Distribution of Antibodies to Influenza A Virus in Chickens in Japan

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\textbf{Abstract.} A serological surveillance was carried out to detect antibody against influenza A virus in chicken sera. A total of 8,850 field samples were collected from 47 prefectures in Japan. Initially, all the sera were screened by agar gel immunodiffusion and those sera showing positive reaction were investigated for haemagglutination-inhibition (HI) and neuraminidase-inhibition antibodies against influenza viruses. Only 6 samples had antibodies; 4 sera had antibodies against human subtype H1N1 virus; with HI activity against strain A/PR/34; three sera had strong HI activity to strain A/Tottori/4/87, which by haemagglutination test is closely related to A/Yamagata/120/86. The remaining two chicken sera had antibodies against avian subtypes H1N4 and H3N6 viruses respectively. — Key words: avian influenza A virus, chicken, human influenza, seroepidemiology, serum antibody.


All the different subtypes of influenza A viruses (H1 to H14 and N1 to N9) were isolated from free-living birds, captive caged birds and domestic birds such as ducks, chickens and turkeys. It is known that only limited strains of the H5 and H7 subtypes cause high mortality in chickens. Since the winter of 1979–1980, we have surveyed for influenza virus migratory water-fowl such as whistling swans, pintsails, tufted ducks and black-tailed gull flying from Siberia and northern China. Many influenza viruses, including H7N7, H5N3, H10N4, H2N2 and H1N4 subtypes have been isolated [12, 16, 17]. Thus it is evident that numerous influenza A viruses are being brought into Japan by these migratory free-living birds although there has been no information about the incidence of any type of avian influenza among chicken flocks kept in this country. In fact, it is believed that since 1925 no fowl plague virus (highly pathogenic avian influenza virus) has caused any disaster in the Japanese poultry industry [15].

In 1983, an influenza outbreak occurred in chickens in the United States of America and the causative agent, H5N2 virus, originated an influenza viruses in wild and domestic species. It gained its virulence against chickens during unrestricted replication in the respiratory tracts of a large number of chickens. A critical mutation occurred in the haemagglutinin gene and produced a virus that was lethal [5]. Quite recently, H5N2 influenza virus has become a serious problem in the chicken industry of Mexico [7, 8]. Avian influenza virus is also able to cause some mortality in mammals [6, 18].

Thus it is worthwhile investigating the incidence of influenza among chicken flocks kept in Japan. We performed a serological surveillance for influenza A virus in commercial chicken flocks.

\textbf{Materials and Methods}

\textit{Field Sera:} A total of 8,850 chicken sera were collected randomly from 47 prefectures in Japan between 1971 and 1987 (Table 2). All of the sera were stored at -20°C until tested.

\textit{Virus:} The strains employed as reference viruses are as follows: A/turkey/England/63 (H7N3), A/chick/Germany/“N”/49 (H10N7), A/duck/England/56 (H11N6), A/duck/Czech/56 (H4N6), A/turkey/South Africa/61 (H5N3), A/shearwater/East Australia/1/72 (H6N5), A/duck/Ukraine/1/63 (H3N8), A/turkey/Canada/1/62 (H8N4), A/turkey/Wisconsin/66 (H9N2), A/swine/Iowa/15/30 (H11N1), A/equine/Prague/56 (H7N7), A/equine/Miami/1/63 (H5N8), A/PR/8/34 (H1N1), A/FM/1/47 (H1N1), A/Singapore/1/57 (H2N2), A/gull/Maryland/70/77 (H3N6). Local strains such as A/tufted duck/Shimane/23/77 (H1N4) [17], A/whistling swan/Shimane/35/80 (H6N6) [16] and A/Tottori/4/87 (H1N1) provided by the Institute of Health of Tottori prefecture were also used. The antigenicity of strain A/Tottori/4/87 (H1N1) closely relates to that of A/Yamagata/120/86 (H1N1) and A/Yokohama/5/86 (H1N1) but it is less related to A/Brazil/11/78 (H1N1) and A/Brazil/10/83 (H1N1) (Table 1).

\textit{Agar Gel Immunodiffusion (AGID) Test:} For preparing an antigen for the AGID test, usually $10^2$–$10^3$ EID$_{50}$ of strain A/whistling swan/Shimane/35/80 (H6N6) (strain 35) [16] was inoculated into the allantoic cavity of 11-day-old embryonated hen’s eggs. The eggs were incubated for 3 days at 34°C. After chilling at 4°C overnight the allantoic fluid was harvested, immediately clarified by centrifugation for 3 min at 5,000 rpm and examined for haemagglutinating activity. The virus was pelleted for 90 min at 28,000 rpm and the virus pellet was resuspended at 100-fold concentration in phosphate-buffered saline solution pH 7.2. Concentrated virus was inactivated by adding a very small amount of polyoxyxylene (10) octylphenyl ether.

The AGID medium [19] was prepared containing 1% polyoxyethylene (10) octylphenyl ether. Positive control antiserum was prepared by infecting SPF chickens intratracheally with strain 35 [10]; negative serum was obtained from SPF chicks. The tests were carried out on
microscope slides as described by Beard [4]. The wells were 4 mm in diameter and their margin 2 mm apart. The precipitin-reacting microscope slide was incubated for 3 days at 37°C. The serum samples which gave precipitin lines were subsequently sero-typed by the haemagglutination-inhibition (HI) and neuraminidase-inhibition (NI) and tests.

Haemagglutination (HA) and HI tests were performed as described by Salk [14] with volumes reduced for the micromethod. Neuraminidase (NA) and NI tests were performed using the method of Aymard-Henry et al. [3].

RESULTS

A total of 8,850 chicken serum samples were collected from 47 prefectures of 9 districts in Japan. The results obtained in the 9 districts are shown in Table 2. Six sera were positive in AGID tests; 1 of 476 serum samples was positive in Hokuriku district; 3 of 1,625 were positive in Tokai district; and 2 of 2,030 in Chugoku district.

Serum samples were collected from chicken flocks in the field over a period from 1971 and 1987 and tested for AGID antibody. The yearly results obtained are shown in Table 3. Since the number of serum samples which showed a positive reaction was so low, the positive rate was scattered.

Chicken sera which showed positive reaction were subsequently sero-typed by the HI and NI tests (Tables 4 and 5). One of the serum samples taken in Okayama prefecture, Chugoku district in 1983 inhibited HA activity of strain A/duck/Ukraine/1/63 (H3N8) and NA activity of A/duck/England/56 (H1N6). Sample No. 6 taken in Ishikawa prefecture, Hokuriku district inhibited HA and NA activities of A/chicken/Germany/849/49 (H1N7) and A/turkey/Ontario/6118/68 (H3N4) respectively. The other four positive chicken sera reacted to human influenza virus of subtype H1 in the HI test; three sera taken in Gifu prefecture in 1983 and 1985 strongly inhibited HA activity of strain A/Tottori/4/87 (H1N1) and weakly that of strain A/FM1/47 (H1N1); and serum taken in Hiroshima prefecture inhibited only A/PR18/34 (H1N1) (Table 4). These four sera inhibited NA activity of A/FM1/47 (H1N1) (Table 5). Thus it could be concluded that four chicken sera possessed antibodies against human influenza virus subtype H1N1 and two sera possessed antibodies against avian influenza virus subtypes H1N4 and H3N6 respectively (Table 2).

DISCUSSION

In the present serological surveillance, chicken serum samples that possessed antibodies against influenza virus were detected, although number of antibody-positive sera was very limited. This is the first report to proving that chickens reared in the field in Japan have antibodies against avian and human influenza viruses.

Antibody against avian influenza virus was found in two serum samples taken in different years in different districts. The affected chickens are thought to have been infected with subtypes H1N4 or H3N6 infection virus separately. In Shimane prefecture, close to Okayama prefecture, we isolated H1N4 viruses from the faeces of whistling swans and pintails in November and December 1983, and subtype H3N6 viruses were also isolated from whistling swans in December 1984 at the same place [12]. Since several kinds of migratory waterfowls including ducks flying from Siberia or northern China stay in Ishikawa and Okayama prefectures
Table 4. HI titres of precipitin-antibody-positive chicken sera to the H1, H2 and H3 subtype strains

<table>
<thead>
<tr>
<th>Serum No.</th>
<th>Location (Prefecture)</th>
<th>Year of collection</th>
<th>H1</th>
<th>H2</th>
<th>H3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Okayama (Chugoku)</td>
<td>1983</td>
<td>&lt;32</td>
<td>&lt;32</td>
<td>&lt;32</td>
</tr>
<tr>
<td>2</td>
<td>Hiroshima (Chugoku)</td>
<td>1971</td>
<td>128*</td>
<td>&lt;32</td>
<td>&lt;32</td>
</tr>
<tr>
<td>3</td>
<td>Gifu (Tokai)</td>
<td>1983</td>
<td>&lt;32</td>
<td>&lt;32</td>
<td>&lt;32</td>
</tr>
<tr>
<td>4</td>
<td>Gifu (Tokai)</td>
<td>1985</td>
<td>&lt;32</td>
<td>&lt;32</td>
<td>64</td>
</tr>
<tr>
<td>5</td>
<td>Gifu (Tokai)</td>
<td>1985</td>
<td>&lt;32</td>
<td>&lt;32</td>
<td>&lt;32</td>
</tr>
</tbody>
</table>

Serum 6 blooded in 1980 in Ishikawa prefecture, Hokuriku district inhibited an HA activity of only strain A/chick/Germany"N"/49 (H1N7).

*: The values are reciprocals of the highest serum dilution causing inhibition of 4 haemagglutinating units of virus.

PR/34: A/PR/8/34 (H1N1); S/I/30: A/swine/Iowa/15/80 (H1N1); FM/47: A/FM/1/47 (H1N1); T/77: A/Tottori/4/87 (H1N1); S/57: A/Singapore/1/57 (H2N2); A/68: A/Aichi/3/68 (H3N2); E/M/63: A/Miami/1/63 (H3N8); D/U/61: A/Ukraine/1/63 (H3N8)

Table 5. NI titres of precipitin-antibody-positive chicken sera to the reference strains

<table>
<thead>
<tr>
<th>Serum No.</th>
<th>Location (Prefecture)</th>
<th>Year of collection</th>
<th>N1</th>
<th>N2</th>
<th>N3</th>
<th>N4</th>
<th>N5</th>
<th>N6</th>
<th>N7</th>
<th>N8</th>
</tr>
</thead>
<tbody>
<tr>
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<td>&lt;12</td>
<td>&lt;12</td>
<td>&lt;12</td>
<td>&lt;12</td>
<td>&lt;12</td>
<td>&lt;12</td>
<td>&lt;12</td>
</tr>
<tr>
<td>2</td>
<td>Hiroshima (Chugoku)</td>
<td>1971</td>
<td>≥1,200*</td>
<td>&lt;12</td>
<td>&lt;12</td>
<td>&lt;12</td>
<td>&lt;12</td>
<td>&lt;12</td>
<td>&lt;12</td>
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<tr>
<td>3</td>
<td>Gifu (Tokai)</td>
<td>1983</td>
<td>≥1,200</td>
<td>&lt;12</td>
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<td>Ishikawa (Hokuriku)</td>
<td>1980</td>
<td>&lt;12</td>
<td>&lt;12</td>
<td>&lt;12</td>
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<td>&lt;12</td>
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</tr>
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</table>

*: NI titre expressed as the reciprocal of the serum dilution causing 50% inhibition of neuraminidase activity.

FM/47: A/FM/1/47 (H1N1); S/57: A/Singapore/1/57 (H2N2); T/E/63: A/Turkey/England/63 (H7N3); T/O/68: A/turkey/Ontario/61/68 (H8N4); S/EA/72: A/Shearwater/E. Australia/1/72 (H6N5); D/E/56: A/duck/England/56 (H1N6); E/P/56: A/equine/Prague/56 (H7N7); E/M/63: A/equine/Miami/1/63

during every winter, the chicken flocks are possibly in contact with migratory waterfowls there. Thus avian influenza viruses such as subtypes H10N4 and H3N6 viruses might have been transmitted from migratory waterfowl to chicken.

We also isolated several strains of influenza virus possessing H5 and H7, fowl-plague-virus-type antigens from whistling swans and black-tailed gulls [12, 16]. It should be noted that there are necessarily opportunities for H7 and H5 viruses to be brought into this country by these migratory waterfowls. Although no outbreaks of fowl plague have been reported in any Japanese chicken flocks for the last 70 years, in the future low pathogenic H5 or H7 influenza virus [11] transmitted from migratory waterfowls to chicken flocks reared in Japan may increase their pathogenicity as the American and Mexican cases [5, 7, 8] and causes big economical loss in the Japanese poultry industry. The incidence of fowl plague in this country must always be watched carefully.

It is interesting that antibodies against human influenza virus was detected in the chicken sera [13]. Four chicken sera collected in Hiroshima prefecture, Chugoku district and Gifu prefecture, Tokai district respectively possessed antibodies against subtype H1N1 virus. However, the reaction of each serum against four strains possessing H1N1 antigen was not necessarily the same. A serum taken in 1971 in Hiroshima prefecture inhibited HA activity of only strain A/PR/34 virus (Table 4). Onta et al. [9] reported that pig, cattle and horse sera possessed H1 antibodies against strain A/PR/34.
Three other chicken sera taken in 1983 and 1985 in Gifu prefecture strongly inhibited the HA activity of strain A/Tottori/4/87. The HA antigenicity of strain A/Tottori/4/87 is different from that of strains A/Brazil/11/78 or A/Brazil/10/83 (Table 1). Thus chicken flocks reared between 1983 and 1985 in Gifu prefecture seemed to have already been infected with human H1N1 influenza virus which occurred as an epidemic among humans in 1987. Unfortunately, the exact geographical location where the chicken flocks that possessed antibody against H1N1 influenza virus were reared was not ascertained; whether or not pig farms existed closely to these chicken flocks; and whether these chicken flocks were reared in the suburbs of a big city or in the countryside. The route of transmission of the human-type influenza virus from mammals to chickens could not be ascertained in this survey. Further investigation is needed to clarify these important points.

It was surprising that only 6 of 8,850 chicken sera possessed antibodies against influenza viruses. The result obtained in this surveillance was quite different from that in Spain [2]. It may be possible to consider that only limited chicken flocks are susceptible to influenza virus infection in the field although various kinds of influenza virus cause some disease in chickens following experimental infection [10, 11]. Nevertheless there is concern about the extreme insensitivity of the AGID to detect antibodies against influenza virus in sera, although the AGID test is expected to detect convalescent levels of avian antibodies against all subtypes of type A influenza [1]. Only very high titres of antibodies against influenza virus in chicken sera were detected in the present AGID tests. More sensitive screening methods to detect antibodies to influenza A virus in chicken serum should be developed. An ELISA seems to have advantages as a screening test, however, since absorbance values in ELISA increase dramatically with age [1], it is difficult to set correct positive-negative cut-off levels when carrying out large scale surveillance for the incidence of influenza in the field. When a better screening method such as ELISA is developed in the future, it may become possible to detect lower level of antibody in chicken sera [1].

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REFERENCES