

Existence of Protection against High Pathogenicity Avian Influenza Virus Subtype H5N1 in a Chinese Population

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A vast number of H5N1 avian influenza outbreaks in poultry have occurred in numerous countries throughout the world, however, so few cases of human infection have been reported, especially in China, suggesting humans may acquire resistance to the H5N1 avian influenza virus. A seroprevalence survey was conducted among humans on high pathogenicity avian influenza (HPAI) virus subtype H5N1. Human serum samples from 915 healthy blood donors, 1,223 pig sera from three pig slaughterhouses and 2,120 chicken eggs from a number of supermarkets in Zhengzhou were collected to test for H5N1 antibodies by Haemagglutination inhibition. Among the human blood donors tested 89% were positive for antibodies against HPAI virus subtype H5N1, with an average titer of 2^{5.8}; the positive rate of 2120 chicken egg samples was 100%; whereas all 1223 porcine serum samples were negative. The results indicate that an outbreak of HPAI in humans is unlikely.

Key words: High pathogenicity avian influenza virus, H5N1 subtype, Serum antibodies, Haemagglutination inhibition.

Since the first human H5N1 avian influenza virus fatality in Hong Kong in 1997¹, more and more cases of human infection have been reported worldwide. Research shows that H5N1 avian influenza virus transmission across the species has been achieved². Since 2003, there have been 650 World Health Organization (WHO)-documented cases of avian H5N1 influenza infections in humans reported from 15 countries³. Of the WHO-confirmed cases, 386 (59.4%) have resulted in death (as of 24 January 2014)³. A vast

number of H5N1 avian influenza outbreaks in poultry have occurred in numerous countries throughout the world. It is unclear why, despite this, so few cases of human infection have been reported, especially in China.

This may indicate that, although the H5N1 avian influenza virus exists in poultry and is transmitted among birds⁴, humans, or at least most humans, have acquired resistance to H5N1 high pathogenicity avian influenza (HPAI) virus. The avian influenza virus surface is covered by hemagglutinin (HA) and neuraminidase (NA) glycoprotein projections. HA is the major antigen that elicits the production of antibodies, which protect against clinical signs and death⁵. Following infection, the host produces mainly neutralizing

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antibodies, which provide effective protection against the pathogenic effects of the virus. It is due to this that a global pandemic has not occurred in humans. The occurrence of human influenza A (H5N1) in Southeast Asia has paralleled large outbreaks of avian influenza A (H5N1). It can be speculated that humans have already acquired resistance to the H5N1 avian influenza virus; however, this remains to be confirmed.

Henan Province has close to 100 million inhabitants and is the site of intense farming, with the number of live pigs, at 108 million and the poultry slaughter volume at 1.42 billion, which is the third highest site for these types of agriculture in China. In 2012, the Province's meat output reached 6.38 million tons and the egg output reached 3.887 million tons, both representing the highest output of these types in China. More than 50% of live pigs are exported from the Province.

To determine the frequency of avian influenza (H5N1) transmission to humans, we conducted a seroepidemiologic investigation of human blood donors, pigs and chickens in Zhengzhou, the capital city of Henan Province.

MATERIALS AND METHODS

Ethics statement

This study was approved by the Henan Agricultural University Institutional Review Committee. All blood donors provided written informed consent for participation in the study and donation of samples.

Samples

Human serum samples were collected from 915 healthy blood donors at the Henan Red Cross center; 1,223 pig sera were collected from three pig slaughterhouses; and 2,120 chicken eggs were randomly selected from a number of supermarkets in Zhengzhou. Sera were separated and frozen at -20°C for later use.

Serum samples were assayed to determine the antibody titers against HPAI virus subtype H5N1 using the hemagglutination inhibition (HI) method.

Antigen and antiserum

The avian influenza H5N1 HI antigen, the Newcastle disease HI antigen and relative positive/negative sera used were provided by the National

Reference Laboratory for Avian Influenza in Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, China.

Haemagglutination and Haemagglutination inhibition

Two-fold serial dilutions of the AIV-subtype H5N1 or Newcastle disease antigen were prepared in PBS in 96-well microtiter plates. Washed red blood cells (RBCs) were added to each well. Plates were incubated for 30 minutes at 37°C before recording the HA activity. The HI antibody titer of each serum sample was also determined according to the protocol set by the World Organization for Animal Health (OIE).

The HI procedures are as follows:

- i) Dispense 0.025 ml of PBS into each well of a plastic V-bottomed microtitre plate.
 - ii) Place 0.025 ml of serum into the first well of the plate.
 - iii) Make twofold dilutions of 0.025 ml volumes of the serum across the plate.
 - iv) Add 4 HAU of virus/antigen in 0.025 ml to each well and leave for a minimum of 30 minutes at room temperature (i.e. about 20°C) or 60 minutes at 4°C.
 - v) Add 0.025 ml of 1% (v/v) chicken RBCs to each well and after gentle mixing, allow the RBCs to settle for about 40 minutes at room temperature, i.e. about 20°C, or for 60 minutes at 4°C if ambient temperatures are high, by which time control RBCs should be settled to a distinct button.
 - vi) Results were recorded when complete button formation observed in the RBC control well and spreading RBC pattern in virus control. The HI titre is the highest dilution of serum causing complete inhibition of 4 HAU of antigen. The agglutination is assessed by tilting the plates. Only those wells in which the RBCs stream at the same rate as the control wells (containing 0.025 ml RBCs and 0.05 ml PBS only) should be considered to show inhibition.
 - vii) The validity of results was assessed against a negative control serum, which did not give a titre $>1/4$ ($>2^2$ or $>2\log_2$ when expressed as the reciprocal), and a positive control serum for which the titre was within one dilution of the known titre.
- HI titers were regarded as positive if there was inhibition at a serum dilution of $1/32$ (2^5 or $5\log_2$ when expressed as the reciprocal) or more against four HAU of antigen.

RESULTS AND DISCUSSION

Of 915 blood donors, 466 (50.9%) were women. The mean age of blood donors was 36.5 years (range, 18–55 years) (Table 1). Among the human serum samples, 814 (89%) were positive (HI titer $\geq 2^5$) and 101 (11%) were negative. The average antibody titer exhibited an age-dependent increase, and the total average antibody titer against HPAI virus was $2^{5.8}$. There was no significant difference in the antibody titers in serum samples from males and female blood donors.

All 1,223 swine serum samples from several designated live pig slaughter houses tested negative for antibodies against the HPAI virus H5N1 subtype in HI assays. In contrast, 100% of the 2,120 chicken egg samples obtained from local supermarkets tested positive. The antibody titer distributions against HPAI virus H5N1 subtype from humans and chicken eggs are shown in Figures 1 and 2, respectively.

The results show that 89% of the serum samples collected randomly from the blood donors were positive for antibodies against HPAI virus subtype H5N1, which is a higher rate than those

reported previously from China and other countries [6–12]. These differences may be due to differences in detection methods and samples from different districts. All of the chicken egg samples from local supermarkets were positive for HPAI virus subtype H5N1 antibodies, while none of the swine serum samples were positive. These results are consistent with those reported by Song et al. [13]. We also detected antibodies against Newcastle disease virus (NDV) in human and porcine serum, as well as chicken eggs. No one positive sample against

Table 1. Characteristics of 915 blood donors at the Henan Red Cross Center, China

Characteristic	No. (%)	HI antibody titer
Sex		
Male	449 (49.1)	5.795
Female	466 (50.9)	5.796
Age, y		
18–20	84 (9.2)	5.494
21–30	338 (36.9)	5.608
31–40	281 (30.7)	5.917
41–50	182 (19.9)	6.025
51–55	30 (3.3)	6.183
Total	915	5.796

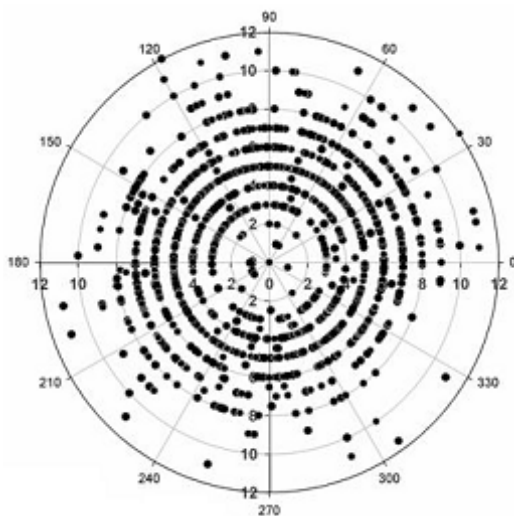


Fig. 1. Antibody titer distribution against HPAI virus H5N1 subtype in human sera. The antibody titer values ranking from 0 to 12 log₂ are marked on the horizontal and vertical axes. Black dots represent the antibody titer values from human serum samples in this study.

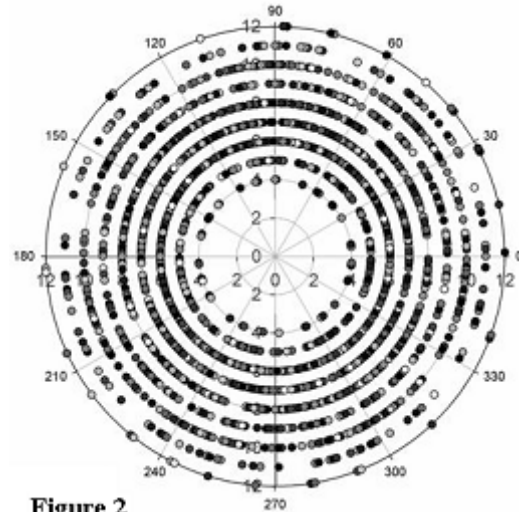


Figure 2
Fig. 2. Antibody titer distribution against HPAI virus H5 subtype in chicken eggs from supermarkets. The antibody titer values ranking from 0 to 12 log₂ are marked on the horizontal and vertical axes. Black dots represent the antibody titer values from egg samples in this study.

NDV was found in human being and swine (data not shown). Based on the serum antibody levels detected, we conclude that humans have acquired protection from the HPAI virus through an unknown mechanism.

On the other hand, China is one of the largest poultry breeding countries in the world. The managing level in poultry industry is in the principle step. The safety of poultry products is a cause for concern. Vaccination is the main approach used for controlling HPAI virus infection in chickens [14], although efficacy of the vaccine against HPAI virus subtype H5N1 is restricted by the risk of virus mutation. The occurrence of high pathogenicity H5N1 AIV infection in migrant waterfowl also represents a global threat [15, 4]. In 2012, China reported only two cases of H5N1 subtype of avian influenza infection. With a total of more than 1.3 billion people in China, the reason for the absence of an outbreak of H5N1 high pathogenicity avian influenza cases in humans in China is puzzling.

In this study, the specified antibody titers indicate that most Chinese people have acquired protection against the HPAI virus, indicating that the level of protection among humans against AIV-H5N1 subtype is satisfactory. It can be speculated that occasional instances of infection occur due to the absence of antibodies against HPAI virus subtype H5N1 in particular individuals. These data indicate that the risk of a bird flu pandemic in China is low and that an HPAI outbreak disaster in humans may be impossible now and in the future. It can be speculated that, with the evolution of influenza A, the human immune system has acquired protection against HPAI virus subtype H5N1 as a result of infection by different evolving human influenza A virus subtypes.

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