Serological Evidence of Influenza A Virus Infection in Kuril Harbor Seals (Phoca vitulina stejnegeri) of Hokkaido, 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 322 Kuril harbor seals in Nosappu (231 sera), Akkeshi (16) and Erino (75), between 1998 and 2005, and were analyzed by ELISA. Antibodies to 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) from seals at Nosappu (231 seals), Akkeshi (16) and Erimo (75), between 1998 and 2005, and were analyzed by ELISA. 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. 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.
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 captured under investigations was 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 KIO4 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 sera 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/
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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 sera 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 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

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.

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

<table>
<thead>
<tr>
<th>Year</th>
<th>Nosappu</th>
<th>Akkeshi</th>
<th>Erimo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>1998</td>
<td>0/2⁴</td>
<td>1/7</td>
<td>–</td>
</tr>
<tr>
<td>1999</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2003</td>
<td>1/31</td>
<td>1/35</td>
<td>–</td>
</tr>
<tr>
<td>2004</td>
<td>3/47</td>
<td>3/32</td>
<td>0/2</td>
</tr>
<tr>
<td>2005</td>
<td>3/45</td>
<td>2/32</td>
<td>0/7</td>
</tr>
<tr>
<td>Total</td>
<td>8/125</td>
<td>7/106</td>
<td>0/9</td>
</tr>
</tbody>
</table>

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**

<table>
<thead>
<tr>
<th>ID of seals</th>
<th>Sampling year</th>
<th>Sex⁴</th>
<th>Maturity⁵</th>
<th>Body length (cm)</th>
<th>OD₄₅₀ ⁶</th>
<th>Hi titers (HA subtype)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NZ9811</td>
<td>1998</td>
<td>F</td>
<td>J</td>
<td>125</td>
<td>0.590</td>
<td>1:80 (H3), 1:80 (H6)</td>
</tr>
<tr>
<td>NZ0348</td>
<td>2003</td>
<td>M</td>
<td>J</td>
<td>118</td>
<td>0.631</td>
<td>–</td>
</tr>
<tr>
<td>NZ0350</td>
<td>2003</td>
<td>F</td>
<td>J</td>
<td>122</td>
<td>0.601</td>
<td>–</td>
</tr>
<tr>
<td>NZ0422</td>
<td>2004</td>
<td>F</td>
<td>A</td>
<td>141</td>
<td>0.486</td>
<td>1:640 (H3)</td>
</tr>
<tr>
<td>NZ0426</td>
<td>2004</td>
<td>F</td>
<td>J</td>
<td>109</td>
<td>0.702</td>
<td>1:640 (H3)</td>
</tr>
<tr>
<td>NZ0429</td>
<td>2004</td>
<td>M</td>
<td>A</td>
<td>135</td>
<td>0.494</td>
<td>1:10 (H3)</td>
</tr>
<tr>
<td>NZ0468</td>
<td>2004</td>
<td>F</td>
<td>A</td>
<td>171</td>
<td>0.630</td>
<td>1:40 (H3)</td>
</tr>
<tr>
<td>NZ0470</td>
<td>2004</td>
<td>M</td>
<td>J</td>
<td>122</td>
<td>1.177</td>
<td>1:80 (H3)</td>
</tr>
<tr>
<td>NZ0475</td>
<td>2004</td>
<td>M</td>
<td>A</td>
<td>138</td>
<td>0.488</td>
<td>1:40 (H3), 1:40 (H6)</td>
</tr>
<tr>
<td>NZ0484</td>
<td>2004</td>
<td>M</td>
<td>A</td>
<td>129</td>
<td>0.658</td>
<td>–</td>
</tr>
<tr>
<td>NZ0509</td>
<td>2005</td>
<td>M</td>
<td>A</td>
<td>151</td>
<td>0.555</td>
<td>1:2560 (H3)</td>
</tr>
<tr>
<td>NZ0511</td>
<td>2005</td>
<td>M</td>
<td>A</td>
<td>167</td>
<td>0.557</td>
<td>1:640 (H3)</td>
</tr>
<tr>
<td>NZ0515</td>
<td>2005</td>
<td>F</td>
<td>A</td>
<td>140</td>
<td>0.518</td>
<td>–</td>
</tr>
<tr>
<td>NZ0519</td>
<td>2005</td>
<td>M</td>
<td>A</td>
<td>147</td>
<td>0.639</td>
<td>1:640 (H3)</td>
</tr>
<tr>
<td>NZ0521</td>
<td>2005</td>
<td>F</td>
<td>J</td>
<td>121</td>
<td>0.563</td>
<td>–</td>
</tr>
</tbody>
</table>

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.
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 seals 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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