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Determination of *N*-Linked Sialyl-Sugar Chains in the Lungs of Domestic Cats and Dogs in Thailand Susceptible to the Highly Pathogenic Avian Influenza Virus (H5N1)

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Abstract: Highly pathogenic and potentially pandemic H5N1 avian influenza A viruses have become endemic and are now residing in Asia, Europe, Africa, and the Middle East. H5N1 viruses have been shown to cross the species barrier and infect both dogs and cats. Domestic cats and dogs in Thailand, which were naturally infected with H5N1, exhibited severe pulmonary edema and peumonia in lung tissue as well as in other tissue dysfunctions. In order to understand the structure and quantity of influenza A receptor sialyl sugar chains in cats and dogs, especially in lung tissue, glycosylation profiles of *N*-glycans were determined from lung tissues of dogs and cats susceptible to H5N1 in Thailand by using multi-dimensional HPLC mapping combined with mass spectrometry. The results demonstrated different *N*-linked glycans composition ratios between dogs and cats. There were a total of 30 kinds of *N*-linked glycans from cat lungs, which were comprised of 11 neutral, 13 mono-, 3 di-, and 3 tri-sialyl sugar chains, and 29 kinds from dog lungs, which were comprised of 16 neutral, 11 mono- and 2 di-sialyl sugar chains. Cat lungs exhibited both 5-*N*-acetylneuraminic acid and 5-*N*-glycolylneuraminic acid sialic acid (Siaα2-3Gal and Siaα2-6Gal), but dog lungs contained only 5-*N*-acetylneuraminic (Siaα2-3Gal and Siaα2-6Gal) molecular species. The composition ratios of molar percentage of Siaα2-3Gal for domestic cat and dog lungs were 21.5 and 9.9, respectively, while the composition ratios of Siaα2-6Gal were 47.1 and 59.2, respectively. These results may indicate that domestic cats are more susceptible than dogs to H5N1 influenza virus infection and also cats and dogs play an important role as "mixing vessels" for the virus re-assortment.

Keywords: Cat, Dog, H5N1, Lung, Sialyl sugar chains, Influenza virus.

INTRODUCTION

Highly pathogenic avian influenza (HPAI) is one of the most widely distributed zoonotic infectious diseases in the world, and furthermore, the pathogenic influenza virus is extremely prone to mutation. The H5N1 avian influenza virus is a highly contagious and deadly pathogen in poultry, which has been transmitted to humans, and has potentially generated mutations during human-to-human propagation [1,2]. Fatal avian H5N1 viruses have been demonstrated to cross the species barrier, and infect humans as well as felines, including domestic cats [3-5], leopards, and tigers [6,7]. The H5N1 viruses have also been isolated from domestic dogs [8,9]. Furthermore, influenza viruses can be isolated from many species, such as humans, pigs, horses, mink, mice, ferrets, cynomolgus macaques, cats, leopards, tigers, marine mammals, and a wide range of domestic birds

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[5,10,11]. Previous experimental studies have revealed viral antigens in lung tissue after intratracheal inoculation with H5N1 virus in 4 to 6 month-old European shorthair cats [3]. Moreover, necropsy of domestic cats and dogs in Thailand found incidences of nasal discharge, severe pulmonary congestion, and pulmonary edema, since the viruses are predominantly attached to the lower respiratory tract, indicating that the important organ of viral infection is the lung [4,12].

With regard to human cases, HPAI H5N1 was first reported in poultry farms and wet markets located in Hong Kong in 1997 [10,13]. The clinical signs of this disease resulted in severe respiratory tract distress and multiple organ dysfunctions, which require hospitalization. Additionally, the autopsy results from a patient that died after the onset of H5N1 infection in Thailand revealed the proliferative phase of diffused alveolar macrophage, interstitial pneumonia, focal hemorrhage, and bronchitis. These results also suggest that virus replication is predominantly in lung tissue [14]. Influenza A virus binding involves interaction between a receptor-binding site on hemagglutinin (HA) and the sialyl receptor, which is known as sialic acid [2]. Results of recent studies have indicated that *N*-linked sialoglycoproteins are required for influenza virus infection and entry into host cells of influenza viruses, at least for influenza A (H1N1 and H3N2) and influenza B viruses [15,16]. The hemagglutinin of the virus recognizes different linkages, dependent on the viral particle encountering avian or mammal hemagglutinin strains. Hemagglutinin of human and avian influenza A isolates recognizes sialic acid with α2-6 and α2-3 linkages, respectively, and HA of type B viruses prefers the α2-6-linked sialic acid [17,18]. Avian strains preferentially bind to α2-3 linkage (Neu5Acα2-3Gal), while human strains have specificity for α2-6 linkages (Neu5Acα2-6 Gal) [2,19,20].

Pigs are susceptible to respiratory infections by both avian and human strains, since the pig trachea contains carbohydrates with both α2-3 and α2-6 linked sialic acids [21,22]. Some investigations have suggested that pigs might attract influenza pandemics by serving as a "mixing vessel", through which antigenically-novel avian-human re-assortant viruses might be generated following co-infections [21,22]. However, there have been no studies, thus, far identifying the structural formula, molecular weight, or characteristics of *N*-linked glycans in domestic animals that are in close contact with humans. This investigation characterized *N*-glycans derived from domestic cats and dogs in Thailand that have been reported to exhibit susceptibility to highly pathogenic avian influenza A viruses [4,12], through utilization of the high-performance liquid chromatography (HPLC) mapping method, which is applicable for determining *N*-glycan profiles in a quantitative manner at molecular, cellular, and tissue levels [23].

MATERIALS AND METHODOLOGY

Tissue Preparation from Cat and Dog Lung Tissue

To study the structure of *N*-linked glycans in the lower respiratory tract of cats and dogs, particularly in the lungs, lung tissue were directly collected from donated animals at the Kasetsart University Veterinary Teaching Hospital (KUVTH), Faculty of Veterinary Medicine, Kasetsart University, Thailand. These animals died without respiratory tract infections, and samples were collected from a total of three dogs and three cats ranging from 2 months to 8 years old.

Freshly harvested lungs were individually weighed and placed in a homogenization tube with miliQ water using sterile technique. The homogenates poured into freeze-drying bottles to lyophilize the lung tissue. Total dry weight tissue of cats and dogs were 1.36 and 2.49 grams, respectively, and 100 mg of the freeze-dried samples were delipidated using 80% ethanol, 100% ethanol, Chloroform/Methanol (2:1, v/v), and Chloroform/Methanol/miliQ water (1:2:0.8, v/v/v) [24]. After lipid removal, the remaining 10 mg was used for the structural analysis of sugar chains as starting materials.

Purification and Characterization of *N*-Linked Glycans

All experimental procedures used, including the enzymatic, chromatographic, and mass spectrometric conditions, have been previously described [25-27]. The lung tissue extractions were proteolyzed with pepsin and further digested with glycoaminidase A to release *N*-glycans. The reducing ends of the released glycans were labeled with fluorescent reagent 2-aminopyridine, under previously described conditions [28]. These pyrimidylamino (PA)-glycans were fractionated on a diethylaminoethyl (DEAE) column (7.5×75 mm, Tosoh, Tokyo, Japan), according to sialic acid contents. Each fraction was separated by an octadecyl silica (ODS) column (6×150 mm, Shimadzu, Kyoto), and the recorded elution time represents the glucose unit (GU) value. The individual fractions were subjected to matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS). Fractions, including some *N*-glycans, were further separated by an amide column (4.6×250 mm, Tosoh) and GU values recorded. The identification of *N*-glycan structures was based on GU and mass values in comparison to PA-glycans in the GALAXY database (http://www.glycoanalysis.info/galaxy2/ENG/systemin1.jsp) [29]. The structures of PA-glycans not previously registered in GALAXY were characterized by exoglycosidase treatments (α-sialidase, α2,3-sialidase, α-fucosidase, α-galactosidase, β-galactosidase, and β-*N*-acetylglucosaminidase) and mass spectrometric analysis, as previously described [25,27].

RESULTS AND DISCUSSION

Isolation of Neutral Glycans and Acidic Glycans from Domestic Cat and Dog Lung Tissue

We performed *N*-glycosylation profiling of domestic dog and cat lung tissues by multi-dimensional HPLC mapping combined with mass spectrometric analysis. The neutral glycans and acidic PA-glycans derived from the lung tissues were separated by a diethylaminoethyl (DEAE) column. There were four peaks of individual *N*-linked glycans, which were comprised of one peak of neutral glycans and three peaks of acidic glycans (mono-sialyl, di-sialyl and tri-sialyl sugar) at 2, 12, 23, and 27 min retention times, respectively (Fig. **1a**). The composition ratios of the molar percentages of the neutral, mono-sialyl, di-sialyl, and tri-sialyl forms of *N*-linked glycans from cat lung tissue were 28, 24.6, 41.3, and 6.1, respectively, and those from dog tissue were 27.9, 16.6, and 55.4, respectively, with dog tissue lacking tri-sialyl *N*-linked glycans (Table **1**).

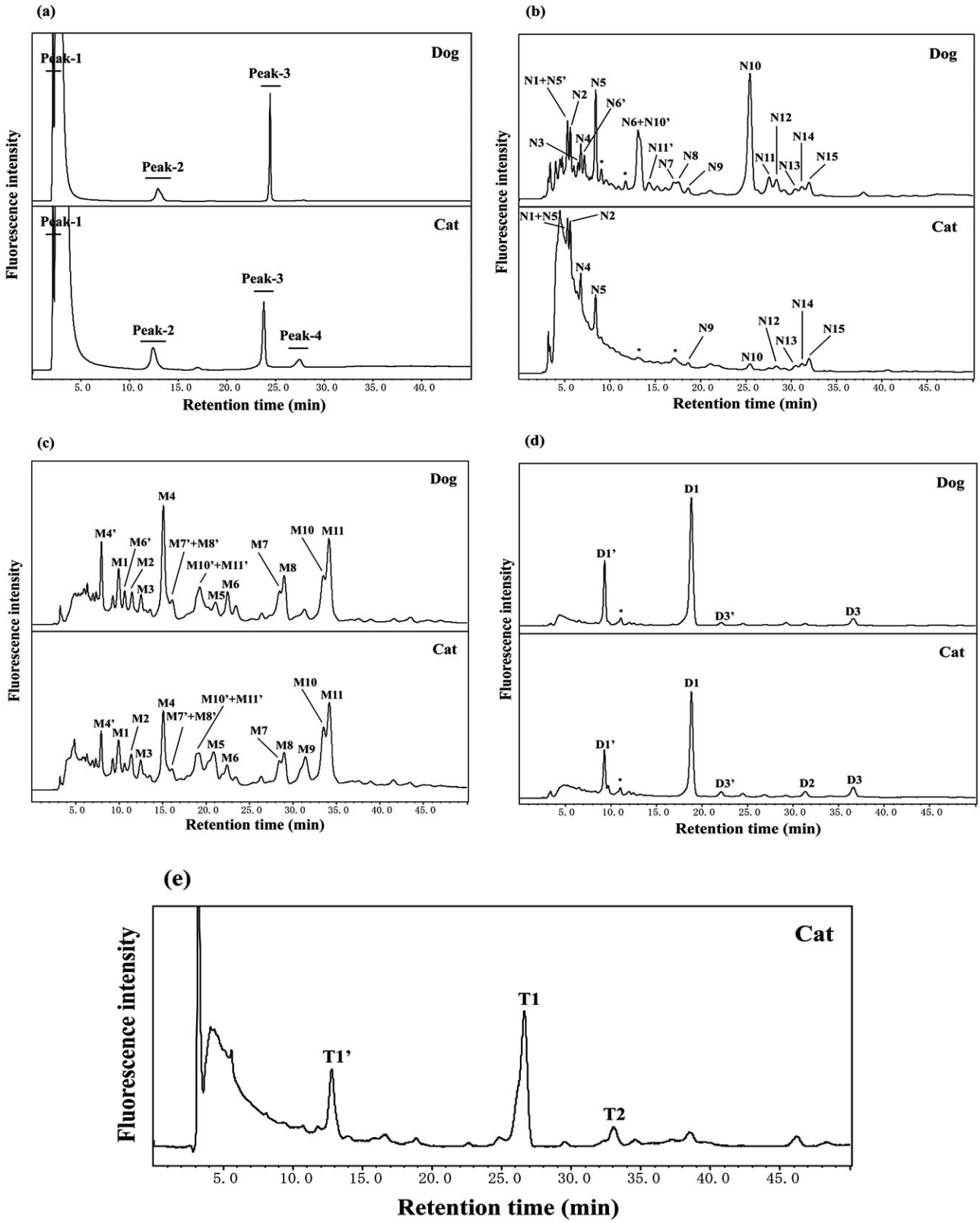


Fig. (1). Comparison of HPLC profiles (**a**-**e**) of pyridylamino (PA) derivatives of *N*-glycans derived from dog and cat lung tissues. The PA-glycans from dog and cat lung tissues were separated on diethylamino ethyl (DEAE) column (**a**). Peaks 1, 2, 3, and 4 indicate the fractions that are neutral, mono-, di-, and tri-sialyl oligosaccharides, respectively. Each peak of the neutral, mono-, di-, and tri-sialyl oligosaccharide was individually applied to an octadecyl silica (ODS) column and given elution profiles of **b**, **c**, **d**, and **e**, respectively. Asterisks indicate the fractions containing no detectable PA-oligosaccharides.

Table 1. Characteristics of Composition Ratios of Glycan Content Between cat and Dog Lung Tissues

Species	Composition Ratios (Molar %)[a]				Sialyl Sugar Chain (Molar %)[a]		Sialyl Sugar Chain (pmol mg^{-1})[b]	
	Neutral Sugar	Mono-sialyl Sugar	Di-sialyl Sugar	Tri-sialyl Sugar	Siaα2-3Gal	Siaα2-6Gal	Siaα2-3Gal	Siaα2-6Gal
Cat	28.0	24.6	41.3	6.1	21.5	47.1	20.9	45.7
Dog	27.9	16.6	55.4	-	9.9	59.2	28.8	167.3

[a]The molar percentage of glycan contents in cat and dog lung tissues was calculated on the basis of the peak areas in Fig. (**1b-e**) by comparison with total glycan content in the lung tissues, respectively.
[b]Total amount of sialyl sugar chain derived from domestic cat and dog lung tissue dry weight (pmol mg^{-1}).

Each fraction separated by the DEAE column from peaks 1, 2, 3, and 4 was individually applied to an ODS column, and the individual sub-fractions were subjected to MALDI-TOF-MS analysis. There were 16 major peaks of neutral, 13 major peaks of mono-sialyl, 3 major peaks of di-sialyl, and 3 major peaks of tri-sialyl glycans (Fig. **1b-e**). The peaks N2, M1, M9, and T1, which included two kinds of *N*-glycans, were further separated utilizing an amide column. The PA-oligosaccharides were identified on the basis of HPLC elution time, normalized by GU and mass values, in comparison to PA-glycans in the GALAXY database. For example, the major *N*-glycan that corresponded to peak D1 was eluted at 11.0 GU on the ODS column. The molecular mass of this glycan was 2302 Da, as determined by MALDI-TOF-MS analysis. The data set was in good agreement with the known reference for di-sialyl glycan,

Neu5Acα2→6Galβ1→4GlcNAcβ1→2Manα1→6(Neu5Acα2→6Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc-PA (code no. 2A1-200.4 in the GALAXY database). The structure of this PA-oligosaccharide was confirmed by co-chromatography. The PA-glycans that corresponded to the fractions N9, N14, N15, M1-2, M2, M3, M5, M8, M9-2, and M11 did not match any of the PA-glycans registered in GALAXY. These compounds were trimmed by exoglycosidase treatments to identify known PA-glycans. The original structures of these PA-glycans were uniquely determined by taking into account the specificities of the used exoglycosidases.

In this study, 35 kinds of *N*-glycans derived from dog and cat lung tissue were identified, which included the epimerization of *N*-glycans. In total, 30 types of *N*-linked glycans (11 neutral glycan, 13 mono-sialyl, 3 di-sialyl, and 3 tri-sialyl sugar chains) existed in cat lungs, and 29 types (16 neutral glycan, 11 mono-sialyl, and 2 di-sialyl sugar chains) in dog lungs. Collectively, the ratio of molar percentage of neutral glycans and acidic glycans was approximately 30:70 for both cat and dog tissues. The reference code numbers, structures, and relative quantities (%) of *N*-linked glycans from dog and cat lung tissues are shown in Table **2**.

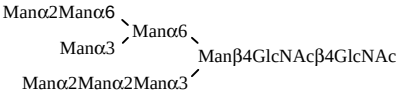
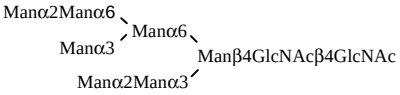
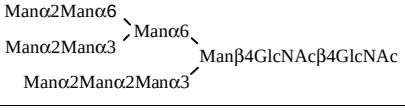
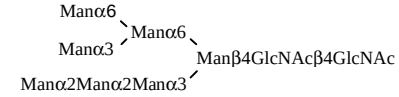
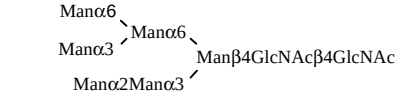
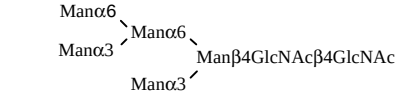
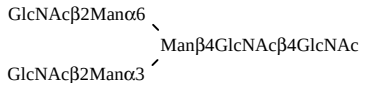
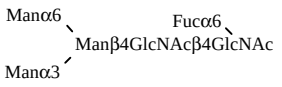
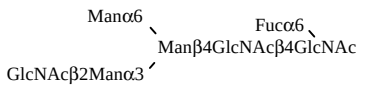
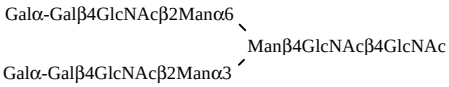
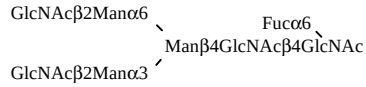
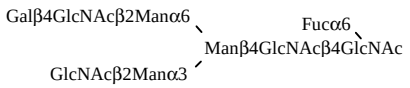
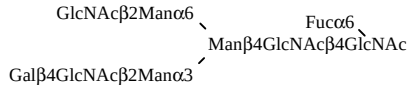
Cat lungs contained both Neu5Ac and Neu5Gc molecular species of sialic acid (Siaα2-3Gal and Siaα2-6Gal), while dog lungs contained only Neu5Ac (Siaα2-3Gal and Siaα2-6Gal). The composition ratios of the molar percentage of Siaα2-3Gal and Siaα2-6Gal in cat lung tissue were 21.5 and 47.1, respectively, while the composition ratios in dog lung tissue were 9.9 and 59.2, respectively (Table **1**). However, the total amounts of Siaα2-3Gal and Siaα2-6Gal derived from cat lungs were 20.9 and 45.7 pmol mg^{-1}, respectively, and the total amounts from dog lungs were 28.8 and 167.3 pmol mg^{-1} of tissue dry weight, respectively (Table **1**). Interestingly, these results demonstrated that only cat lung tissue possessed α2-3 Neu5Ac and α2-3 Neu5Gc residues of mono-sialyl PA-glycan, which corresponded to peak M9. Moreover, the Neu5Acα2-3Gal and Neu5Acα2-6Gal in the same glycan of the di-sialyl (peak D2) and tri-sialyl sugar chain (peaks T1, T1', and T2) were also detected only in cat lung tissue (Table **2**).

Previous studies demonstrated that the distribution of sialic acid species varies in different animals. For example, pig and horse tissue contain large amounts of Neu5Gcα2-3Gal, while chicken tissue does not have Neu5Gc, and humans are the mammal that lack Neu5Gc expression in normal tissue [30]. More than 90% of the horse trachea epithelial cells contain Neu5Gc [2], and previous studies indicated that the Neu5Gc α2-3Gal moiety is critical for influenza A replication in horse and duck species [2,31]. Neu5Gc was also found in cat lung tissue, however, the total composition of Neu5Gc was less than Neu5Ac. Moreover, the higher ratio of the Siaα2-3Gal and avian-type receptor sialyl-sugar linkage in cat lung tissue (21.5%) compared to dog lungs (9.9%) (Table **1**) may indicate the reason that cats are more susceptible than dogs to avian influenza infection. Therefore, we suggest that domestic cats and dogs as well as pigs, chickens, and quails, which have already been reported as "mixing vessel" animals for the virus re-assortment, should be part of the alert or there should be special monitoring during H5N1 outbreaks.

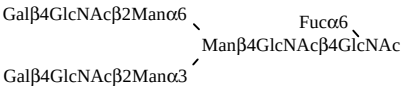
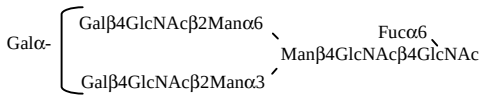
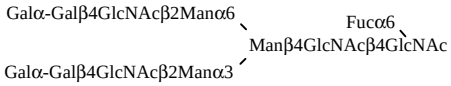
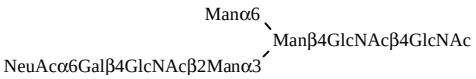
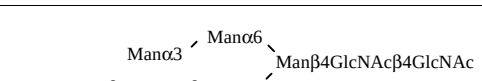
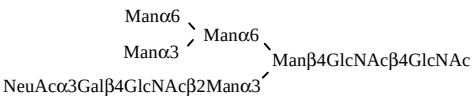
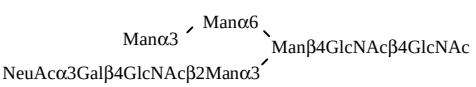
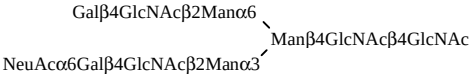
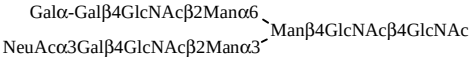
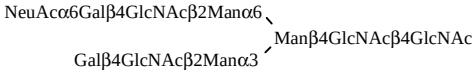
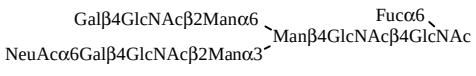
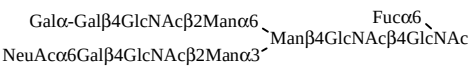
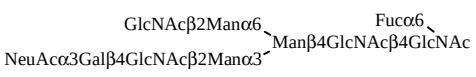
In addition, H5N1 viruses can be found in other organs, such as heart, intestine, placenta, trachea, brain [32,33], cerebrospinal fluid [34], and faeces [32]. Furthermore, H5N1 viruses were also found in fetal mononuclear cells, macrophages in the liver, and the fetal placenta [33]. These results indicate that all of these organs possess avian influenza virus receptors and the virus could be transmitted from mother to fetus by the transplacental route. With respect to these results, further investigations should focus on characterizing the structures of avian influenza virus receptors and demonstrating the vertical transmission in animals.

In summary, domestic cats and dogs in Thailand were demonstrated to have both *N*-linked Siaα2-3Gal and Siaα2-6Gal linkages in lower respiratory tract lung tissue, indicating that cats and dogs might play an important role as "mixing vessels" for the virus re-assortment. The composition ratio of molar percentages of Siaα2-3Gal and Siaα2-6Gal

Table 2. Structure of *N* -Glycans Derived from Dog and Cat Lung Tissues, and Prevalence (mol %)

Peak Code No. [a]	GU (ODS)	Molecular Mass (Da) [b]	Structure	Relative Quantity (%) [c]	
				Dog	Cat
Neutral glycans					
N1 M8.1	4.8	1801		0.3	1.7
N2-1[d] M7.2	5.1	1638		0.3	2.2
N2-2[d] M9.1	5.1	1962		0.7	2.3
N3 M7.1	5.9	1638		0.4	—
N4 M6.1	6.1	1476		0.8	3.2
N5+N5' M5.1	7.2	1314		3.9	7.2
N6+N6' 200.1	9.3	1396		3.0	—
N7 010.1	10.5	1135		0.6	—
N8 110.2	10.7	1338		0.6	—
N9	11.0	2044		0.2	0.7
N10+N10' 210.1	13.0	1542		11.4	1.6
N11+N11' 210.2	13.7	1704		1.6	—
N12 210.3	14.0	1704		1.1	0.7

(Table 2). Contd…..

Peak Code No. [a]	GU (ODS)	Molecular Mass (Da) [b]	Structure	Relative Quantity (%) [c]	
				Dog	Cat
N13 210.4	14.7	1866		0.4	1.5
N14	15.0	2028		0.7	2.3
N15	15.3	2190		0.9	3.7
Others				1.0	0.9
Total				27.9	28.0
Monosialylated glycans					
M1-1[d] 1A1-100.4	8.1	1646		0.3	0.4
M1-2[d]	8.1	1808		0.6	0.8
M2	8.8	1970		0.3	0.8
M3	9.2	1808		0.3	0.5
M4+M4' 1A1-200.4	10.1	2011		4.3	4.3
M5	11.8	2173		0.3	1.6
M6+M6' 1A2-200.4	12.2	2011		1.2	0.5
M7 1A1-210.4	14.2	2157		1.1	1.3
M8+M8'	14.4	2319		1.8	1.9
M9-1[d] 1A2-210.3	15.3	1995		—	1.3

(Table 2). Contd…..

Peak Code No. [a]	GU (ODS)	Molecular Mass (Da) [b]	Structure	Relative Quantity (%) [c]	
				Dog	Cat
M9-2[d]	15.3	2335	Galα-Galβ4GlcNAcβ2Manα6 (Fucα6) Manβ4GlcNAcβ4GlcNAc; NeuGcα3Galβ4GlcNAcβ2Manα3	—	0.7
M10+M10' 1A3-210.4	16.1	2157	Galβ4GlcNAcβ2Manα6 (Fucα6) Manβ4GlcNAcβ4GlcNAc; NeuAcα3Galβ4GlcNAcβ2Manα3	1.9	3.8
M11+M11'	16.4	2319	Galα-Galβ4GlcNAcβ2Manα6 (Fucα6) Manβ4GlcNAcβ4GlcNAc; NeuAcα3Galβ4GlcNAcβ2Manα3	3.6	5.7
Others				0.9	1.0
Total				16.6	24.6
Disialylated glycans					
D1+D1' 2A1-200.4	11.0	2302	NeuAcα6Galβ4GlcNAcβ2Manα6; NeuAcα6Galβ4GlcNAcβ2Manα3 Manβ4GlcNAcβ4GlcNAc	49.9	33.6
D2 2A3-210.4	15.0	2448	NeuAcα3Galβ4GlcNAcβ2Manα6; NeuAcα6Galβ4GlcNAcβ2Manα3 (Fucα6) Manβ4GlcNAcβ4GlcNAc	—	1.6
D3+D3' 2A4-210.4	17.0	2448	NeuAcα3Galβ4GlcNAcβ2Manα6; NeuAcα3Galβ4GlcNAcβ2Manα3 (Fucα6) Manβ4GlcNAcβ4GlcNAc	3.5	4.2
Others				2.1	1.9
Total				55.4	41.3
Trisialylated glycans					
T1-1+T1-1'[d] 3A3-300.8	13.4	2958	Neu5Acα3Galβ4GlcNAcβ2Manα6; Neu5Acα3Galβ4GlcNAcβ4 (Manα3); Neu5Acα6Galβ4GlcNAcβ2 Manβ4GlcNAcβ4GlcNAc	—	1.0
T1-2+T1-2'[d] 3A2-300.8	13.4	2958	Neu5Acα6Galβ4GlcNAcβ2Manα6; Neu5Acα3Galβ4GlcNAcβ4 (Manα3); Neu5Acα6Galβ4GlcNAcβ2 Manβ4GlcNAcβ4GlcNAc	—	4.2
T2 3A1-300.8	15.7	2958	Neu5Acα6Galβ4GlcNAcβ2Manα6; Neu5Acα6Galβ4GlcNAcβ4 (Manα3); Neu5Acα6Galβ4GlcNAcβ2 Manβ4GlcNAcβ4GlcNAc	—	0.4
Others				—	0.5
Total				—	6.1

[a]PA-oligosaccharides are coded according to the literature (Takahashi & Kato, 2003).
[b]Average mass calculated from the *m/z* values of $[M+Na]^+$ and $[M-H]^-$ ions for neutral and sialyl oligosaccharides, respectively.
[c]Molar percentages of glycan content in dog and cat lung tissues were calculated on the basis of peak areas in Fig. (**1b-e**) by comparison with total glycan content in the dog and cat lung tissues, respectively.
[d]Fractions N2, M1, M9, and T1 from the ODS column was separated into two sub-fractions on the amide column. The molar percentage of each glycan was calculated on the basis of peak areas in the elution profile on the amide column.

was 21.5:47.1 in domestic cat and 9.9:59.2 in dog lung tissue. Neu5Gc and tri-sialyl sugar chains were found in domestic cat, but not in domestic dog lung tissue. These results indicate that domestic cats are more susceptible than dogs to H5N1 influenza virus infection. The structure of receptor sialyl sugar chains in different host species may be useful for the development of new anti-influenza drugs and to clarify the mechanism or pathogenesis of H5N1 infection and viral tropism in other mammals.

ABBREVIATIONS

Da	=	dalton
DEAE	=	diethylaminoethyl
Gal	=	galactose
GALAXY	=	Glycoanalysis by the three axes of mass spectrometry and chromatography
GlcNAc	=	*N*-acetylglucosamine
GU	=	glucose unit value on the ODS and amide column
HPLC	=	high-performance liquid chromatography
HPAI	=	highly pathogenic avian influenza
MALDI-TOF-MS	=	matrix-assisted laser desorption/ionization-time of flight-mass spectrometry
Neu5Ac	=	5-*N*-acetylneuraminic acid
Neu5Gc	=	5-*N*-glycolylneuraminic acid
ODS	=	octadecyl silica
PA	=	pyrimidylamino
Sia	=	sialic acid

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