are highlighted in green. Scale bar indicates distance unit between sequence pairs.

RBSs of the HA and in the M2, NP, PA, PB1, and PB2 proteins.

Falconry is a prominent pastime in the Middle East, and when smuggled birds of different species are transported together and used for training raptors, they increase the risk of host range transmission to humans and spread to wild and domestic avian species.¹⁸ The isolation of H9N2 influenza viruses from quail, pheasants, and WBB used for falconry in the UAE supports that smuggling of infected birds is a potential mechanism for the mixing and spread of H9N2 influenza viruses. There are no reports on the presence of H9N2 influenza viruses in WBBs in nature. These birds are possibly infected with H9N2 viruses when they are held and transported with other infected birds with H9N2 thereby providing optimal conditions for genetic mixing of influenza viruses and interspecies transmission. Further studies are required to test the hypothesis because this mode of interspecies transmission can facilitate rapid adaptation of influenza viruses to newer host species.

The close antigenic and molecular similarity between H9N2 influenza viruses from WBB and pheasants is suggestive that the virus infecting WBB may come from pheasants, but further studies are necessary to establish this hypothesis. Their antigenic and molecular distinctness from the quail isolate indicates that multiple different H9N2 viruses are cocirculating in the UAE domestic poultry or being imported from neighboring countries. Additionally, it is possible that the H9N2 viruses circulating in the predator species such as

raptors are distinct and not related to the poultry species so it would be interesting to do a detailed phylogenetic analyses to test whether they are unique or not.

Although the H9N2 influenza virus was isolated from a WBB that showed signs of disease, the symptoms may have been the consequence of underlying secondary infection, for the experimental infection of chickens with H9N2 virus was asymptomatic. The replication efficiency of the quail isolate was lower than that of the WBB isolate, and there were no visible signs of disease in the infected chickens.

The continual acquisition of substitutions in the HA and internal gene segments of H9N2 that are considered avian–human signatures emphasizes the need for continued surveillance and characterization of H9N2 influenza viruses. As H9N2 influenza viruses cause limited signs of disease, they receive less attention in veterinary and human public health, but their pandemic potential should be not be ignored especially taking into account the H9N2 contribution to the Asian H5N1 and the co-circulation of H5N1 and H9N2 in the Middle East. Also, as recently shown in the contribution of H9N2 in the emergence of Chinese H7N9 human influenza.⁹

The importance of H9N2 influenza viruses has been largely underestimated in both veterinary and human public health; however, the emergence of the H7N9 influenza virus that caused lethality in humans and possesses six gene segments from H9N2⁹ should alert us to the unknown consequences of spreading H9N2 in birds used for falconry practices.

Table 2. "Avian-human signature" residues in H9N2 influenza viruses isolated from UAE*

HA	Glycosylation site		NA Hemadsorbing site	M1 V15I	M2 Drug Resistance S31N	NS (227) motif	NP R214K	PA S409N	PB1 L13P	PB2 G590S	A661T	K702R						
	HA RBS	HA RBS																
H9	191	234	HA1/HA2 Cleavage site	403														
H3	183H	Q226L																
A/WBB/UAE/ D1520/2011**	H L		R5SR	No	No	W	I	N	G	GSEV	R	D	N	P	S	E	T	R
A/Pheasant/ UAE/D1521/2011**	H L		R5SR	No	No	W	I	N	G	GSEV	R	D	N	P	S	E	T	R
A/Pheasant/ UAE/D1307/B/2011**	H L		R5SR	No	No	W	I	N	G	GSEV	R	D	N	P	S	E	T	R
A/Qual/UAE/ D1556/2011**	H Q		R5RR	Yes	No	W	I	S	K	KPEI	K	D	S	P	G	K	V	K

*Critical amino acids defined by Shaw et al.,¹⁴ Pan et al.¹⁵ supporting mammalian replication.

**The nucleotide sequence of each virus has been submitted to Genbank Accession numbers KC555075-KC555106.

Red amino acids indicate human-like changes.

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References

- 1 Fusaro A, Monne I, Salviato A *et al.* Phylogeography and evolutionary history of reassortant H9N2 viruses with potential human health implications. *J Virol* 2011; 85:8413–8421.
- 2 Peiris M, Yuen KY, Leung CW *et al.* Human infection with influenza H9N2. *Lancet* 1999; 354:916–917.
- 3 Butt KM, Smith GJ, Chen H *et al.* Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. *J Clin Microbiol* 2005; 43:5760–5767.
- 4 Cong YL, Pu J, Liu QF *et al.* Antigenic and genetic characterization of H9N2 swine influenza viruses in China. *J Gen Virol* 2007; 88(Pt 7):2035–2041.
- 5 Peiris JS, Guan Y, Markwell D *et al.* Cocirculation of avian H9N2 and contemporary “human” H3N2 influenza A viruses in pigs in south-eastern China: potential for genetic reassortment? *J Virol* 2001; 75:9679–9686.
- 6 Matrosovich MN, Krauss S, Webster RG. H9N2 influenza A viruses from poultry in Asia have human virus-like receptor specificity. *Virology* 2001; 281:156–162.
- 7 Xu KM, Li KS, Smith GJ *et al.* Evolution and molecular epidemiology of H9N2 influenza A viruses from quail in southern China, 2000–2005. *J Virol* 2007; 81:2635–2645.
- 8 Guan Y, Shortridge KF, Krauss S *et al.* Molecular characterization of H9N2 influenza viruses: were they the donors of the “internal” genes of H5N1 viruses in Hong Kong? *Proc Natl Acad Sci USA* 1999; 96:9363–9367.
- 9 Gao R, Cao B, Hu Y *et al.* Human infection with a novel avian-origin influenza A (H7N9) virus. *N Engl J Med* 2013; 368:1888–1897.
- 10 Brown IH, Banks J, Manvell RJ *et al.* Recent epidemiology and ecology of influenza A viruses in avian species in Europe and the Middle East. *Dev Biol (Basel)* 2006; 124:45–50.
- 11 Lee DH, Song CS. H9N2 avian influenza virus in Korea: evolution and vaccination. *Clin Exp Vaccine Res* 2013; 2:26–33.
- 12 Abbas MA, Spackman E, Swayne DE *et al.* Sequence and phylogenetic analysis of H7N3 avian influenza viruses isolated from poultry in Pakistan 1995–2004. *Virology* 2010; 7:137.
- 13 Sorrell EM, Wan H, Araya Y, Song H, Perez DR. Minimal molecular constraints for respiratory droplet transmission of an avian-human H9N2 influenza A virus. *Proc Natl Acad Sci USA* 2009; 106:7565–7570.
- 14 Shaw M, Cooper L, Xu X *et al.* Molecular changes associated with the transmission of avian influenza A H5N1 and H9N2 viruses to humans. *J Med Virol* 2002; 66:107–114.
- 15 Pan C, Cheung B, Tan S *et al.* Genomic signature and mutation trend analysis of pandemic (H1N1) 2009 influenza A virus. *PLoS ONE* 2010; 5:e9549.
- 16 Monne I, Fusaro A, Al-Blawi MH *et al.* Co-circulation of two sublineages of HPAI H5N1 virus in the Kingdom of Saudi Arabia with unique molecular signatures suggesting separate introductions into the commercial poultry and falconry sectors. *J Gen Virol* 2008; 89(Pt 11):2691–2697.
- 17 Aamir UB, Wernery U, Ilyushina N *et al.* Characterization of avian H9N2 influenza viruses from United Arab Emirates 2000–2003. *Virology* 2007; 361:45–55.
- 18 Bertran K, Busquets N, Abad F *et al.* Highly (H5N1) and Low (H7N2) Pathogenic Avian Influenza Virus Infection in Falcons Via Nasochoanal Route and Ingestion of Experimentally Infected Prey. *PLoS ONE* 2012; 7:e32107.
- 19 World Health Organization Global Surveillance Network. Manual for the laboratory diagnosis and virologic surveillance of influenza. 2011. Available at http://whqlibdoc.who.int/publications/2011/9789241548090_eng.pdf (Accessed 6 November 2013).