

Serological Analysis of Serum Samples from Humans Exposed to Avian H7 Influenza Viruses in Italy between 1999 and 2003

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(See the editorial commentary by Hayden and Croisier, on pages XXX–XX.)

We evaluated the potential for avian-to-human transmission of low pathogenic avian influenza (LPAI) and highly pathogenic avian influenza (HPAI) H7N1 and LPAI H7N3 viruses that were responsible for several outbreaks of influenza in poultry in Italy between 1999 and 2003. A serological survey of poultry workers was conducted by use of a combination of methods. Evidence of anti-H7 antibodies was observed in 3.8% of serum samples collected from poultry workers during the period in 2003 when LPAI H7N3 virus was circulating. These findings highlight the need for surveillance in people occupationally exposed to avian influenza viruses, so that they can be monitored for the risk of avian-to-human transmission during outbreaks of avian influenza caused by both LPAI and HPAI viruses.

Birds, particularly those in the order Anseriformes, are the natural reservoir of all influenza A viruses, and this supports the hypothesis that new pandemic strains in humans are of avian origin [1]. The introduction of avian influenza viruses (AIVs) from the wild bird reservoir to domestic poultry may

be of concern, because of the increased opportunity for human transmission, either through a process of genetic reassortment between avian and human strains during mixed infections or by direct transmission from birds to humans. Indeed, during the past 8 years, infection of humans with AIVs of 3 subtypes (H5, H7, and H9) has been described [1]. In particular, during an outbreak of avian influenza (AI) in poultry farms, highly pathogenic AI (HPAI) H7N7 virus caused 89 human infections and 1 death in The Netherlands in 2003 [2]. Human infections with H7N3 virus were also reported in British Columbia in 2004 [3] and were associated with outbreaks of influenza caused by both low pathogenic AI (LPAI) and HPAI viruses in poultry in the same Canadian regions. Serological evidence of bird-to-human transmission of AIVs has been described [1, 4].

Between 1999 and 2003, several outbreaks of influenza caused by LPAI and HPAI H7N1 and by LPAI H7N3 viruses occurred in poultry in regions of northern Italy where the majority of the national commercial poultry is raised [5]. To evaluate the possibility of transmission of AIVs to humans, we performed a serological analysis of serum samples from individuals exposed to AIVs during outbreaks of AI caused by LPAI and HPAI H7N1 and LPAI H7N3 viruses.

Subjects, materials, and methods. Between August 1999 and July 2003, a total of 983 serum samples were collected from workers in several categories of labor at different farms located in the Veneto and Lombardy regions of Italy (table 1). Serum samples were collected as part of either the ongoing public health measures to evaluate the possibility of bird-to-human transmission of AIVs or a European collaborative study to conduct serosurveillance in poultry workers. Informed consent was obtained from subjects, and ethical approval was obtained from local ethics committees in Lombardy and Veneto for the retrospective serosurveillance studies. All subjects were asked to complete a questionnaire for which they noted the type of work they did with poultry and any respiratory tract illnesses they had during the AI epizootics. For epizootic 4, serum samples were collected >1 year after the last outbreak of AI caused by an H7N1 LPAI virus in Veneto (table 1), because of delays in obtaining ethical approval and permission for the data and serum sample collection.

Serum samples were collected at least 15 days after the onset of each epizootic and were stored at -20°C until tested for antibodies against H7N1 (A/Ty/It/2676/99 and A/Ty/It/3889/99) or H7N3 (A/Ty/It/214845/02) virus. H7 viruses selected for testing were egg-grown LPAI viruses that were antigenically representative of viruses circulating at the relevant farms during the period in question.

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Table 1. Avian influenza epizootics in Italy and serological results.

Epizootic no.	Region	Period of epizootic	Circulating virus (pathogenicity)	Time of serum sample collection	Antigens used in the assays	Serum samples, no.	Positive results, no.		
							By HI assay ^a	By MN assay ^b	By SRH assay
1	Lombardy	29 Mar 1999–17 Dec 1999	H7N1 (LP)	4 Aug 1999–15 Oct 1999	A/Ty/Ilt/2676/99 (H7N1), A/Ty/Ilt/3889/99 (H7N1)	85	0	0	0
2	Lombardy	17 Dec 1999–5 Apr 2000	H7N1 (HP)	6 Mar 2000–14 Apr 2000	A/Ty/Ilt/2676/99 (H7N1), A/Ty/Ilt/3889/99 (H7N1)	513	0	0	0
3	Veneto	17 Dec 1999–5 Apr 2000	H7N1 (HP)	14 Feb 2000–9 May 2000	A/Ty/Ilt/2676/99 (H7N1), A/Ty/Ilt/3889/99 (H7N1)	159	0	0	0
4	Veneto	14 Aug 2000–March 2001	H7N1 (LP)	21–23 Oct 2002	A/Ty/Ilt/3889/99 (H7N1), A/Ty/Ilt/214845/02 (H7N3)	41	0	0	NT
5	Lombardy	16 Oct 2002–30 Sep 2003	H7N3 (LP)	25–26 Mar 2003–1 Apr 2003	A/Ty/Ilt/3889/99 (H7N1), A/Ty/Ilt/214845/02 (H7N3)	43	1 (C03)	4 (B03, C01, C03, C08)	NT
6	Veneto	16 Oct 2002–30 Sep 2003	H7N3 (LP)	July 2003	A/Ty/Ilt/3889/99 (H7N1), A/Ty/Ilt/214845/02 (H7N3)	142	3 (87, 90, 102)	3 (87, 90, 102)	NT

NOTE. HI, hemagglutination inhibition; HP, highly pathogenic; LP, low pathogenic; MN, microneutralization; NT, not tested; SRH, single radial hemolysis.

^a Identification numbers of serum samples that were confirmed as being reactive by Western blot analysis against H7N1 and H7N3 are given in parentheses.

^b Identification numbers of serum samples that were confirmed as being reactive by Western blot analysis against H7N1 and H7N3 are given in parentheses. In serum samples with positive results, titers ranged from 30 to >300.

Serum samples were assayed using several different serological techniques. Each serum sample was tested by hemagglutination inhibition (HI) and microneutralization (MN) assays [6, 7]. If a serum sample was considered to be positive for either test, Western blot (WB) analysis by use of purified egg-grown H7 virus and/or baculovirus-expressed H7 hemagglutinin (HA) was performed.

Each serum sample was tested at least twice in separate MN assays that were performed in duplicate with a starting dilution of 1:10, in accordance with methods described elsewhere [6], and serum samples that repeatedly had titers >20 were considered to be reactive in the MN assays. All MN assays were performed with MDCK cells derived from the European Cell Culture Collection lineage. The HI test was performed with live viruses and horse red blood cells (HRBCs), in accordance with procedures described elsewhere [7]. A titer of 10 was considered to be a positive result in the HI assays. The single radial hemolysis (SRH) assay was performed using turkey erythrocytes sensitized with A/Ty/It/2676/99 antigen, as described elsewhere [8]. Positive and negative control serum samples were included for each batch of plates in each assay, and serum samples were tested in parallel with control plates, which were prepared with all components except virus. Serum samples were considered to be positive only if they had reactive zones with diameters >3.5 mm. Antiserum from immunized animals (goat, ferret, sheep, chicken, rabbit, and turkey) were used as positive controls in the HI, MN, and SRH assays, and monoclonal antibody 2E6G7 specific for H7 HA was also used in the WB analysis.

In the WB analysis, samples containing 150 µg of viral protein were heated in SDS sample-reducing buffer at 70°C for 10 min and were loaded into each well of an SDS-PAGE system (4%–12% Bis-Tris gel, NuPage Novex, in MOPS running buffer and the XCell SureLock Mini-Cell system; all from Invitrogen). After electrophoresis, the gels were blotted onto pre-cut Hybond ECL Nitrocellulose membranes (Amersham Life Sciences) by use of the XCell II Blot Module (Invitrogen), in accordance with the manufacturers' instructions. The blots were incubated with either subjects' serum samples at a dilution of 1:200 or monoclonal antibody at a dilution of 1:10, and then they were incubated with horseradish peroxidase-conjugated anti-species antibody at a dilution of 1:2000, and antigen-antibody binding was detected with the ECL fluorescence system (Amersham Life Sciences).

Results. Serum samples were tested against H7N1 and H7N3 viruses (table 1). When tested by MN, HI, and SRH assays, all 798 serum samples collected between 1999 and 2002 from epizootics 1–4, which involved H7N1 viruses, showed no evidence of antibody to H7N1 virus. H7N3 viruses were not circulating during this time period. When tested by MN and HI assays, serum samples collected after epizootic 4, which involved H7N1 virus, showed no evidence of antibody to H7N1 or H7N3 virus. A total of 185 serum samples collected during epizootics 5 and

6, which involved H7N3 virus, were also tested against H7N1 and H7N3 viruses by MN and HI assays. Seven serum samples (3.8%) were clearly reactive as assessed by MN assays to both viruses, with higher titers to H7N3, and 4 of 7 serum samples were reactive as assessed by HI assays, with higher titers to H7N3. When these 7 serum samples were tested in WB analysis against purified H7N1, H7N3, and baculovirus-expressed HA from H7N1 A/Ty/It/3889/99 or H7N3 A/Ty/It/214845/02, all 7 also showed clear reactivity to H7 HA, in contrast to serum samples that did not show any reactivity by HI and MN assays (figure 1). We therefore concluded that these subjects showed unequivocal serological evidence of exposure to or infection with H7 viruses. Questionnaire data for the seropositive subjects indicated that 3 were male and 3 were female, and all were 35–62 years old. No questionnaire data were available for 1 of these 7 seropositive subjects.

The seropositive subjects came from different farms in a single province of Brescia (Lombardy) or in a single province of Verona (Veneto). All of the seropositive subjects had close direct physical contact with either turkeys or chickens in poultry housing, which was described as being a dusty environment. Only 1 had clinical symptoms of conjunctivitis at the time of the AI epizootics. Six of the seronegative subjects reported a history of conjunctivitis but did not show any serological reactivity to H7 HA. None of the 7 seropositive subjects reported a history of influenzalike illness (ILI) during the AI epizootics, although 14 seronegative subjects reported ILI symptoms. No data were collected regarding sickness due to any other cause during the AI epizootics.

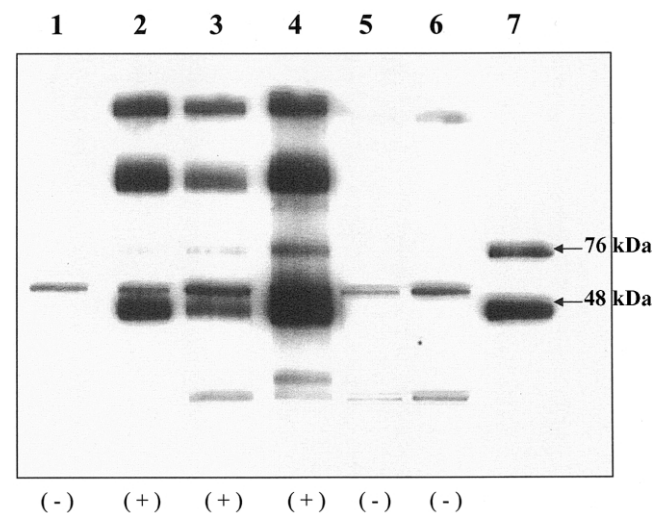


Figure 1. Italian serum samples vs. purified H7N3. Shown are responses from serum samples 77 (lane 1), 87 (lane 2), 90 (lane 3), 102 (lane 4), 106 (lane 5), and 110 (lane 6) and from monoclonal antibody 2E6G7 raised against A/Ty/It/3889/99 reactive with hemagglutinin of H7N1 and H7N3 (lane 7). +, neutralizing antibody detected; –, no neutralizing antibody detected.

Discussion. Beginning in March 1999, an LPAI H7N1 virus circulated mainly in Veneto and Lombardy; from the end of 1999 to April 2000, an HPAI H7N1 virus, which evolved from the LPAI virus by mutation, circulated and was responsible for devastating outbreaks of influenza in Italian poultry [5]. Two additional epizootics caused by LPAI H7N1 viruses occurred in turkey flocks, between August 2000 and March 2001, in Veneto [9]. In October 2002, an LPAI H7N3 virus spread rapidly in farms rearing turkeys and chickens in the same regions that were devastated by the previous epizootics caused by H7N1 viruses, and it circulated until September 2003 [9, 10]. Previous studies showed that the H7N1 and H7N3 viruses were clearly distinguishable at the antigenic and molecular levels [11].

After these epizootics, a seroepidemiological investigation was conducted to determine the risk of transmission of AIVs to humans who had been in contact with the birds in the affected regions. Because of the insensitivity of the classic HI assay, we analyzed serum samples by use of a combination of serologic assays: the MN assay, which is recommended for the detection of specific antibodies against AIVs in human serum samples [6], and a modified HI assay performed with HRBCs, which has been shown to increase the sensitivity of the traditional HI assay using AIVs, because of sialic acid receptors present on the surface of the HRBCs [7]. A confirmatory H7-specific WB analysis was also performed.

Of 185 serum samples collected in 2003 during the epizootic caused by an LPAI H7N3 virus, 7 were found to be clearly and concordantly reactive, showing evidence of infection with or exposure to H7 viruses, because all serum samples reacted in MN assays to both H7N3 and H7N1 viruses, with higher titers to H7N3, which is consistent with exposure to H7N3 and cross-reaction to the antigenically related H7N1. All of the seropositive subjects had close direct physical contact with either turkeys or chickens in poultry housing, which was described as being a dusty environment. Of the seropositive subjects, only 1 had a clinical history of conjunctivitis at the time of the AI epizootics.

However, no seroreactivity was found in subjects exposed to the H7N1 viruses circulating in 1999–2001. It is possible that the risk of transmission to humans could be different between H7N1 and H7N3 virus subtypes and may be related, at least in part, to the neuraminidase (NA) of these viruses. As a consequence of H1N1 circulating during the last 30 years, most humans possess antibodies against N1 NA, and these antibodies may have provided some protection during the outbreaks of AI caused by H7N1 viruses, although the human deaths caused by infection with H5N1 virus in Asia in 2004 do not fully support this hypothesis [12]. Because the genetic determinants responsible for bird-to-human transmission are still undetermined, the ability of different strains to infect humans is not understood, and genetic differences between the 2 virus groups may be im-

portant. In this regard, the H7N1 viruses show several amino acid changes in the HA and the internal protein genes, compared with the sequences in both Italian H7N3 and Dutch H7N7 viruses (L.C., unpublished data).

To our knowledge, this is the first serological evidence of transmission of LPAI viruses to humans during an epizootic in domestic poultry. So far, reports of human infection with AIVs have been linked to outbreaks of AI caused by HPAI viruses in The Netherlands, Vietnam, Thailand, Cambodia, Hong Kong, and Canada.

The seropositivity rate in the 185 serum samples collected from workers exposed to AIVs during the outbreak of AI caused by H7N3 virus in Italy in 2003 was 3.8%. This result is consistent with that in a retrospective cohort study conducted in poultry workers during the AI outbreaks caused by H5N1 viruses in Hong Kong in 1997 [13].

A serosurvey conducted by Meijer et al. [14] using modified HI assays to detect anti-H7 antibody in poultry workers exposed to AIVs during the epizootic caused by H7N7 virus in The Netherlands in 2003 found that approximately half of the individuals exposed to poultry and the household contacts of infected persons had anti-H7 antibodies. It should be emphasized that the present study likely underestimates the real seroconversion rate in exposed individuals, because serum samples were considered to be positive for antibodies to H7 viruses only if they repeatedly gave unequivocally positive results by use of at least 2 different serological techniques, including the MN assay. Furthermore, the delay between the onset of some of the epizootics (e.g., epizootic 4) and the collection of the serum samples might have significantly reduced the chance of detecting antibodies to H7 viruses.

In conclusion, the serologic evidence for infections in poultry workers reported here further demonstrates the potential of AIVs to infect humans and suggests that permanent serosurveillance studies both in animals and in humans should be implemented to gain more knowledge about the crossing of the species barrier, which appears to be a means of generating a virus with pandemic potential. Although all the individuals who were found to be seropositive in this study were exposed only to LPAI viruses, our findings highlight the risk of the emergence of a potentially pandemic strain, as a result of reassortment of avian and contemporaneously circulating human strains during outbreaks of AI caused by LPAI viruses, and emphasize the importance of strengthening specific surveillance systems not only during outbreaks of AI caused by HPAI viruses but also when LPAI viruses are circulating.

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References

1. Nicholson KG, Wood JM, Zambon M. Influenza. *Lancet* **2003**;362:1733–45.
2. Koopmans M, Wilbrink B, Conyn M, et al. Transmission of H7N7 avian influenza A virus to human beings during a large outbreak in commercial poultry farms in The Netherlands. *Lancet* **2004**;363:587–93.
3. Tweed SA, Skowronski DM, David ST, et al. Human illness from avian influenza H7N3, British Columbia. *Emerg Infect Dis* **2004**;10:2196–9.
4. Shu LL, Zhou NN, Sharp GB, et al. An epidemiological study of influenza viruses among Chinese farm families with household ducks and pigs. *Epidemiol Infect* **1996**;117:179–88.
5. Capua I, Mutinelli F, Dalla Pozza M, Donatelli I, Puzelli S, Cancellotti FM. The 1999–2000 avian influenza (H7N1) epidemic in Italy: veterinary and human health implications. *Acta Trop* **2002**;83:7–11.
6. Rowe T, Abernathy RA, Hu-Primmer J, et al. Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays. *J Clin Microbiol* **1999**;37:937–43.
7. Stephenson I, Wood JM, Nicholson KG, Zambon MC. Sialic acid receptor specificity on erythrocytes affects detection of antibody to avian influenza haemagglutinin. *J Med Virol* **2003**;70:391–8.
8. Donatelli I, Campitelli L, Di Trani L, et al. Characterization of H5N2 influenza viruses from Italian poultry. *J Gen Virol* **2001**;82:623–30.
9. Capua I, Marangon S, Bonfanti L. Eradication of low pathogenicity avian influenza of the H7N3 subtype from Italy. *Vet Rec* **2004**;154:639–40.
10. Capua I, Marangon S, Cordioli P. H7N3 avian influenza in Italy. *Vet Rec* **2002**;151:743–4.
11. Campitelli L, Mogavero E, De Marco MA, et al. Interspecies transmission of an H7N3 influenza virus from wild birds to intensively reared domestic poultry in Italy. *Virology* **2004**;323:24–36.
12. Centers for Disease Control and Prevention. Cases of influenza A (H5N1)—Thailand. *MMWR Morb Mortal Wkly Rep* **2004**;53:100–3.
13. Buxton Bridges C, Lim W, Hu-Primmer J, et al. Risk of influenza A (H5N1) infection among poultry workers, Hong Kong, 1997–1998. *J Infect Dis* **2002**;185:1005–10.
14. Meijer A, van de Kamp E, Wilbrink B, du Ry van Beest Holle M, Koopmans M. Development and validation of a hemagglutination inhibition assay for detection of human antibodies against the Dutch HPAI A (H7N7) virus (abstract 03-1). Program and abstracts of the Second European Congress of Virology (Madrid). 5–9 September **2004**.