NOTE *Virology*

A Sero-Survey of Subtype H3 Influenza A Virus Infection in Dogs and Cats in Japan

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(Received 28 September 2010/Accepted 23 November2010/Published online in J-STAGE 7 December 2010)

ABSTRACT. A sero-epidemiological survey of human and equine H3 influenza A virus infections in dogs and cats using the hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests was conducted. Serum samples were collected from 582 dogs and 237 cats in Japan during the periods 2002–2008 and 1997–2008, respectively. Although no HI antibodies against equine H3 virus were detected, 9 (3.8%) from cats and 12 (2.1%) from dogs were HI-positive against human H3 virus. Only one serum each from dogs and cats was NI-positive against N2 virus. These findings suggest that although equine H3 influenza virus infections have not been prevalent in companion animals, human H3N2 influenza A virus infections have occurred in dogs and cats in recent years in Japan.
KEY WORDS: canine, feline, influenza A virus, sero-surveillance.

J. Vet. Med. Sci. 73(4): 541–544, 2011

Influenza A virus, a member of the *Orthomyxoviridae*, is one of the etiological agents of the highly contagious disease of influenza. Influenza A virus has 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes which are classified serologically. Many animals including birds, mammals and human are known to be susceptible to the virus infection, and particular HA subtypes tend to be found in particular host species except in waterfowl which are susceptible to all subtypes [14]. Influenza A virus infection has been well studied in domestic animals but relatively little is known about its occurrence in companion animals such as dogs and cats. Because companion animals are usually in close contact with human and domestic animals, influenza A virus infection in dogs and cats has important implications for both veterinary medicine and public health. In fact, several cases of interspecies transmission of influenza A virus from other animals including human to companion animals have been reported. Recently, equine-derived influenza A virus infections of the H3N8 subtype emerged in dogs in the United States [18] as a result of interspecies transmission from horses. The virus has been prevalent in dogs in the United States and was also reported in the United Kingdom [4]. Other studies have reported transmission of human H3N2 viruses [2] to dogs. In 1978, human H3 virus infection in cats was reported based on serological evidence [10]. In addition, the susceptibility of cats to human H3N2 influenza A virus infection has been confirmed by experimental infections [12]. Other subtypes that have been transmitted include highly virulent avian influenza H5N1 virus infections from birds to dogs and cats [5], and pandemic H1N1 influenza A virus infections in dogs and cats [6]. These findings are causing increasing concern about the prevalence or emergence of influenza A virus as a result of interspecies transmission involving dogs and cats. However, no serological or etiological studies have been carried out on these animals in recent years in Japan.

In the present study, we examined the prevalence of H3 subtype infection in dogs and cats in Japan because interspecies transmissions of the H3 virus from equine and human species to these companion animals have been reported and some of them have been serious problems in other countries in recent years. The study was carried out with a sero-epidemiological survey using the hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests.

Serum samples from 582 dogs and 237 cats from at least 44 and 25 prefectures of Japan, respectively, were obtained from the Veterinary Medical Centre of Tottori University, and kindly provided from Kyoritsu Seiyaku Corporation (Tokyo, Japan) and Kyoto Biken Laboratories, Inc. (Kyoto, Japan). The dog samples were collected from 2002 to 2008 and the cat samples were collected from 1997 to 2008. Influenza A viruses A/Tottori/45989/97 (H3N2); A/equine/Kentucky/1/94 (H3N8) and A/duck/Ukraine/63 (H3N8) were used in the HI test. In the NI test, A/Tottori/45989/97 (H3N2) and A/turkey/Wisconsin/1/66 (H9N2), were used to detect NI antibodies against N2 subtype. HI tests were carried out according to the World Organization for Animal Health (OIE) Manual of Diagnostic Tests and Vaccines for

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Terrestrial Animals [9] with some modifications. In the HI test, sera were treated with receptor-destroying enzyme (RDE) (DENKA SEIKEN Co., Ltd., Tokyo, Japan) prior to conducting the assay to destroy non-specific inhibitors as described [11]. For the detection of HI antibodies against equine H3N8 virus and human H3N2 virus, 0.6% chicken red blood cells (RBCs) (Nippon Bio-test Laboratories, Tokyo, Japan) and 0.75% guinea pig RBCs (Nippon Bio-test Laboratories) were used, respectively. The reciprocal dilution of the sera that showed complete inhibition of hemagglutination was recorded as the HI titer. NI tests were carried out as described by Aymard-Henry *et al.* [1], but all reagents used in the test were reduced to 1/10 volume of the original amounts. NA activity was measured as the optical density (OD) at 549 nm by using a spectrophotometer (Ultro 1100 pro, GE healthcare, England). In every test, phosphate buffered saline (PBS) was used instead of the serum samples as a negative control. The reciprocal dilution of the sera which showed 50% inhibition of neuraminidase activity was recorded as the NI titer. Anti-A/Aichi/2/68 (H3N2)-hyperimmune; anti-A/duck/Ukraine/63 (H3N8)-hyperimmune and, anti-A/turkey/Wisconsin/1/66 (H9N2)-hyperimmune chicken sera prepared basically according to previous report [7] were used as positive controls in each test.

Tracheal tissues obtained from dead cats donated from an animal shelter by permission of the Eastern Tottori General Office of Tottori Prefecture were used for lectin staining to reveal the distribution of receptor molecules for influenza A virus in their tracheal epithelium. The tissues were unfixed, frozen and embedded in optimal cutting temperature compound, and sectioned at 5 μm with a cryostat (Leica, CM1850 UV, Nussloch, Germany). Lectin staining of the feline tracheal tissues was performed according to Suzuki *et al.* [13] with some modifications. The sections were blocked with 5% bovine serum albumin (Wako Pure Chemical industries, Ltd., Osaka, Japan) in PBS for 1 hr at room temperature (RT), washed three times with 100 mM HEPES (pH7.5), mounted with FITC-labeled *Sambucus nigra* (SNA) lectin (1:400, Vector Laboratories, Burlingame, CA, U.S.A.) and biotinylated *Maackia amurensis* (MAA) lectin (1:400, Vector Laboratories) for detection of sialic acid α2,6 galactose (SAα2,6Gal) and sialic acid α2,3 galactose (SAα2,3Gal), respectively, kept at 4ºC overnight, washed three times with HEPES, incubated with Alexa Fluor 594-conjugated streptavidin (1:800, Molecular Probes, Inc., Eugene, OR, U.S.A.) for 2 hr at RT, washed three times with HEPES, counterstained with 4',6-diamino-2-phenylindole, dihydrochloride (DAPI; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) and observed with an FSX-100 Olympus microscope (Olympus, Tokyo, Japan). To confirm the specificity of SNA and MAA lectins binding to the SAα2,6Gal and SAα2,3Gal, respectively, control experiments were performed in which tracheal tissues from the two animal species were depleted of sialic acids by treatment with 25 unit/μl neuraminidase from *Clostridium perfringens* (New England Biolabs, Beverly, MA, U.S.A.) and incubated under humid conditions at 37ºC for 24 hr before staining with lectins as described [17].

All of the tested sera taken from either animal showed HI titers less than 16 against the equine H3N8 virus. On the other hand, of the 582 dog samples, 12 samples (2.1%) gave HI titers ranging from 16 to 64 against the human H3N2 strain. Of the 237 cat samples, 9 samples (3.8%) gave HI titers ranging from 32 to 128 (Table 1). HI tests using these 12 dog and 9 cat sera showed HI titers less than 16 against avian H3 virus. The year and prefecture of these sera taken, and breed, sex and age of each donor animal of the sera were also shown in Table 1. The positive rate of HI against human H3 in cats was similar to the rate in cats reported in 1978 (5.8%) [10]. On the other hand, the positive rate in dogs was different from the previous report [10] which showed no HI antibodies against human H3 virus in 126 dogs. Subsequently, these 21 sera were subjected to NI tests against human H3N2 and avian H9N2 strains. Although only one of the dog samples, No.11458 from Hokkaido in 2008 with an HI titer of 16 against human H3N2 virus showed NI titer against H3N2 (a titer of 6.5), NI titer of this sample against H9N2 was less than 3.2 ($10^{0.5}$). Only one of the cat samples, No. BF-72 from Hokkaido in 1998 with an HI titer of 128 against human H3N2 virus, showed NI titers against H3N2 (a titer of 25) and H9N2 (a titer of 31) (Table 1). In lectin staining, we were unable to determine whether SAα2,3Gal was present in the cat trachea because pretreatment with neuraminidase did not affect the MAA lectin staining. On the other hand, FITC-labeled SNA lectin reacted with the cat epithelial cells indicating the presence of SAα2,6Gal, which is thought to be a major receptor for human influenza A virus [3], in cat. This reaction was considered as specific because the reaction was disappeared by pretreatment with neuraminidase (data not shown).

Only two sera, one from a dog and one from a cat, were HI-positive against human H3 virus and NI-positive against N2 viruses. On the other hand, HI antibodies against equine and avian H3 viruses were not detected in these sera. These results imply that the cat and the dog were previously exposed to currently circulating H3N2 human influenza A virus rather than to equine or avian viruses. In lectin staining, SAα2,6Gal, which is thought to be a major receptor for human influenza A virus, was detected in the cat tracheal epithelial cells. Furthermore, recent studies on the distribution of receptor molecules in the canine respiratory tract [4, 8] detected SAα2,3Gal and SAα2,6Gal. Although SAα2,3Gal was the dominant receptor, SAα2,6Gal was detected in the trachea, bronchus and bronchiole. These reports, together with our detection of SAα2,6Gal in the cat, are consistent with the hypothesis that the sero-positive cat and dog found in this study were infected with H3N2 influenza A virus from human sources.

The NI tests employed in this study were designed to detect antibodies against the N2 subtype of influenza A virus because all sera used in the NI tests showed HI activities against human H3 virus but not to equine and avian H3

Table 1. HI and NI titers of the 21 samples positive in HI tests against A/Tottori/45989/97 (H3N2), year and prefecture of the sera taken, and breed, sex and age of each donor animal of the sera

Species	Sample ID	Year	Prefecture	Breed	Sex	Age (Year)	HI titer against[a)]			NI titer against[b)]	
							human H3[c)]	equine H3[d)]	avian H3[e)]	H3N2[c)]	H9N2[f)]
Dog	Kc139	2005	Akita	Golden Retriever	–	–	64	<16	<16	<3.2	< 3.2
	c53	2006	Tottori	Corgi	Male	4	32	<16	<16	<3.2	< 3.2
	c49	2006	Tottori	Miniature Dachshund	Female	12	16	<16	<16	<3.2	< 3.2
	c151	2007	Hiroshima	–	–	–	64	<16	<16	<3.2	< 3.2
	11520	2008	Aichi	–	–	11	64	<16	<16	<3.2	< 3.2
	11458	2008	Hokkaido	Siberian Husky	–	1	16	<16	<16	6.5	< 3.2
	c137	2008	Tottori	West Highland White Terrier	Male	13	64	<16	<16	<3.2	< 3.2
	c139	2008	Tottori	Kishu Inu	Female	10	64	<16	<16	<3.2	< 3.2
	c150	2008	Tottori	Siberian Husky	Female	16	64	<16	<16	<3.2	< 3.2
	11008	–	Gifu	American Cocker	–	4	64	<16	<16	<3.2	<3.2
	11949	–	Kanagawa	Pug	Female	7	32	<16	<16	<3.2	<3.2
	10953	–	Mie	Labrador Retriever	Female	10	64	<16	<16	<3.2	< 3.2
Cat	BF–72	1998	Hokkaido	Japanese Cat	–	2	128	<16	<16	25	31
	F5	2006	Tottori	Japanese Cat	Female	6	128	<16	<16	<3.2	< 3.2
	F11	2006	Tottori	–	–	–	64	<16	<16	<3.2	< 3.2
	BF6	2007	Chiba	Japanese Cat	–	–	128	<16	<16	<3.2	< 3.2
	c144	2007	Tottori	Japanese Cat	Female	16	128	<16	<16	<3.2	< 3.2
	F111	2007	Tottori	–	–	–	64	<16	<16	<3.2	< 3.2
	F115	2007	Tottori	–	–	–	64	<16	<16	<3.2	< 3.2
	c141	2008	Tottori	–	Male	11	64	<16	<16	<3.2	< 3.2
	c146	2008	Tottori	Japanese Cat	Male	3	32	<16	<16	<3.2	< 3.2

a) Haemagglutination inhibition (HI) titers are expressed as a reciprocal of the dilution that completely inhibits hemagglutination of 4 HA units of the tested antigens. b) Neuraminidase inhibition (NI) titers are expresssed as the serum dilution that gave 50% inhibition of neuraminidase activity against the tested antigen. c) A/Tottori/45989/97 (H3N2). d) A/equine/Kentucky/1/94 (H3N8). e) A/duck/Ukraine/63 (H3N8). f) A/turkey/Wisconsin/1/66 (H9N2). –: no data.

viruses as described above. However, only 2 samples gave positive in NI tests against the N2 subtype. Although it is unclear why no NI antibodies were detected in the other 19 sera, A/Tottori/45989/97 (H3N2) used in this study may not be suitable for detection of the antibodies raised by the recent influenza A virus infection. Other H3N2 viruses isolated in recent years may be more suitable for detection of NI antibodies against the N2 subtype. Another possibility is that the NI negative sera against the N2 subtype have antibodies against other NA subtypes of influenza A viruses. Unfortunately, we could not employ the NI tests against NA subtypes other than N2 because the amounts of sera obtained for this study were not enough to conduct the NI tests.

In this study, no antibodies were detected in dog sera against the equine H3N8 strain, which is the same subtype prevalent in dogs in the United States [18] and the United Kingdom [4] as a result of interspecies transmission from horses. From mid to late August 2007, there were outbreaks of H3N8 subtype infection in horses in Japan [16]. Subsequently, interspecies transmission of the Japanese isolate A/equine/Ibaraki/1/07 from horses to dogs was confirmed under experimental conditions [15]. However, all of the sample sera from dogs collected in this period were negative. This raises the possibility that the H3N8 infection, which is prevalent in other countries, has not been prevalent in Japan.

In the present study, antibodies against the human H3 and N2 subtypes of influenza A virus were detected in dog and cat sera examined. This raises the possibility that dogs and cats are important in the epidemiology of influenza A virus in Japan. In addition, we conducted preliminary sero-survey using the same samples with the HI test against another HA subtype of H1 human influenza A virus. The HI tests showed HI antobodies against human H1 virus in dogs and cats in Japan (data not shown). Since influenza A virus infection has emerged unexpectedly in dogs and cats in other countries, continuous and extensive surveillance is needed to control and prevent widespread outbreaks of influenza A in these animals.

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