NOTE *Pathology*

Distribution of Influenza Virus Sialoreceptors on Upper and Lower Respiratory Tract in Horses and Dogs

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ABSTRACT. It is strongly suspected that equine influenza virus (EIV) is the origin of canine influenza virus (CIV, H3N8), which was first isolated in U.S.A. in 2004, on the basis of phylogenetic analyses. Although the distribution of influenza virus sialoreceptors seems to be associated with this interspecies transmission, there have been scant data of comparison about distributions of sialoreceptors on the whole respiratory tract between horses and dogs. We examined the histological distribution of influenza virus sialoreceptors on the upper and lower respiratory tract in detail in both animals using double lectin staining with *Maackia amurensis* (specific for SAα2,3Gal) and *Sambucus sieboldiana* (specific for SAα2,6Gal). SAα2,3Gal was observed on the surface of ciliated epithelial cells in the nasal mucosa, trachea and bronchus in both animals. The results may indicate that dogs are susceptible to EIV without alteration of receptor binding specificity.

KEY WORDS: canine influenza virus, equine influenza virus, sialoreceptor.

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Influenza A viruses (IVs) are members of the *Orthomyxoviridae*, and are known to cause acute respiratory disease in humans, pigs, fowl and horses [13]. IVs are classified into subtypes on the basis of the two main surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). HA binds specifically to cell surface sialic acid (SA) as a receptor when IVs infect target cells. After viral replication in the infected cell, NA is responsible for cleaving the SA that binds the mature viral particles, for releasing progeny virus from the cell. Although all IVs recognize oligosaccharides containing a terminal SA, the specificity of the HA towards these molecules differs by their linkage positions and the sequences of the sialyloligosaccharides in the target cells. For example, avian influenza viruses preferentially bind to SAα2,3-galactose (Gal) linkages (SAα2,3Gal), while human influenza viruses preferentially recognize SAα2,6Gal [5, 7, 11].

Recently, influenza A virus has become prevalent among the dog population in the United States and the United Kingdom [1–3]. Since phylogenetic analyses of nucleotide sequences of HA indicated that the canine influenza virus (CIV, H3N8) was strongly related to contemporary equine influenza virus (EIV, H3N8), it is considered that CIV was derived from EIV by interspecies transmission [1, 2]. To understand the mechanism of interspecies transmission, it is important to clarify the histological distribution of influenza virus sialoreceptors on the respiratory tract in those animals. Several studies on the distribution of the sialoreceptors along the respiratory tract of either horses or dogs have been reported [2, 9, 10, 12]. However, there have been scant studies on the systematical comparison about distribution of sialoreceptors on the airway between horses and dogs. Therefore, we examined the histological distribution of influenza virus sialoreceptors in the respiratory tract in both animals in detail by double lectin staining with *Maackia amurensis* (MAM: specific for SAα2,3Gal) and *Sambucus sieboldiana* (SSA: specific for SAα2,6Gal) [8].

Ten thoroughbred horses, including 5 males and 5 females (6.8±6.4 years old), that were euthanized because of severe locomotor disability but had normal respiratory systems and four healthy beagles, including 1 male and 3 females (all 11 months old), were used in this study. Samples of the nasal mucosa (respiratory region), trachea, bronchus and lung parenchyma were taken from these animals. The tissue samples were fixed at room temperature in 4% paraformaldehyde for 24 hr, dehydrated using alcohol, embedded in paraffin wax and cut into 6-μm-thick sections. The sections were deparaffinized in xylene and rehydrated. Lectin staining for SAα2,3Gal or SAα2,6Gal was carried out according to Suzuki *et al.* with a slight modification [12]. Non-specific reaction was blocked by the treatment with 2.5% bovine serum albumin and 2.5% normal goat serum in PBS at room temperature for 30 min. The sections were incubated with biotin-labeled MAM (J-Oil Mills, Tokyo, Japan) and FITC-labeled SSA (J-Oil Mills) at 4°C overnight, rinsed, reacted with TRITC-labeled streptavidin (TRITC-StAv) at 4°C for 20 min, and then washed with PBS and mounted using buffered glycerol (pH 9.0) for observation. All sections were examined with a confocal laser scanning microscope (FLUOVIEW 3500, OLYMPUS,

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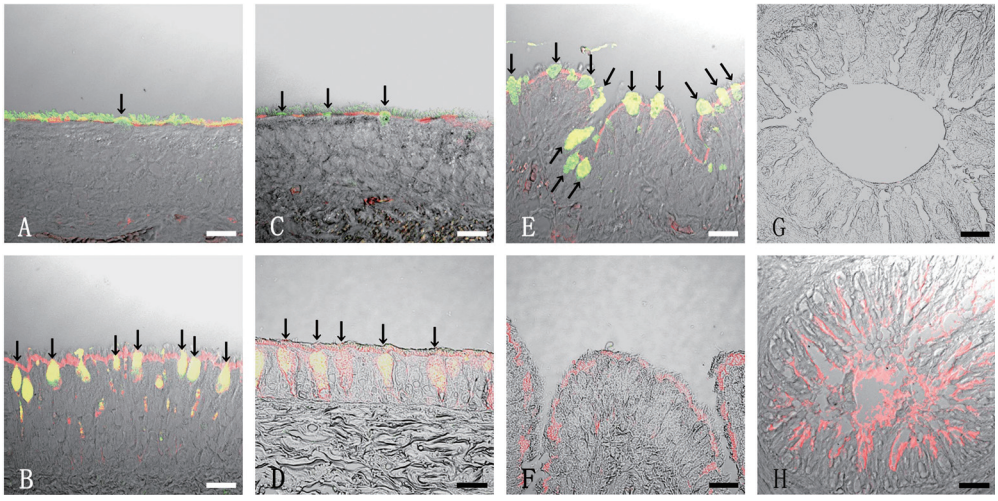

Fig. 1. Distribution of SAα2,3Gal and SAα2,6Gal containing sialoglycans in horse and dog tissues. A, Horse nasal mucosa; MAM lectin signal is detected on the surface of horse ciliated cells, while SSA lectin signal is detected in goblet cells and cilia, respectively. B, Dog nasal mucosa; only MAM lectin signal is detected on the surface of dog ciliated cells, while both MAM and SSA lectin signals are detected in goblet cells. C, Horse trachea; MAM lectin signals is detected on the surface of horse ciliated cells, while SSA lectin signal is detected in goblet cells and cilia, respectively. D, Dog trachea; only MAM lectin signal is detected on the surface of dog ciliated cells, while both MAM and SSA lectin signals are detected in goblet cells. E, Horse bronchus; MAM lectin signal is detected on the surface of horse ciliated cells, while SSA lectin signal is detected in goblet cells and cilia, respectively. F, Dog bronchus; MAM lectin signal is detected on the surface of dog ciliated cells. G, Horse bronchiole; neither MAM nor SSA lectin signal is detected in horse epithelial cells. H, Dog bronchiole; MAM lectin signal is detected on the surface of dog epithelial cells. SSA and/or MAM lectin positive goblet cells are indicated by arrows. Bars, 20 μm.

Tokyo, Japan); FITC signals for SSA were detected in green using an Ar laser (488 nm) and TRITC signals for MAM were detected in red using a HeNe laser (543 nm).

By examination of horse respiratory tract, MAM lectin was detected on the surface of ciliated epithelial cells and nasal gland in the nasal mucosa (Fig. 1A), on the surface of ciliated epithelial cells and tracheal gland in the trachea (Fig. 1C) and on the surface of ciliated epithelial cells in the bronchus (Fig. 1E). SSA lectin was detected in cilia and goblet cells in the nasal mucosa (Fig. 1A), in cilia and goblet cells in the trachea (Fig. 1C) and in cilia and goblet cells in the bronchus (Fig. 1E). Neither MAM nor SSA lectins were detected in horse bronchiole (Fig. 1G). By examination of dog respiratory tract, meanwhile, MAM lectin was detected on the surface of ciliated epithelial cells and goblet cells in the nasal mucosa (Fig. 1B), on the surface of ciliated epithelial cells, goblet cells and tracheal gland in the trachea (Fig. 1D) and on the surface of ciliated epithelial cells in the bronchus (Fig. 1F). SSA lectin was detected in goblet cells in the nasal mucosa (Fig. 1B) and in goblet cells and tracheal gland in the trachea (Fig. 1D). On the surface of bronchiolar epithelium, only MAM lectin was detected, but SSA lectin was not detected (Fig. 1H). No sex difference was observed in these findings. In other words, SAα2,3Gal was observed on the surface of ciliated epithelial cells in the nasal mucosa, trachea and bronchus in both horses and dogs. However, on the surface of the bronchiole, SAα2,3Gal was strongly seen in dogs while neither SAα2,3Gal nor SAα2,6Gal was seen in horses. Only SAα2,6Gal was expressed on cilia in the nasal mucosa, trachea and bronchus in horses, while it was not expressed in dogs. Both sialoreceptors were expressed in goblet cells of the nasal mucosa and trachea in horses, while only SAα2,6Gal was expressed in those of dogs (Table 1). These results were almost identical in each organ of dogs and horses, although individual variability in the intensity of expression was slightly observed.

A few previous studies of sialoreceptors in horses or dogs have been reported. Suzuki *et al*. [12] carried out single lectin staining with *Maackia amurensis* agglutinin (MAA: specific for SAα2,3Gal) or *Sambucus nigra* agglutinin (SNA: specific for SAα2,3Gal) in the trachea of horses, and reported that only SAα2,3Gal was expressed on the surface of the tracheal epithelium. Song *et al*. [10] carried out single lectin staining with MAA or SNA in the trachea and lung of dogs and reported that only SAα2,3Gal was expressed on the surface of the tracheal, bronchial and bronchiolar epithelia. Additionally, Daly *et al*. [2] carried out single lectin staining with MAA or SNA in the trachea of horses and dogs and reported that SAα2,3Gal was expressed on the surface of the tracheal epithelium of horses and dogs. These previous reports only examined solo species, and/or limited area of respiratory tract, and/or limited type of cells, and not included goblet cells, accessory gland epithelial cells whose functions are different from ciliated epithelial cells. We systematically investigated the distribution of IV sialoreceptors in respiratory epithelium from the nasal mucosa to the lung

Table 1. Summary of lectin binding intensities in respiratory tract of horses and dogs in this study

		Nasal mucosa				Trachea				Bronchus			Bronchiole
		cilium	ciliated cell	goblet cell	nasal gland	cilium	ciliated cell	goblet cell	tracheal gland	cilium	ciliated cell	goblet cell	epithelial cell
Horse (n=10)	MAM (Saα2,3Gal)	−	+	−	+	−	+	−	+	−	+	−	−
	SSA (Saα2,6Gal)	+	−	+	+	+	−	+	−	+	−	+	−
Dog (n=4)	MAM (Saα2,3Gal)	−	+	+	−	−	+	+	+	−	+	−	+
	SSA (Saα2,6Gal)	−	−	+/−	−	−	−	+	+	−	−	−	−

in horses and dogs. Our study revealed that SAα2,3Gal expression was widespread on the surface of the respiratory tract epithelium in horses and dogs. It was previously reported that EIV preferentially recognized SAα2,3Gal [12]. The expression of SAα2,3Gal on the surface of respiratory epithelium from the nasal mucosa to the bronchus in both animals might indicate that EIV can attach to this epithelium of dogs. Yamanaka *et al*. [14] reported that dogs can be susceptible to EIV on close contact with experimentally IV infected horses. Additionally, Kirkland *et al.* [4] reported that EIV infection occurred naturally in some dogs kept near to infected horses during the 2007 EIV outbreak in Australia. Our results of sialoreceptor distribution together with these previous reports indicate that dog is susceptible to EIV without alteration of receptor binding specificity.

Furthermore, SAα2,6Gal was expressed in cilia, goblet cells and accessory glands from the nasal mucosa to the bronchus. The goblet cells and the cilia on the respiratory epithelium play important roles in host defense against inhaled viruses [6]. The goblet cells trap inhaled viruses by mucus secretion and the cilia can transport the mucus effectively against gravity by ciliary motility. Our findings that SAα2,6Gal was expressed in cilia, goblet cells and accessory glands may be related to the clearance of IV, which can bind specifically to SAα2,6Gal at least.

We have conclusively demonstrated the histological distribution of influenza virus sialoreceptors in horse and dog respiratory epitheliums. This study revealed that canine respiratory epithelia from the nasal mucosa to the bronchiole have SAα2,3Gal receptors which may contribute a direct interspecies transmission of EIV between horses and dogs. To obtain strong evidence of the direct transmission and to reveal the relationship between the distributions of sialoreceptors and pathogenicity, studies on experimental infection of EIV and CIV in horses and dogs are necessary.

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