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Prevalence of multiple subtypes of influenza A virus in Japanese wild raccoons

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ABSTRACT

Raccoons (*Procyon lotor*), which are not native to Japan, have been suspected to transmit various pathogens by frequent intrusion into agricultural and residential areas. To determine influenza A virus seropositivity in raccoons in Japan, we examined a total of 634 raccoons captured in 19 towns (A–S) from 2009 to 2012. Agar gel precipitation tests showed that the antibody prevalence was 1.89% (12/634). All positive raccoons were captured in three towns (A–C) located within a radius of approximately 30 km, and 75% had antibodies to multiple subtypes (H1, H3–5, N1, N6, and N8). H3 and N8 antibodies were most frequently detected (75%). Among all the raccoons captured, 67% (8/12) were found in town A in 2009 and 2010, and all five raccoons captured in 2010 had H3 and N8 antibodies, suggesting that transmission of the subtype might occur. H5 and N1 antibodies were also detected in two raccoons captured in town A. Virus neutralization tests examining the highly pathogenic avian influenza virus (HPAIV) H5N1 subtype (four isolates of which have been detected in Japan to date) and the low PAIV (LPAIV) H5N3 subtype showed that raccoon sera highly cross-reacted with three H5N1 strains (clade 2.5: Ck/Yamaguchi/7/04; clade 2.3.2.1: Whooper swan/Hokkaido/1/08 and Whooper swan/Hamanaka/11), while they displayed a low cross-reactivity with the antisera to the clade 2.2 virus (Ck/Miyazaki/K11/07) and H5N3 LPAIV (Whistling swan/Shimane/499/83). Thus, the origin of the H5N1 virus was not clearly defined. The viral M gene was detected in four antibody-negative raccoons captured in three towns by real-time reverse transcription-polymerase chain reaction (rRT-PCR) with high Ct values, although no virus was isolated. This study is the first report showing that raccoons of Japan were infected with multiple subtypes of influenza A virus, including H5N1. It remains to be elucidated how raccoons play a role in persistence of influenza A virus in nature and if they could pose risks to animal and human health, for example, by playing a role as "mixing vessel" to generate novel strains of influenza A virus.

1. Introduction

Influenza A virus is classified into subtypes based on the external viral proteins, hemagglutinin (HA) and neuraminidase (NA). To date, 18 HA and 11 NA subtypes have been found (Spackman, 2008; Tong et al., 2013). The principal natural hosts of influenza A virus are thought to be water birds of the orders *Anseriformes* and *Charadriiformes*. However, this virus has been reported to infect not only birds, but also various species of mammals, including humans, pigs, horses, and many others (Webster et al., 1992).

Highly pathogenic avian influenza viruses (HPAIVs) have been regarded as agents causing public health problems in addition to great economic damage to poultry farming. In Japan, HPAI outbreaks have occurred successively almost every year since 2007 (OIE, 2013a, 2013b). Although the invasion route of HPAIV into poultry farms has not been determined, an epidemiological survey suggested that transmission occurs through wild animals that

Abbreviations: HA, hemagglutinin; NA, neuraminidase; HPAIV, highly pathogenic avian influenza virus; VN, virus neutralization; VTM, virus transfer medium; AGP, agar gel precipitation; CAMs, chorioallantoic membranes; HI, hemagglutination inhibition; NI, neuraminidase inhibition; RDE, receptor destroying enzyme; PBS, phosphate-buffered saline; AF, allantoic fluid; LPAIV, low pathogenic avian influenza virus; rRT-PCR, real-time reverse transcription–polymerase chain reaction.

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invade farms, such as mice and rats in addition to wild birds (Food Safety and Consumer Affairs Bureau, Ministry of Agriculture et al., 2007).

It has been reported that various animal species can be infected with HPAIV in natural and laboratory conditions (Klopfleisch et al., 2007; Reperant et al., 2008; Zhou et al., 2009), suggesting that they can become HPAIV carriers. Raccoons (*Procyon lotor*), species native to North America, are an invasive species in Japan; recently, these animals have rapidly and widely distributed throughout Japan after their introduction in the field in the 1960s (Yanagihara-Agetsuma, 2004). Raccoons have caused many problems, including damage to crops and have adverse effects on the ecosystem by predation on native animals. Transmission of pathogens is an additional consequence of the presence of raccoons (Ikeda et al., 2004). Many pathogens, including *Leptospira* (Koizumi et al., 2009), raccoon roundworm (Jacobson et al., 1982), and Japanese encephalitis virus (Ohno et al., 2009), have been reported to infect raccoons. Influenza A virus is one of the pathogens that can infect raccoons, which are omnivores that are abundant near water, and may have chance to prey on wild water birds, which are known to be the natural hosts of influenza A virus. Hall et al. (2008) and Roberts et al. (2009) detected antibodies to influenza A viruses from raccoons in the United States (U.S.). Horimoto et al. (2011) also reported that raccoons captured during 2005–2008 in Japan might be infected with H5N1 HPAIV following the detection of specific antibodies (0.9% of 1088) by virus neutralization (VN) tests.

Since it was reported that raccoons invaded poultry farms and homes (Yamazaki et al., 2009), HPAIV infection in raccoons may represent a significant concern for agriculture and human health. Experimental studies showed that raccoons were susceptible to both human H3N2 and avian H4N8 influenza virus infections and have human and avian type cellular receptors, suggesting that raccoons may play a role as mixing vessels of influenza A virus, similar to pigs (Hall et al., 2008). However, information about the role of raccoons in the propagation and transmission of influenza A virus, and the risks associated with infected raccoons to animal and human health has been limited (Hall et al., 2008; Horimoto et al., 2011; Roberts et al., 2009; Root et al., 2010). Therefore, further and continuous investigations on the host–parasite relationship between influenza A viruses and raccoons is necessary to elucidate those critical points.

In Japan, there have been no reports on prevalence of influenza A virus in wild raccoons since 2008, although one serological surveillance study was conducted during 2005–2008 (Horimoto et al., 2011). In this study, we conducted a serological, virological, and genetical survey in raccoons and identified multiple subtype infections of influenza A virus, including H5N1.

2. Materials and methods

2.1. Samples

Sera and nasal and rectal swabs were collected from raccoons captured under the official eradication program in the eastern Japan (towns A–S) during 2009–2012 (Table 1).

Sera were collected from 634 raccoons and stored at −20 °C or −80 °C until use. Nasal swabs were collected from 131 raccoons, and rectal swabs from 129 raccoons. Swabs were placed in 1 mL of virus transfer medium (VTM) consisting of Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) including penicillin G (final concentration of 1000 U/mL), streptomycin (1 mg/mL), gentamycin (100 μg/mL), and amphotericin B (10 μg/mL). These samples were kept at room temperature for 2 h and stored at −80 °C until use.

Table 1
Detection of AGP antibody to influenza A virus in raccoons.

Town	Year	No. positive/ no. of sera tested	Prevalence (%)
A	2009	3/49	6.12
	2010	5/58	8.62
	2011	0/21	0.00
Subtotal	2009–2011	8/128	6.25*
B	2009	1/72	1.39
	2010	0/32	0.00
	2011	1/64	1.56
Subtotal	2009–2011	2/168	1.19
C	2009	1/36	2.78
	2010	0/25	0.00
	2011	1/4	25.00
Subtotal	2009–2011	2/65	3.08
D–S	2009–2012	0/273	0.00
Total		12/634	1.89

* $P < 0.05$ (among three towns [A–C] from 2009 through 2011).

2.2. Serological assay

2.2.1. Agar gel precipitation (AGP) test

Sera were tested for antibodies against influenza A virus by AGP tests, as described in a previous report (Beard, 1970). AGP antigens were prepared as described previously (Beard, 1970). Briefly, chorioallantoic membranes (CAMs) were collected from 10-day-old embryonated chicken eggs infected with A/budgerigar/Aichi/97 (H3N8), which was kindly provided by the National Institute of Animal Health (NIAH), Japan. The membranes were cut into small pieces and homogenized in phosphate-buffered saline (PBS, pH 7.4). The homogenates were treated with 0.1% formalin for inactivation of the virus. AGP-positive control serum was kindly provided by NIAH, Japan.

2.2.2. Hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests

The following reference viruses and antisera, which were kindly provided by Dr. H. Kida, Hokkaido University, Japan, were used in this study: A/swine/Hokkaido/1/81 (H1N1), A/duck/Hong Kong/278/78 (H2N9), A/duck/Hokkaido/5/77 (H3N2), A/duck/Czechoslovakia/56 (H4N6), A/duck/Hong Kong/820/80 (H5N3), A/shearwater/South Australia/1/72 (H6N5), A/duck/Hong Kong/301/78 (H7N2), A/turkey/Ontario/6118/67 (H8N4), A/duck/Hong Kong/448/78 (H9N2), A/chicken/Germany/N/49 (H10N7), A/duck/England/1/56 (H11N6), A/duck/Alberta/60/76 (H12N7), A/gull/Maryland/704/77 (H13N6), A/mallard/Astrakhan/263/82 (H14N5), and A/duck/Australia/341/83 (H15N8).

HI tests were conducted according to the World Health Organization (WHO) Manual on Animal Influenza Diagnosis and Surveillance (WHO Manual) by using 0.5% chicken blood cells. Briefly, sera were treated with receptor destroying enzyme (RDE) (Denka Seiken, Tokyo, Japan) to remove nonspecific inhibitors, and were serially diluted to 2-fold in PBS starting from 1:16.

NA activity of the reference viruses (N1–N9) was measured as described previously (Imai et al., 2012) to determine the optimal virus dilution giving a value of 0.5 units of OD_{549} for NI tests. Briefly, 10 μL of the reference virus (OD = 0.5) was mixed with an equal volume of the serum sample, which was diluted at 1:10 in PBS, and then 20 μL of fetuin solution was added (Sigma, St. Louis, MO). The tubes containing the mixtures were incubated at 37 °C for 18 h. The tubes were cooled to room temperature, and then 20 μL of periodate reagent was added to each tube, followed by shaking and incubation at room temperature for 20 min. The reaction was stopped by the addition of 200 μL of arsenite reagent. Then, 500 μL of thiobarbituric acid reagent was added to each tube followed by 15 min incubation at 100 °C. After cooling the tubes on