北海道沿岸のゼニガタアザラシ(Phoca vitulina stejnegeri)のインフルエンザAウイルス感染に関する血清学的調査

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FULL PAPER *Virology*

Serological Evidence of Influenza A Virus Infection in Kuril Harbor Seals (*Phoca vitulina stejnegeri*) of Hokkaido, Japan

Kei FUJII[1], Chiharu KAKUMOTO[2], Mari KOBAYASHI[3], Sachiko SAITO[4], Tatsuya KARIYA[5], Yukiko WATANABE[6], Yoshihiro SAKODA[7], Hiroshi KIDA[7] and Masatsugu SUZUKI[1]

[1]*Laboratory of Wildlife Biology, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818,* [2]*Eco Friends, Sapporo 002–0851,* [3]*Laboratory of Aqua Resource Management, Department of Aqua Bioscience and Industry, Tokyo University of Agriculture, Abashiri 099–2422,* [4]*Laboratory of Veterinary Anatomy and* [5]*National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080–8555,* [6]*Institute for Raptor Biomedicine Japan, Kushiro 084–0922, and* [7]*Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan*

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ABSTRACT. For proper management and conservation of the Kuril harbor seal (*Phoca vitulina stejnegeri*) through disease control, serological analysis was performed for influenza A virus infection in free-ranging seals in Hokkaido, Japan. Serum samples were collected from seals at Nosappu (231 seals), Akkeshi (16) and Erimo (75), between 1998 and 2005, and were analyzed by ELISA. Antibodies to the influenza A virus were detected only in seals from Nosappu. The incidences were 11% (1/9), 3% (2/66), 12% (7/59) and 6% (5/77) in 1998, 2003, 2004 and 2005, respectively. These suggest sporadic infection. Because antibody-positive seals included juvenile seals in each year, the infections were considered to have been circulated since no later than the late 1990s until recent years. ELISA-positive sera were analyzed by hemagglutination inhibition (HI) tests to determine the subtypes. Antibodies to the H3 and H6 subtypes were detected in 10 and 2 sera, respectively. Two of the sera that had antibodies to the H6 subtype also had antibodies to the H3 subtype. These two seals were considered to have been infected with both the H3 and H6 subtypes. This is the first investigation to find antibodies to the H6 subtype in seals. Although the H6 subtype had been isolated only from avians, genetic analysis had suggested that the H6 subtype could become a novel mammalian pathogen. For definitive diagnosis, detection of the virus from the tissue or mucus of seals is required.

KEY WORDS: H6 subtype, influenza A virus, Japan, *Phoca vitulina stejnegeri*, seroepidemiology.

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The influenza A virus infects various avian and mammalian species, including humans and marine mammals such as seals and cetaceans [20, 34]. Waterfowls are the natural primary hosts for all the strains of influenza A virus that have been introduced into domestic poultry, mammals and humans. It has been experimentally demonstrated that in human pandemics, pigs serve as intermediate hosts [17]. Monitoring of viral infections in domestic and feral birds and mammals is important for the control of animal disease and for the prevention of human pandemics.

Mass mortality associated with pneumonia occurred in Western Atlantic harbor seals (*Phoca vitulina concolor*) on the northeast coast of the U.S.A. in 1979–1980 [8]. Six hundred seals, approximately 20% of the regional harbor seal population, died. The A/seal/Massachusetts/1/80 (H7N7) influenza virus was isolated from dead animals during that outbreak [19, 35]. The H4N5 [10], H4N6 and H3N3 [2] viruses were isolated from dead seals in subsequent epizootics of pneumonia in the same location in 1982–1983 and 1991–1992. All of these seal viruses were of avian origin [2, 10, 16, 35]. Serological investigation of seals in the Bering Sea [6], Barents Sea [30], Alaska [5], Arctic Canada [22], Caspian Sea [24], Lake Baikal and Kara Sea [23] revealed viral infections.

The Kuril harbor seal (*P. v. stejnegeri*) is one of five harbor seal subspecies [1, 18]. Kuril harbor seals are distributed from the coast of Hokkaido along the Northwestern Pacific and northward along the Kuril Islands and eastern Kamchatka, as far north as the Commander Islands. The population of Kuril harbor seals in Hokkaido dramatically declined from hunting; the number of hauling-out sites decreased [12]. Although the population has been rebounding for the past 20 years, exceeding 900 individuals in 2002, the number of hauling-out sites has not recovered [27]. Over 60% of Kuril harbor seals in Hokkaido are concentrated at two hauling-out sites (Daikoku Island, Akkeshi and Erimo) of the seven in the prefecture [4, 13, 27]. Overpopulation at hauling-out sites increases the opportunity for pathogenic infection. For proper management and conservation of seals, information on the prevalence of the influenza A virus, which can cause mass mortality of seals, is indispensable. However, investigation of influenza A virus infection in Kuril harbor seals of Hokkaido had not been done. In this study, serological analysis was performed for influenza A virus infection in Kuril harbor seals in Hokkaido, Japan.

MATERIALS AND METHODS

Samples: Serum samples were collected from 322 Kuril harbor seals in Nosappu, Akkeshi and Erimo (Fig. 1). All samples from Nosappu (231 sera) and Akkeshi (16) were

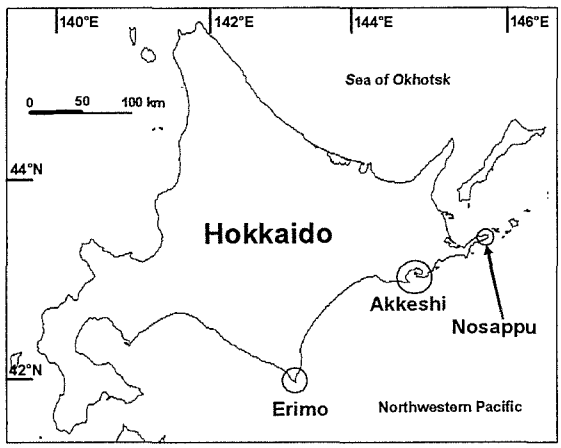

Fig. 1. Map of the sampling area. Sample sera were collected from Kuril harbor seals at Nosappu (231 sera), Akkeshi (16) and Erimo (75), between 1998 and 2005.

obtained from seals by-caught in salmon fixed nets between late August and November. Of the samples from Erimo, 46 samples were collected from seals captured under investigative capturing from late June to the beginning of July, and 29 samples were collected from seals by-caught in salmon fixed nets between late-August and November. The body length (nose to tail-tip) of all seals was measured as an index of age [7, 21, 31]. Seals which were 125 cm or below were judged to be juvenile, that is less than 2 years of age.

Viruses: Influenza viruses A/swine/Iowa/15/30 (H1N1), A/Singapore/1/57 (H2N2), A/Aichi/2/68 (H3N2), A/duck/Czechoslovakia/56 (H4N6), A/duck/Pennsylvania/56 (H5N2), A/shearwater/Australia/1/72 (H6N5), A/seal/Massachusetts/1/80 (H7N7), A/turkey/Ontario/6118/67 (H8N4), A/chicken/Hong Kong/G24/98 (H9N2), A/chicken/Germany/N/49 (H10N7), A/duck/England/1/56 (H11N6), A/duck/Alberta/60/76 (H12N5), A/gull/Maryland/704/77 (H13N6), A/mallard/Astrakhan/263/82 (H14N5) and A/duck/Australia/341/83 (H15N8) were obtained from the repository of the Department of Disease Control in the Graduate School of Veterinary Medicine at Hokkaido University. The viruses were propagated in the allantoic cavity of 10- to 11-day-old embryonated hen' eggs at 35°C for 48 hr. Allantoic fluid infected with each virus was treated with 0.1% formalin and 0.08% NaN_3 at 4°C for one week to inactivate the virus. A/Aichi/2/68 (H3N2) virus was purified by high-speed centrifugation of the allantoic fluid followed by differential centrifugation through a 10 to 50% sucrose density gradient [15] and used as the antigen in enzyme-linked immunosorbent assay (ELISA). The inactivated fluid samples were added to glycerol at 1:1 volume, and used as antigens in a hemagglutination-inhibition (HI) test.

Enzyme-linked immunosorbent assay (ELISA): ELISA was performed according to the modified procedure described previously [16, 24]. Purified A/Aichi/2/68 (H3N2) virus was disrupted in 0.5 M Tris pH 7.8 containing 0.5% Triton X-100 and 0.6 M KCl and diluted to 2 μg/ml in phosphate-buffered saline (PBS). As antigen, the disrupted virus was absorbed into each well (50 μl/well) of a 96-well microtiter plate at 4°C over 2 hr. After the wells were blocked with 1% bovine serum albumin (BSA) in PBS for one hour at room temperature (RT), they were washed with 0.05% Tween 20 in PBS (PBST). Fifty μl of sera diluted at 1:100 in PBS containing 0.5% BSA and 0.05% Tween 20 (BSA-PBST) was added to each well. After incubation at RT for one hr, the plates were washed with PBST. Fifty μl of Peroxidase-conjugated Protein G (Sigma) diluted at 1:200 in BSA-PBST was added to each well, and the plate was then incubated for 1 hr at RT. The plates were washed with PBST, and 100 μl of substrate solution (0.05 M citrate buffer pH 4.0, 0.008% hydrogen peroxide; 40 mM 2.2'-azino-di-3-ethyl-benzothiazobine-6-sulfuric acid) was added to each well. After incubation for 30 min at RT, the optical density (OD) of each well was read with a spectrophotometer using a 405 nm filter. The cutoff value was set at 0.48. This value was taken from the OD of apparently negative samples collected in this study. Distribution of OD for the samples was unimodal, with the mode around 0.2. The mean OD of the negative sera plus three standard deviations was 0.48.

Hemagglutination-inhibition (HI) test: An HI test was carried out with the reference influenza A virus strains of the known hemagglutinin (HA) subtypes (H1-H15). Sera were treated with trypsin and KIO_4 according to Stuart-Harris and Schild [29] to inactivate nonspecific hemagglutination inhibitors. Then, to exclude nonspecific hemagglutinations, the sera were incubated with chicken red blood cells (RBC). HI tests were performed by a microtechnique described by Sever [28]. Briefly, 25 μl of serial twofold dilution of the treated serum samples was mixed with 4HA units of virus in microtiter plates and incubated at RT for 30 min. Then 50 μl of 0.5% chicken RBCs was added to each well and incubated on ice for 45 min. The HI titer was expressed as the reciprocal of the highest serum dilution that completely inhibited hemagglutination of 4HA units of the virus. Serum samples indicating <10 HI titer was regarded as negative.

RESULTS

In ELISA, positive sera were detected only in serum samples from Nosappu (Table 1). The incidences were 11% (1/9), 3% (2/66), 12% (7/59) and 6% (5/77) in 1998, 2003, 2004 and 2005, respectively. The range of OD for ELISA-positive sera was 0.486–1.177 (Table 2). There was no difference in incidence between males and females (Fisher's exact test, $P>0.05$). In each year, one or two ELISA-positive seals were ≤125 cm in body length; these were judged to be juveniles (Table 2). There was no difference in the incidence between juveniles and adults (Fisher's exact test, $P>0.05$).

All ELISA-positive sera were used for the HI tests. One serum out of 1 in 1998, 6 out of 7 sera in 2004 and 3 out of 5 sera in 2005 inhibited hemagglutination of the A/Aichi/2/

Table 1. Number of antibody-positive sera to influenza A virus collected from Kuril harbor seals at Nosappu, Akkeshi and Erimo, Hokkaido, Japan

Year	Nosappu		Akkeshi		Erimo	
	Male	Female	Male	Female	Male	Female
1998	0/2[a)]	1/7	–	–	–	–
1999	–	–	–	–	0/6	0/2
2003	1/31	1/35	–	–	0/9	0/6
2004	4/47	3/32	0/2	0/2	0/16	0/12
2005	3/45	2/32	0/7	0/5	0/15	0/9
Total	8/125	7/106	0/9	0/7	0/46	0/29

a) Number of sera containing antibodies to influenza A / Number of sample sera.

Table 2. Hemagglutination-inhibition (HI) titers of ELISA-positive sera collected from Kuril harbor seals at Nosappu, Hokkaido, Japan

ID of seals	Sampling year	Sex[a)]	Maturity[b)]	Body length (cm)	OD_{450}[c)]	Hi titers (HA subtype)
NZ9811	1998	F	J	125	0.590	1:80 (H3), 1:80 (H6)
NZ0348	2003	M	J	118	0.631	–
NZ0350	2003	F	J	122	0.601	–
NZ0422	2004	F	A	141	0.486	1:640 (H3)
NZ0426	2004	F	J	109	0.702	1:640 (H3)
NZ0429	2004	M	A	135	0.494	1:10 (H3)
NZ0468	2004	F	A	171	0.630	1:40 (H3)
NZ0470	2004	M	J	122	1.177	1:80 (H3)
NZ0475	2004	M	A	138	0.488	1:40 (H3), 1:40 (H6)
NZ0484	2004	M	A	129	0.658	–
NZ0509	2005	M	A	151	0.555	1:2560 (H3)
NZ0511	2005	M	A	167	0.557	1:640 (H3)
NZ0515	2005	F	A	140	0.518	–
NZ0519	2005	M	A	147	0.639	1:640 (H3)
NZ0521	2005	F	J	121	0.563	–

a) M or F indicates male or female, respectively.
b) Seals measuring 125 cm or less, were judged to be juvenile (age < 2 years). J or A indicates juvenile or adult (age ≥ 2 years), respectively.
c) Optical density read in ELISA with 405 nm filter.

68 (H3N2) virus at HI titers in the range of 10–2560 (Table 2). One serum out of 1 in 1998 and 1 out of 7 sera in 2004 inhabited hemagglutination of the A/shearwater/Australia/1/72 (H6N5) virus at HI titers in the range of 40–80. Two sera responding to the H6 subtype also contained antibodies to the H3 subtype. The ELISA-positive sera did not inhibit hemagglutination of other subtypes than H3 and H6.

DISCUSSION

The present results provide serological evidence for sporadic influenza A virus infection in Kuril harbor seals from Nosappu, Hokkaido, Japan. Antibodies to influenza A virus were detected in both juvenile and adult seals. In each year, antibody-positive seals included juveniles under 2 years of age. Because more than 3 months had passed since birth [21], the detected antibodies were not considered as maternal antibodies [26, 36]. These results indicate that sporadic infection in Kuril harbor seals have bees recurring since no later than the late 1990s. It is thought that the seals inhabiting around the south Kuril Islands may be exposed to influenza A virus, as the Hokkaido seals have been exposed, because most of the seals by-caught in salmon fixed nets at Nosappu are considered to originate from the Habomai Islands [9, 33].

In HI tests, 10 of the 15 ELISA-positive sera inhibited hemagglutination of the influenza A virus. It is generally agreed that ELISA using purified influenza A virus as the antigen is more sensitive than HI test. Therefore, it is possible that ELISA might show a positive for the virus without the subtype being identifiable by HI test. Previous serological studies on influenza A virus infection have shown that the same sample can test positive by ELISA but negative by HI test [22–24]. In this study, antibodies to H3 and H6 subtypes of influenza A virus were detected from sera collected from Kuril harbor seals at Nosappu. H3 subtypes of influenza A virus have been isolated from dead harbor seals from Cape Cod, U.S.A. [2]. Antibodies to the H3 subtype were detected in the seals from the Bering Sea [6]. H3 subtypes commonly infect other species including humans, pigs, horses and birds [25, 32]. Antibodies to the H6 subtype were detected in 2 sera. Additionally, neutralization test

showed antibodies to the H6 subtype in the 2 sera (data not shown). As the 2 seals whose sera contained antibodies to the H6 subtype also contained antibodies to H3 subtype, the seals were considered to have been infected with both subtypes. This is the first investigation to find antibodies to the H6 subtype in seals. The H6 subtype has not yet been isolated from mammals, but only from avians, including turkeys, shorebirds, ducks, geese and chickens [14, 25]. Genetic analysis has suggested that the H6 subtype isolated from teals can become a novel mammalian pathogen [3, 11]. For definitive diagnosis, detection of the virus from the tissue or mucus of seals is required.

Antibodies to the influenza A virus were only detected in Kuril harbor seals from Nosappu. The distance from Erimo to Akkeshi is about 170 km, and no hauling-out sites exist between the two areas [27]. Analysis of mtDNA has shown that there is little movement of Kuril harbor seals between Erimo and southeastern Hokkaido (from Akkeshi to Nosappu) (Watanabe, undergraduate thesis, Obihiro University of Agriculture and Veterinary Medicine, 2000). The difference in the incidence trends between Nosappu and Erimo may relate to the exiguity of contact between the Kuril harbor seals inhabiting southeastern Hokkaido and the Kuril harbor seals inhabiting Erimo.

Many waterfowls, including geese, gulls and cormorants, are found at habitats of Kuril harbor seals, and hauling-out sites are contaminated by feces from these birds. Though the origin of the influenza viruses in the seals reported in this study is not clear, the seals may be exposed to viruses in seabird feces. Previous studies on the influenza A virus in seals stranded on the northwest coast of the U.S.A. have shown that these seal viruses originated from birds [2, 10, 16, 35].

Our findings suggest that influenza A virus infection has occurred in marine mammals in Hokkaido and the south Kuril Islands. However, mass mortalities from viral infection have not been reported for marine mammals in this area. Ongoing monitoring is required for the management and conservation of these animals and the prevention of zoonoses.

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