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Sialic acid profiles in the respiratory tracts of selected species of raptors: evidence for potential binding sites for human and avian influenza A viruses

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Abstract

Context. The ability of influenza A viruses to recognise and bind to cell surface receptors such as sialic acid linked to galactose by an α2,3 linkage (SAα2,3-gal) and sialic acid linked to galactose by an α2,6 linkage (SAα2,6-gal) is a major determinant of influenza A virus infection. Although the epidemiological surveys of influenza A virus infection in raptors suggest that some raptor species are susceptible to influenza A viruses under natural conditions, the sialic acid profiles in the respiratory and intestinal tracts of raptors are unknown.

Aims. To examine the sialic acid receptor profiles in the respiratory tracts of the selected raptor species and assess the potential susceptibility of raptors to avian and human influenza viruses and the role of raptors in the epidemiology and evolution of influenza A viruses.

Methods. The lectin immunohistochemistry staining method was used to examine the sialic acid profiles in the respiratory tracts of eight different species of raptors.

Key results. A strong staining with *Maackia amurensis* agglutinin (MAA), specific for sialic acid linked to galactose by an α2,3 linkage (SAα2,3-gal), was observed in the epithelial cells of the respiratory tract of *Accipiter nisus* and *Falco tinnunculus*. However, a positive staining for both MAA and *Sambucus nigra* agglutinin (SNA), specific for sialic acid linked to galactose by an α2,6 linkage (SAα2,6-gal), was detected in the epithelial cells of the upper respiratory tract of *Accipiter gularis*, *Buteo buteo*, *Otus sunia*, *Bubo bubo* and *Asio otus*, and in the epithelial cells of the alveoli of *Buteo buteo*, *Falco peregrinus*, *Otus sunia* and *Bubo bubo*.

Conclusions. Both avian and human influenza A virus receptors are expressed in six species of raptors examined. There are some variations in the type and distribution of sialic acid receptor expression among different raptor species. No correlation between phylogeny of birds and their sialic acid receptor distributions was observed.

Implications. Since SAα2,3-gal and SAα2,6-gal are often considered as the primary receptors for avian influenza A viruses and human influenza A viruses, respectively, our data suggest that raptors could be a potential host for avian and human influenza A viruses.

Additional keywords: influenza A virus, MAA, raptor, SNA, SAα2,3-Gal, SAα2,6-Gal.

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Introduction

Wild aquatic birds serve as the natural reservoirs of all known hemagglutinin (HA, H1–H16) and neuraminidase (NA, N1–N9) subtypes of influenza A viruses (Webster *et al.* 1992; Horimoto and Kawaoka 2005). Some of these viruses are non-pathogenic in their natural reservoir hosts. However, these natural hosts may transmit influenza viruses to other species, including humans (Guo 2002; Fouchier *et al.* 2004; Tweed *et al.* 2004). Four human influenza pandemics were caused by viruses derived partially or entirely from avian influenza viruses in the last century (Kawaoka *et al.* 1989; Webster *et al.* 1992; Taubenberger *et al.* 1997; Cameron *et al.* 2000). Most recent data suggest that all the fatal human influenza cases result from the direct transmission of virus from birds to humans (Gambotto *et al.* 2008).

The ability of the influenza A virus to recognise and bind to cell surface receptors, such as sialic acid linked to galatose by an α2,3 linkage (SAα2,3-gal) and sialic acid linked to galatose by an α2,6 linkage (SAα2,6-gal), is a major determinant of influenza virus

infection. The distribution and abundance of sialic acid differs among species of animals and even among different tissues from the same animal species (Suzuki *et al.* 2000). Moreover, virus strains isolated from different host species recognise different linkages between sialic acid and galactose. Influenza A viruses isolated from avian and equine species preferentially bind to sialic acid receptors that are linked to galactose by Neu5Ac-2,3Gal residues, whereas human strains preferentially bind the Neu5Ac-2,6Gal-terminated sugar chains (Rogers and Paulson 1983; Rogers and D'Souza 1989; Connor *et al.* 1994). Therefore, the receptor types expressed in a potential host are a critical factor in determining whether that host is susceptible to infection with influenza viruses. The human upper respiratory tract mainly expresses SAα2,6-gal, while the lower respiratory tract expresses abundant SAα2,3-gal. This may partially explain why avian influenza viruses are not directly transmissible from human to human (Kogure *et al.* 2006; Shinya *et al.* 2006; Nicholls *et al.* 2007; van Riel *et al.* 2007).

Of the 432 species of raptors distributed worldwide, 298 are Falconiformes (e.g. falcons and hawks) and 134 are Strigiformes (owls). All raptors found in China are nationally protected animals. With environmental deterioration and the rapid urban spread into wildlife habitats, opportunities for contact between humans, domestic animals and wild animals increase. Epidemiological surveys of influenza virus infection in raptors suggest that some raptor species are susceptible to both highly pathogenic and low pathogenic influenza viruses under natural conditions (Manvell *et al.* 2000; Aly *et al.* 2010; Van Borm *et al.* 2005; Khan *et al.* 2009; Marjuki *et al.* 2009; Goyal *et al.* 2010). Viruses are usually isolated from both tracheal and cloacal swabs of naturally or experimentally infected raptors (Khan *et al.* 2009; Hall *et al.* 2009). However, the sialic acid profiles in the respiratory and intestinal tracts of raptors are unknown. The goal of this study was to examine the sialic acid profiles of the respiratory tract of the selected raptor species, which will provide information on the potential susceptibility of raptors to avian and human influenza viruses and on the role of raptors in the epidemiology and evolution of influenza A viruses.

Materials and methods

Animals

Tissues were obtained from birds that were killed following rescue from the wild. These birds were diagnosed with conditions that had a poor prognosis for recovery to allow them to return to a successful future in the wild (Table 1). Birds were killed with an overdose of sodium pentothal, under conditions strictly in keeping with international standards of animal welfare. Carcasses were stored at 4°C until necropsy within 24 h.

Tissue preparation

Tissue samples of the pharynx, trachea and lung were collected at the time of post-mortem examination. The quality of the collected tissues was good. Trimmed samples were fixed in 10% neutral buffered formalin for 24 h, then fixation in fresh 10% neutral buffered formalin for another 24 h. Following fixation, tissues were dehydrated, embedded and sectioned. Tissue sections were 3 microns thick.

Table 1. Raptors used in the present study

No.	Species	Ages	Cause of death
1	*Accipiter gularis*	Adult	Killed due to bone fracture
2	*Accipiter gularis*	Adult	Died from traumatic injury
3	*Accipiter nisus*	Adult	Died from traumatic injury
4	*Accipiter nisus*	Adult	Killed due to bone fracture
5	*Buteo buteo*	Adult	Killed due to bone fracture
6	*Buteo buteo*	Adult	Killed due to bone fracture
7	*Falco peregrinus*	Adult	Killed due to chronic pododermatitis
8	*Falco peregrinus*	Young	Killed due to severe bilateral pododermatitis
9	*Falco tinnunculus*	Adult	Killed due to pododermatitis
10	*Falco tinnunculus*	Young	Killed due to bone fracture
11	*Asio otus*	Adult	Killed due to bone fracture
12	*Asio otus*	Adult	Killed due to bone fracture
13	*Bubo bubo*	Adult	Killed due to bone fracture
14	*Bubo bubo*	Adult	Killed due to bone fracture
15	*Otus sunia*	Adult	Killed due to bone fracture
16	*Otus sunia*	Adult	Killed due to bone fracture

Lectin immunohistochemistry

Formalin-fixed paraffin-embedded tissue sections were deparaffinised with xylene and rehydrated by gradient alcohol. The influenza virus receptors were detected with a Dig Glycan Differentiation Kit (Roche Applied Science, www.roche-applied-science.com) according to the manufacturer's instructions with minor modifications. Briefly, deparaffinised sections were incubated with blocking solution and AP-blocking reagent (GMS12107 Genmed, www.sh-genmed.com), respectively, for 30 min at room temperature. Then the sections were stained with digoxigenin (DIG)-labelled *Maackia amurensis* agglutinin (MAA) or DIG-labelled *Sambucus nigra* agglutinin (SNA) for 1 h at 37°C. After washing with Tris-buffered saline (TBS, pH 7.5) three times, sections were incubated with sheep anti-DIG Fab fragments conjugated with alkaline phosphatase for 1 h at 37°C. Finally, tissues were incubated with the substrate NBT/BCIP to allow visualisation of the labels. TBS was used instead of DIG-labelled lectin in the negative control sections. The duck's intestinal tract and pigeon's respiratory tract, in which the distribution of sialic acid receptors had been confirmed previously, were used as positive controls for MAA and SNA, respectively (Liu *et al.* 2009). Sections were examined under a light microscope (BX-41, Olympus, http://cn.olympus.com) and photographed at a 400× magnification. Two similar sections from each tissue of each tested bird were examined for the expression of MAA or SNA in the respiratory tract.

Results

MAA and SNA staining in Falconiformes

Tissue sections of tracheae, pharynges, and lungs were incubated with MAA (specific for SAα2,3-gal linkage) or SNA (specific for SAα2,6-gal linkage) and the staining was examined under microscope. As shown in Fig. 1 and Table 2, the epithelial cells of tracheal and pharyngeal tissues from *Accipiter gularis* (Japanese sparrow hawk) were stained strongly positive for both MAA and SNA, whereas the

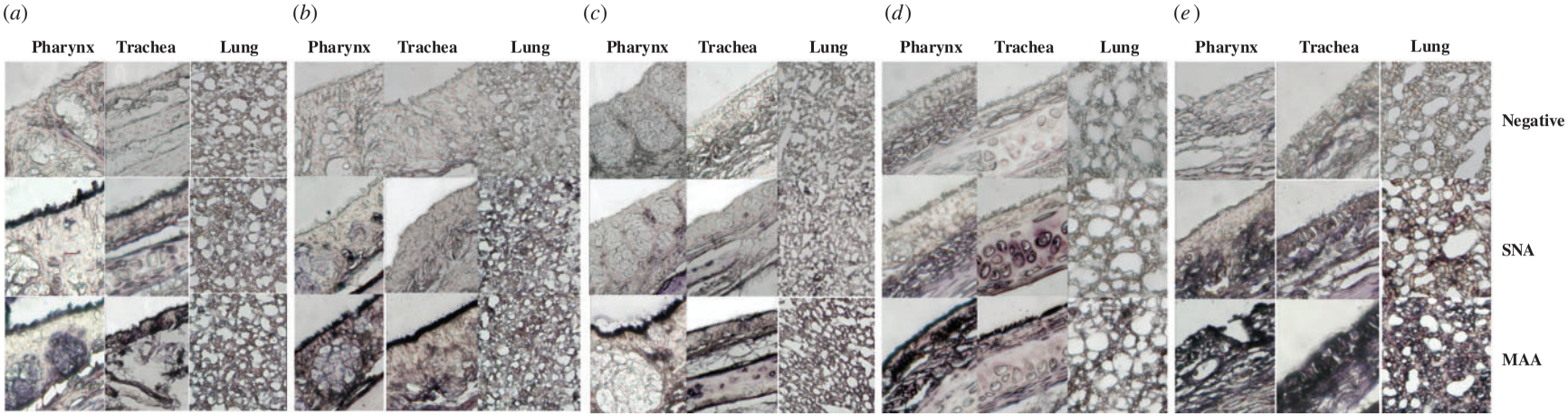

Fig. 1. Immunohistochemical staining of sialic acid receptors in the Falconiformes raptor's respiratory tract epithelium. Representative results of two birds for each species are shown. (*a*) *Accipiter gulars*, (*b*) *Falco peregrines*, (*c*) *Falco tinnunculus*, (*d*) *Accipiter nisus*, (*e*) *Buteo buteo*. Magnifications × 400.

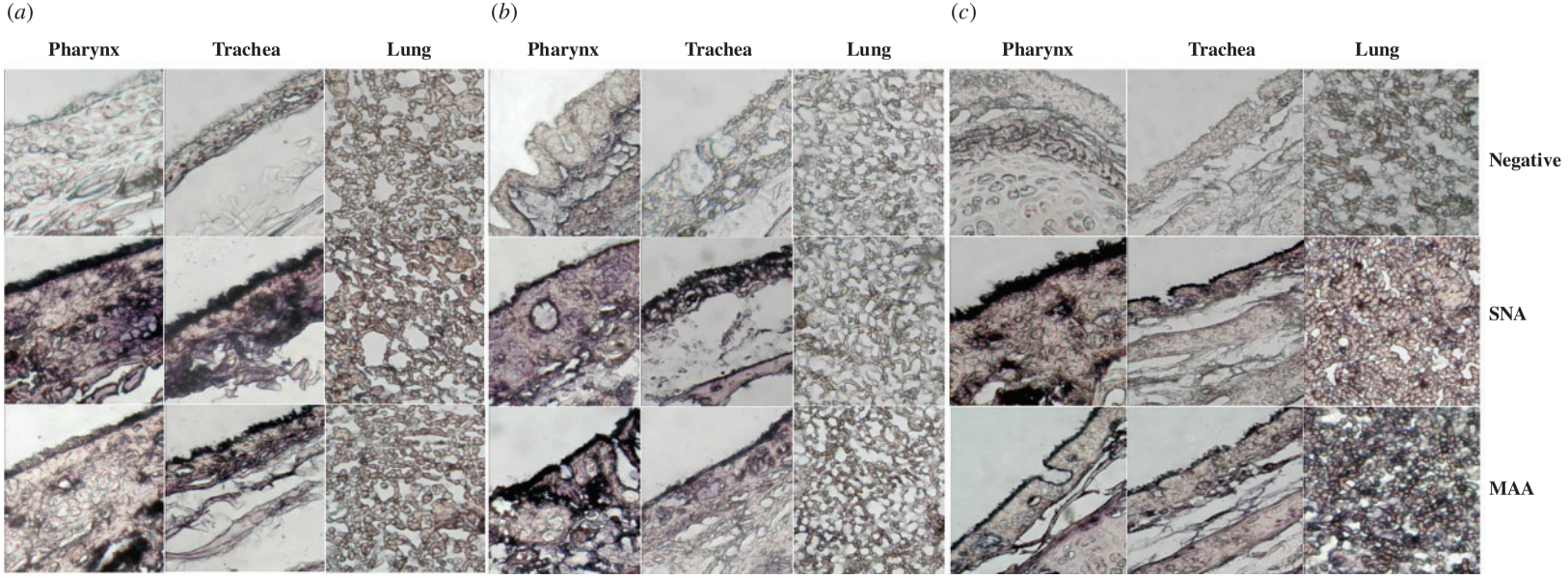

Fig. 2. Immunohistochemical staining of sialic acid receptors in the Strigiformes raptor's respiratory tract epithelium. Representative results of two birds for each species are shown. (*a*) *Bubo bubo*, (*b*) *Asio otus*, (*c*) *Otus sunia*. Magnifications × 400.

Table 2. *Maackia amurensis* agglutinin (MAA) and *Sambucus nigra* agglutinin (SNA) staining profiles in the respiratory tract of selected raptor species

+: positive staining; –: negative staining

Raptor species	Tissues	Staining	
		MAA	SNA
Accipiter nisus	Pharynx	+	–
	Trachea	+	–
	Alveolus	+	–
Accipiter gularis	Pharynx	+	+
	Trachea	+	+
	Alveolus	+	–
Buteo buteo	Pharynx	+	+
	Trachea	+	+
	Alveolus	+	+
Falco peregrinus	Pharynx	+	–
	Trachea	+	–
	Alveolus	+	+
Falco tinnunculus	Pharynx	+	–
	Trachea	+	–
	Alveolus	+	–
Otus sunia	Pharynx	+	+
	Trachea	+	+
	Alveolus	+	+
Bubo bubo	Pharynx	+	+
	Trachea	+	+
	Alveolus	+	+
Asio otus	Pharynx	+	+
	Trachea	+	+
	Alveolus	+	–

epithelia cells of those same tissues from *Accipiter nisus* (Eurasian sparrow hawk), *Falco tinnunculus* (common kestrel), and *Falco peregrines* (Peregrine falcon) showed staining with MAA only (Fig. 1, Table 2). Strong positive staining for MAA and weak positive staining for SNA were observed in the tracheal and pharyngeal epithelial cells of *Buteo buteo.*

The epithelial cells of the alveoli of *A. gularis*, *A. nisus*, and *F. tinnunculus* reacted weakly with MAA, indicating that these tissues only expressed low levels of SAα2,3-gal. In the alveoli of *Buteo buteo* (Common Buzzard) and *F. peregrines*, both MAA and SNA exhibited a similar positive staining pattern.

MAA and SNA staining in Strigiformes

The positive stainings of both MAA and SNA were observed in the tracheal and pharyngeal epithelial cells of *Otus sunia* (Oriental Scops Owl) (Fig. 2, Table 2). The MAA staining was dominant in the epithelial cells of the alveoli of *Otus sunia*, with few SNA staining.

In tracheal and pharyngeal tissues of *Bubo bubo* (Eurasian Eagle Owl), the SNA staining was dominant in epithelial cells, with limited MAA staining. The epithelial cells of the alveoli of *Bubo bubo* exhibited a similar staining intensity for both MAA and SNA.

The epithelial cells of the pharyngeal and tracheal tissues of *Asio otus* (Long-Eared Owl) were stained strongly with MAA and weakly with SNA. Only MAA staining was observed in the epithelial cells of the alveoli. No SNA staining was detected in the epithelial cells of the alveoli.

Discussion

Several recent epidemiological studies suggest that raptors are naturally susceptible to influenza A virus infection. A highly pathogenic H7N3 influenza A virus was isolated from a dead Peregrine falcon in Dubai (Manvell *et al.* 2000). The H5N1 influenza A virus was isolated from Peregrine falcons in Hong Kong and the Slovak Republic, and from a common buzzard in Denmark (Alexander 2007). Low-pathogenic influenza viruses were also detected from some raptor species (Hall *et al.* 2009; Khan *et al.* 2009; Goyal *et al.* 2010). However, the sialic acid receptor profiles, the primary determinants of the hosts' susceptibility to influenza A virus, of the respiratory and intestinal tracts of raptors have not been studied yet. In this study, we examined the sialic acid receptor distribution in the respiratory tract of raptors using lectin immunohistochemistry labelling techniques as described previously (Ito *et al.* 2000; Gambaryan *et al.* 2002; Kuchipudi *et al.* 2009; Liu *et al.* 2009; Pillai and Lee 2010).

Our results showed that the upper and lower respiratory tracts of all tested raptors expressed avian-type receptor SAα2,3-gal as evidenced by the strong MAA staining, implying that avian influenza viruses may initiate infections in these raptors by utilising these receptors for virus binding and entry. This result further confirms the findings of epidemiological studies showing the infection of raptors with avian influenza A viruses. The upper respiratory tract of *Bubo bubo*, *Otus sunia*, and *Accipiter gulars* stained strongly with SNA, suggesting the expression of SAα2,6-gal, a primary receptor for the human influenza A virus. The high levels of expression of human influenza virus receptors in the trachea and pharynx of *Bubo bubo*, *Otus sunia*, and *Accipiter gulars* suggest that those species of raptors may be susceptible to human influenza A virus infection. We observed some variations in the type and distribution of sialic acids receptor expression among different raptor species. No correlation between phylogeny of birds and their sialic acid receptor distributions was observed.

Both MAA and SNA positive staining were present in the upper respiratory tracts of five species raptors (Table 2). These findings were similar to those observed in pigs, chicken, ducks, turkeys and quails (Gambaryan *et al.* 2002; Ma *et al.* 2008; Pillai and Lee 2010). The human influenza A virus receptor, SNA, is also found in other wild birds, such as mallard ducks (*Anas platyrhynchos*), black-headed gulls (*Larus ridibundus*), Dunlin (*Calidris alpina*), mew gulls (*Larus canus*), herring gulls (*Larus argentatus*) and common murres (*Uria aalge*) (Ellström *et al.* 2009). *In vitro* experiments demonstrated that avian and human influenza A viruses can bind to plasma membranes of epithelial cells derived from chicken and quail tracheae (Gambaryan *et al.* 2002; Wan and Perez 2006). Under natural infection conditions, researchers isolated genetically reassorted viruses that are a combination of avian H1N1 and human H3N2 viruses in pigs from European in 1993 and 1998, respectively (Castrucci *et al.* 1993; Brown *et al.* 1998; Ito *et al.* 1998). Previously, we successfully reisolated influenza viruses from the trachea of chickens infected with avian influenza viruses, whereas reisolation failed in pigeons (Liu *et al.* 2007). This difference was at partially attributed to the fact that pigeons do not express the avian influenza receptor (Liu *et al.* 2009). Taken together, these studies indicated the distribution and type of receptors in the

upper respiratory tract coincide with the type of virus that infects and replicates in the host. Nevertheless, further studies are needed to determine whether some human influenza viruses may actually infect some of the raptor species.

Conclusion

We examined the expression of avian and human influenza A virus receptors in the upper and lower respiratory tracts of eight species of raptors by using the lectin immunohistochemistry staining method. Both avian and human influenza virus receptors are expressed in the six species of raptors examined, suggesting that these species of raptors may be susceptible to both avian and human influenza A virus and could serve as a natural reservoir for influenza A viruses. It will be interesting to determine the sialic acid profiles in the intestinal tracts of raptors in future studies once influenza viruses are isolated from cloacal swabs.

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