NOTE Avian Pathology

Isolation of Myxoviruses from Migratory Waterfowls in San-in District, Western Japan in Winters of 1997–2000

Yu SHENGQING1), Kyoko SHINYA3), Koichi OTSUKI2), Hiroshi ITO1) and Toshihiro ITO1)*

1) Departments of Veterinary Public Health and 2) Veterinary Microbiology, Faculty of Agriculture, Tottori University, Tottori 680–8553, Japan
3) Institute of Medical Science, The University of Tokyo, Tokyo 118–8639, Japan

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ABSTRACT. Between November 1997 and February 2000, winter migratory waterfowls of several species staying in San-in district, western Japan were surveyed for influenza A virus and paramyxovirus at four stations. A total of 18 influenza A viruses was isolated from 1,404 fecal samples of whistling swans, pintails, mallards, and white-fronted geese. Five different hemagglutinins and eight neuraminidases were identified in the viruses isolated, in 11 different combinations, including H7N8 related to a subtype of a highly pathogenic chicken virus. In 2000, five lentogenic (non-pathogenic) Newcastle disease viruses were also isolated from white-fronted geese. These results suggested that possible precursor viruses for highly pathogenic avian myxoviruses are still brought into Japan by migratory waterfowls. The results also support the contention that continued surveillance of wild waterfowl population should be an integral part of control policies for these serious poultry diseases.

KEY WORDS: influenza A virus, migratory waterfowl, Newcastle disease virus.

Since December 1979, we have surveyed for influenza A virus at a few stations of San-in district western Japan. Several subtypes of influenza A viruses have been isolated from a few species of migratory waterfowls flying from Siberia or northern China [21–24, 28]. Specifically, interesting subtypes of influenza viruses such as H7N7 from whistling swans and black-tailed gulls were isolated in the winter of 1979–1980 [28], and H5N3 and H10N4 from whistling swans in 1983–1984 [24]. In January 1980 on the coast of the northern Atlantic Ocean in the United States, H7N7 influenza viruses were isolated from the lungs of dead seals together with mycoplasma [10]. An outbreak of fowl plague (highly pathogenic avian influenza) caused by the H5N2 virus occurred in the U.S.A. in October 1983 [15] and an influenza epizootic with H10N4 virus occurred in mink farms in southern Sweden in October 1984 [17]. All of the causative agents of the above diseases are thought to have been brought in by free-living waterfowls.

Highly pathogenic avian influenza viruses, which cause systemic, lethal infection in poultry and cause considerable economic losses in the poultry industry, are restricted to the H5 and H7 subtypes. In the last 20 years, highly pathogenic avian influenza viruses have appeared periodically around the world: the United States (H5 in 1983–84; 15), Ireland (H5 in 1983–84; 16), Australia (H7 in 1985 and 1994; 19, 25), England (H5 in 1991; 3), Mexico (H5 in 1995; 9), and Pakistan (H7 in 1995; 18). In 1997, when an H5N1 avian influenza outbreak occurred among chickens in the New Territories of Hong Kong, the virus was transmitted directly to humans, infecting 18 humans, 6 of whom died [7]. This was the first case in which humans were affected by avian influenza viruses. During 1999–2000, highly pathogenic avian influenza appeared in Italy, having mutated from a mildly pathogenic avian influenza virus [6]. This H7N1 virus was eradicated in April 2000, but the mildly pathogenic H7N1 virus re-emerged in poultry of northern Italy during August 2000. Such viruses found in domestic poultry are often thought to originate from migratory waterfowl [13, 29]. Recently, we experimentally demonstrated that non-pathogenic H5 viruses maintained in wild waterfowl in nature have the potential to become highly pathogenic after several cycles in chickens [14]. Thus, non-pathogenic H5 or H7 viruses harboring in wild waterfowl could well be precursors for highly pathogenic derivatives.

In addition to avian influenza, Newcastle disease is one of the most important diseases impacting international trade in poultry and poultry products. The causative agent, Newcastle disease virus (NDV), has been isolated from a variety of species of wild, domestic, and cage birds around the world [2]. As with avian influenza viruses, the majority of NDVs isolated from wild birds, especially waterfowl, are non-pathogenic (lentogenic), causing no clinical diseases. However, recent genetic comparison between NDVs in domestic poultry and feral waterfowl suggested the velogenic viruses arise from avirulent strains originating from wild birds [8, 12]. These findings indicate that it is important to survey for NDVs harboring in wild waterfowl as well as avian influenza viruses. This paper describes the isolation of influenza A viruses and NDVs from migratory waterfowl of several species staying in San-in district, western Japan in the winters of 1997–2000.

Fresh fecal samples from these birds were collected individually into screw-capped tubes (15 × 80 mm). The specimens were stored at −80°C until assayed. Each fecal sample was suspended to a concentration of 20–30% in phosphate-buffered saline (pH 7.2) containing penicillin at 8,000 units
per ml and streptomycin at 8,000 mg per ml. The suspension was centrifuged at 2,500 rpm for 20 min. One tenth ml of the supernatant was inoculated into the allantoic cavities of two 10-day-old fertile hen’s eggs. The eggs were incubated at 35°C for 2 to 3 days unless death of the embryo was detected. At the end of the incubation period or upon embryo death, the allantoic fluids were tested for hemagglutinating activity. All hemagglutinating agents were identified in hemagglutination-inhibition (HI; 26) and neuraminidase-inhibition (NI; 5) tests using specific antiserum to the following virus strains: influenza A virus strains, A/PR/8/34 (H1N1), A/FM/1/47 (H1N1), A/Singapore/1/57 (H2N2), A/Aichi/2/68 (H3N2), A/swine/Iowa/15/30 (H1N1), A/equine/Prague/1/56 (H7N7), A/equine/Miami/1/63 (H3N8), A/duck/Ukraine/1/63 (H3N8), A/duck/Czech/56 (H4N6), A/tern/South Africa/61 (H5N3), A/turkey/Ontario/61/188/68 (H8N4), A/turkey/Wisconsin/66 (H9N2), A/chicken/Germany F'/N'/49 (H10N7), A/duck/England/56 (H11N6), and A/gull/Maryland/704/77 (H13N6), and a NDV strain, Miyadera. The hyperimmune antiserum against each strain were used as previously prepared and described [28].

Plaque assays were performed with Madin-Darby canine kidney (MDCK) cells [27], which were grown in Eagle’s minimum essential medium (EMEM) with 10% calf serum, L-glutamine, and antibiotics. After 1-hr adsorption of virus, the inoculum was removed and the cells were then overlaid with EMEM containing 1% Bacto-Agar (Difco) in the absence or presence of trypsin (2.5 µg/ml). After incubation at 37°C for 2 days in a 5% CO₂ atmosphere, cells were overlaid again with EMEM containing 1% Bacto-Agar and 0.005% neutral red, followed by plaque count.

Mean death time (hr) at minimum lethal dose (MDT/MLD) of chicken embryos was measured to assess the virulence of NDV isolates [4].

From November 1997 to February 2000, a total of 1,404 fecal samples, 930 from whistling swans (Cygnus columbianus), 89 from mallards (Anas platyrhynchos) and 92 from white-fronted geese (Anser albifrons) were collected at four stations in Shimane and Tottori prefectures, western Japan (Table 1). From these samples, 18 strains of influenza A virus, 4 from pintails and 14 from whistling swans, and 5 strains of Newcastle disease virus from white-fronted geese were isolated (Table 1). No virus was isolated from mallard samples.

Antigenic characterization of these isolates was carried out by HI and NI tests. As shown in Table 2, subtypes of the influenza virus isolates from whistling swans were H2N3 and H11N2 in 1997, H2N1, H2N4, H2N6, H2N8, H7N8, and H11N9 in 1999, and H11N9 in 2000. Subtypes of the influenza virus isolates from pintail ducks were H10N7 in 1997, H11N9 in 1998, H1N1 and H10N4 in 1999. Different subtypes of influenza viruses were isolated in different years in the same station. These results indicate that different subtypes of influenza A viruses co-circulate in waterfowl populations flying from Siberia or northern China and wintering in Japan.

Jirulence of the H7 isolate was assessed by plaque forming ability. The strain did not form plaques in the absence of trypsin in MDCK cells, but did so in the presence of trypsin. The results indicate that the H7 isolate is avirulent.

The types of influenza virus isolated from feces of migratory birds have changed over the years [22–24, 28]. However, limited subtypes of influenza A virus have repeatedly appeared. For example, subtype H7 viruses isolated from whistling swans, tufted ducks, and black-tailed gulls in 1979–1980 [28] and from whistling swans in 1982–1983 [23] were isolated again in the present study (Table 2). Specifically, the H7N8 virus was isolated from whistling swans in rice fields in the suburbs of Yasugi city, Shimane Prefecture in 1999. Although the isolation rate is low, possible precursor H7 viruses for highly pathogenic derivatives have been brought into Japan repeatedly by migratory waterfowl. Okazaki et al. [20] reported that non-pathogenic H5N3 and

### Table 1. Isolation of myxoviruses from fecal samples of waterfowls in San-in District, West Japan in 1997–2000

<table>
<thead>
<tr>
<th>Location</th>
<th>Species</th>
<th>No. of samples with virus/Total no. of samples tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rice field in the suburbs of</td>
<td>Whistling swan</td>
<td>2/0/115/342/174</td>
</tr>
<tr>
<td>Yasugi city, Shimane Pref.</td>
<td>White-fronted goose</td>
<td>2/0/115/174</td>
</tr>
<tr>
<td>Coast of Lake Nakaumi</td>
<td>Pintail duck</td>
<td>1/0/105/80</td>
</tr>
<tr>
<td>Shimane Pref.</td>
<td></td>
<td>0/96</td>
</tr>
<tr>
<td>Tenjin River, Tottori Pref.</td>
<td>Mallard duck</td>
<td>0/20</td>
</tr>
<tr>
<td>Pond Nikko, Tottori Pref.</td>
<td>Mallard duck</td>
<td>0/69</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>3/151/1443/13451/6539</td>
</tr>
</tbody>
</table>

No. of each antigenic subtype of the isolates is as follows: a) H2N3 and H11N2, b) H2N1, H2N4, H2N6, H2N8, H7N8, and H11N9, c) H11N9, d) NDV, e) H10N7, f) H11N9, and g) H1N1 and H10N4.
poultry diseases. They should be an integral part of control policies for both serious and mild infections. Continued surveillance of wild waterfowl populations is essential to monitor the emergence of new viruses and the circulation of existing ones.

H5N4 viruses were isolated from fecal samples of ducks in Hokkaido, northern Japan in October 1996. These findings suggest that it is important for the poultry industry to minimize the risk of introduction of viruses from wild birds to domestic poultry.

It should also be noted that ten out of the 18 influenza virus strains isolated in the present study were identified as subtype H2. The H2 viruses were also isolated in both winters of 1982–1983 [23] and 1983–1984 [24] from whistling swans and a pintail respectively. Human H2 viruses, called "Asian" type, caused a pandemic from 1957 to 1968; more than 30 years have passed since its disappearance from human populations. Recent repeated isolation of H2 viruses from wild birds indicates that they can transmit to humans and thus may cause another pandemic among humans in the near future [1, 11, 30].

Virulence of NDV isolates was assessed by the MDT/MLD test with chicken embryos. The MDT in eggs of all 5 isolates was over 120 hr, typical results for lentogenic viruses. It was reported that a benign NDV recently became virulent after transmission to and circulation in chicken populations [69, 70, 71]. Therefore, virulent NDV could arise from these non-pathogenic precursor viruses. In fact, we experimentally demonstrated that avirulent viruses, maintained in wild waterfowl in nature, have the potential to become highly pathogenic after transmission to and circulation in chicken populations [Shengqing et al., submitted for publication]. Therefore, continued surveillance of wild waterfowl populations should be an integral part of control policies for both serious poultry diseases.

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