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Measurement of antibodies to avian influenza virus A(H7N7) in humans by hemagglutination inhibition test

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Abstract

During the epizootic of highly pathogenic avian influenza A(H7N7) in 2003 in The Netherlands, RT-PCR and culture confirmed infection was detected in 89 persons who were ill. A modified hemagglutination inhibition (HI) test using horse erythrocytes and 2 hemagglutinating units of virus was applied to assess retrospectively the extent of human (subclinical) infection. Validation of the HI-test with sera from 34 RT-PCR and culture confirmed A(H7) infected persons and sera from 100 persons from a human influenza vaccine trial in autumn 2002 showed that this HI-test had a sensitivity of 85% and a specificity of 100% when using a cut-off titer of ≥ 10 . Using this cut-off value, A(H7) specific antibodies were detected in 49% of 508 persons exposed to poultry and in 64% of 63 persons exposed to A(H7) infected persons. Correlation of seropositivity with the occurrence of eye symptoms in exposed persons who had not received antiviral prophylaxis and of reduced seropositivity with taking antiviral prophylaxis provided further evidence that the A(H7) HI antibody titers were real. In conclusion, by applying an HI-test using horse erythrocytes human antibodies against the avian A(H7N7) virus were detected with high sensitivity and specificity in an unexpectedly high proportion of exposed persons.

1. Introduction

In 2003, The Netherlands experienced an epizootic of highly pathogenic avian influenza (HPAI) A(H7N7) among poultry in which 233 commercial and 22 pet poultry holdings were infected and over 30 million animals were culled (Den Boer et al., 2004). Active case finding among the population at risk by providing diagnostic tests for ill persons (conjunctivitis and/or influenza-like illness) detected A(H7) infection in 89 persons using RT-PCR and virus culture (Fouchier et al., 2004 and Koopmans et al., 2004). Among these were three possible human-to-human transmissions without any known exposure to poultry, and one fatal case. To understand completely the zoonotic implications of this epizootic we wanted to perform sero-epidemiological studies among humans exposed to infected poultry or RT-PCR and culture confirmed A(H7) infected persons (subsequently in this paper referred to as A(H7) infected persons).

Evidence that humans can mount a serological response to avian influenza A viruses of the H1–H13 subtype has been demonstrated in a survey among rural dwellers in Southern China, but this may have occurred after passage of the virus through pigs (Shortridge, 1992). In that study, antibody responses were detected by the laborious single radial hemolysis assay. The hemagglutination inhibition test (HI-test), which is used most often for sero-epidemiological studies of human influenza, however has been found relatively insensitive for the detection of mammalian antibodies against avian influenza viruses, even after natural or experimental infection or vaccination (Beare and Webster, 1991, Lu et al., 1982, Rowe et al., 1999 and Stephenson et al., 2003a). Consequently, a combination of microneutralization or ELISA and Western blot assays was recommended for definitive proof of detection of human antibodies induced by avian influenza virus (Rowe et al., 1999). This resulted in documentation of serological evidence for asymptomatic poultry-to-human and human-to-human transmission during the epizootic of the highly pathogenic avian influenza (HPAI) virus A(H5N1) in Hong Kong in 1997 (Buxton Bridges et al., 2000, Buxton Bridges et al., 2002 and Katz et al., 1999), whereas standard hemagglutination inhibition was insensitive to measure those anti-H5 responses (Stephenson et al., 2003a). Similarly, human antibody responses to H9 hemagglutinin have also been found after natural infection in one of two culture confirmed A(H9N2) cases (Uyeki et al., 2002), and in approximately 30% of poultry workers in Hong Kong (Eick et al., 2000). However, the use of HI-tests is not completely unsuccessful for detection of antibodies to avian influenza viruses. Guo et al. (1999) found 19% of humans tested in China by standard HI positive for anti-H9 antibodies. In addition, during a trial with an A(H9N2) vaccine, pre-existing anti-H9 antibodies were found in the general population of the UK born before 1969, and people without pre-existing antibodies mounted an antibody response after vaccination, as measured using standard HI and microneutralization (Stephenson et al., 2003b). Antibody responses were not detected to A(H7) subtype avian influenza viruses using standard HI in three (Campbell et al., 1970, DeLay et al., 1967, Kurtz et al., 1996 and Webster et al., 1981) and with a very low HI-titer in one (Taylor and Turner, 1977) of four culture confirmed influenza virus A(H7) infected human cases reported previously. In addition, in a large cohort study carried out among 759 persons exposed to infected poultry during the 1999–2000 avian influenza A(H7N1) epizootic in Italy, antibodies against this virus could not be detected by microneutralization and single radial hemolysis assays (Capua et al., 2002). However, clearly detectable antibody responses were found recently in two culture confirmed influenza virus A(H7N2) infected persons, one in Virginia, and one in New York, USA, using microneutralization confirmed by Western blot, and ELISA (Terebuh et al., 2003 and ProMED-mail, 2004). In addition, anti-H7 antibodies have been demonstrated in people without associated disease in 90 of 1318 persons tested in China and in 1 of 32 persons tested in Memphis, TN, USA, using single radial hemolysis or ELISA (Shortridge, 1992 and Zhou et al., 1996).

Recently, Stephenson et al. (2003a) showed that the sensitivity of the HI-test for detection of human antibodies against the avian A(H5N1) virus, and of antibodies from other mammals (ferret, rabbit, goat, sheep) against avian A(H6N5), A(H11N6), and A(H13N6) and equine A(H7N7) influenza viruses, could be improved considerably using horse instead of turkey erythrocytes to levels comparable of those for microneutralization and single radial hemolysis assays (Stephenson et al., 2003a). This difference was attributed to the greater density of the receptor for avian influenza virus, the $\alpha(2,3)$ Gal-linked sialic acids, on horse as compared with turkey erythrocytes.

This report describes the adjustment and application of the HI-test using horse erythrocytes for detection of antibodies against the HPAI A(H7N7) virus in serum of persons exposed to infected poultry or A(H7) infected persons during the

A(H7N7) epizootic of 2003 in The Netherlands. In addition, the report describes the comparison of this HI-test with microneutralization assay.

2. Materials and methods

2.1. Sera

Four serum banks were used for the study: (i) 34 sera from A(H7) infected persons collected during the HPAI A(H7N7) epizootic in The Netherlands in spring 2003, (ii) 508 sera from poultry-exposed persons (of which 469 with direct contact with infected poultry), (iii) 63 sera from persons exposed to an A(H7) infected person (of which 56 reported explicitly having no contact with poultry), and (iv) 100 sera that had been collected during a vaccine trial in autumn 2002 performed by Solvay Pharmaceuticals B.V. (Weesp, The Netherlands) to comply with regulatory requirements in the European Union. The vaccine used was Influvac 2003/2004 and consisted of hemagglutinin and neuraminidase of A/Panama/2007/99 RESVIR17 reassortant (H3N2) virus, A/New Caledonia/20/99 IVR-116 reassortant (H1N1) virus, and B/Shangdong/7/97 virus. The same vaccine was used to vaccinate persons exposed to A(H7N7) virus during the epizootic. The sera from groups i–iii had been collected as part of two cohort studies. The detailed epidemiological analyses of these cohorts will be reported elsewhere. Data on symptoms were derived from the questionnaires used in these studies, and used to assemble a serum bank of persons with known clinical picture following A(H7N7) exposure.

Local public health services or general practitioners collected the blood specimens. Single blood specimens were taken at least 3 weeks after possible exposure to A(H7) infected persons or poultry (Table 1). The blood specimens were sent to the National Institute of Public Health and the Environment (RIVM), Bilthoven, The Netherlands, by regular mail. Upon arrival, serum was separated from the blood clot and sera were stored frozen at -20°C until analysed. Dr. I.A. de Bruijn, Solvay Pharmaceuticals B.V., Weesp, The Netherlands kindly provided the vaccine trial sera. Characteristics of the four serum banks are summarized in Table 1.

Table 1.

Characteristics of groups of persons of which sera were analysed in this study

Description	Group			
	A(H7) infected persons	Poultry-exposed persons ^a	Persons exposed to A(H7) infected persons	Persons from vaccination study
Number of sera	34	508	63	100
Women	3	66	37	50
Men	31	441	26	50
Age				
Mean	34	43	27	38
Range	13–59	16–73	3–61	18–59
Time between first day of illness/exposure to A(H7N7) virus and day of collection of serum	Mean: 6 weeks, range: 4–13 weeks	Mean: 22 weeks ^b , range: 16–40 weeks	Mean: 6 weeks ^c , range: 4–13 weeks	No exposure to A(H7N7) virus

^a Of one person the gender and date of birth were missing.

^b Probably overestimated. The time was chosen relative to the start of culling

activities at 4 March 2003 as the exact day of individual first and last day of exposure could not be traced back. Culling activities lasted about 10 weeks.

^c Sera collected on the same day as from the A(H7) infected person in the household, time relative to the first day of illness of the A(H7) infected person.

The Dutch Medical Ethics Committee approved the studies and each person participating in the study gave informed consent.

2.2. Reference materials

The A/Netherlands/33/03 (H7N7) influenza virus was isolated previously at the RIVM from the index case of the A(H7N7) outbreak among humans (Koopmans et al., 2004). Turkey erythrocytes were derived from turkeys held at the RIVM. Dr. L.S. Goehring from the Animal Medicine faculty of the Utrecht University, Utrecht, The Netherlands kindly provided horse and pig erythrocytes. Dr. J.C. de Jong from the National Influenza Centre, Erasmus Medical Centre, Rotterdam, The Netherlands kindly provided human A/New Caledonia/20/99 (H1N1) and A/Panama/2007/99 (H3N2) influenza viruses and ferret antisera raised against these viruses. Dr. C. van Maanen from the Animal Health Service, Deventer, The Netherlands kindly provided avian A/Parrot/Northern Ireland/VF-73-67/73 (H7N1) influenza virus and chicken antiserum raised against this virus.

2.3. Serology

Sera were tested for antibodies against the A(H7N7), A(H1N1), and A(H3N2) viruses. All sera were treated with Receptor Destroying Enzyme (prepared "in house" from *Vibrio cholerae*) and adsorbed with turkey or horse erythrocytes to remove non-specific hemagglutination as described (World Health Organization, 2002), before testing by HI. HI was carried out essentially as described elsewhere (World Health Organization, 2002). Briefly, a two-fold serial dilution series of serum (1:10–1:640) was mixed 1:1 with 25 µl of virus, incubated at 37 °C for 1 h after which 50 µl of erythrocytes were added, mixed, incubated for 1 h at 4 °C, and agglutination patterns were read within 10 min. For the human viruses, 0.5% turkey erythrocytes and 4 hemagglutinating units (HAU) of virus were used. For optimisation of the HI-assay with the A(H7N7) virus, erythrocytes from turkey (0.5%) and horse (1%), and 1, 2 or 4 HAU of virus were used. Final analysis of all sera was performed using 1% horse erythrocytes and 2 HAU of A(H7N7) virus. All final tests were done in "V" bottom microtiter plates and carried out in duplicate. The HI-titer was defined as the reciprocal of the last dilution of serum that inhibits completely hemagglutination.

Neutralizing antibodies were measured using a microneutralization assay essentially as described elsewhere (World Health Organization, 2002) using the same viruses as for HI, Madin Darby Canine Kidney (MDCK) clone CB4 cell monolayers and ELISA readout using anti-nucleoprotein antibodies (Meijer et al., 2004). Briefly, a two-fold serial dilution series of serum (1:20–1:2560) was mixed 1:1 with 60 µl of virus, incubated at 37 °C for 2 h after which 100 µl of the mixture was transferred to washed MDCK cell monolayers and incubated for 2 h at 37 °C. The serum-virus mixture was removed, the cells washed, virus growth medium with trypsin added, and the cells were incubated at 37 °C for 18 h. After fixation of the cells, influenza virus growth was detected by ELISA. The amount of virus used in the assays was 100 tissue culture infectious dose. All virus neutralization tests were carried out in duplicate. The result of a particular dilution of a serum was considered positive when $A_{450\text{ nm}} < [((\text{mean } A_{450\text{ nm}} \text{ of virus control} - \text{mean } A_{450\text{ nm}} \text{ of cell control})/2) + \text{mean } A_{450\text{ nm}} \text{ cell control}]$; i.e.

reduction of viral antigen >50%. The neutralization titer was defined as the reciprocal of the last dilution of serum that had a positive neutralization result.

2.4. Statistical analysis

Data were entered in MS Excel and analysed using Statistica'99 edition, StatSoft Inc., Tulsa, USA and GraphPad Prism, version 3, GraphPad Software Inc., San Diego, USA. The Spearman test was used to analyse correlation and the two-tailed Fisher exact test to analyse contingency tables. A p -value < 0.05 was considered statistically significant. The 95% confidence intervals for sensitivity and specificity were calculated using the web based Clinical Research Calculator 1 at <http://faculty.vassar.edu/lowry/VassarStats.html>.

3. Results

3.1. Optimisation and evaluation of HI

Initial tests with sera from A(H7) infected persons and the routinely used set-up of the HI-test using 0.5% turkey erythrocytes and 4 HAU of A(H7N7) virus were all negative (titer < 10). Prompted by the publication of detection of human HI antibodies against the HPAI A(H5N1) virus with enhanced sensitivity by using horse erythrocytes (Stephenson et al., 2003a), the tests were repeated with 4, 2 and 1 HAU of virus and 1% horse erythrocytes on sera from 22 A(H7) infected persons. The percentage sera with a titer of ≥ 10 increased from 0% with turkey erythrocytes and 4 HAU to 55, 86 and 100% with horse erythrocytes and 4, 2 and 1 HAU, respectively. To control for false positive results, especially when using 1 HAU, the sensitivity and specificity of the HI-assay were determined with 1 and 2 HAU using the results of all 34 A(H7) infected persons and of sera from 100 participants in a human influenza virus vaccine trial (Table 2). The correlation between the titers found with 1 and 2 HAU was highly significant (Spearman $r = 0.61$; 95% CI = 0.46–0.73; p [two-tailed] < 0.0001). Because it is easier to prepare reproducibly a 2 HAU virus stock suspension than a 1 HAU virus stock suspension and because of the absence of false positives in the vaccine trial sera when using 2 HAU and 1% horse erythrocytes, all subsequent tests were done using these conditions.

Table 2.

Characteristics of hemagglutination inhibition assay using 1% horse erythrocytes for detection of human antibodies against the A/Netherlands/33/03 (H7N7) influenza virus

Assay	Characteristics	
	Sensitivity (%) (95% CI) ^a	Specificity (%) (95% CI)
One hemagglutinating unit of virus		
Cut-off ≥ 10	100 (87–100)	82 (73–89)
Cut-off ≥ 20	97 (83–100)	99 (94–100)
Cut-off ≥ 40	91 (75–97)	100 (95–100)
Two hemagglutinating units of virus		
Cut-off ≥ 10	85 (68–95)	100 (95–100)
Cut-off ≥ 20	53 (35–70)	100 (95–100)
Cut-off ≥ 40	6 (1–21)	100 (95–100)

^a CI: confidence interval.

Initially, HI was carried out with either 0.2% (v/v) liquid bovine serum albumin (Organon Teknika, nr 40023) or 0.2% (w/v) bovine serum albumin (Sigma, A3803) in phosphate-citrate buffered saline following Mc Ilvaine (pH 7.2, by mixing 0.1 M citric acid with 0.2 M Na_2HPO_4) with 0.01% sodiumazide, the buffer system routinely used in our laboratory for HI-assays. However, results with horse erythrocytes were difficult to read using this buffer system, regardless whether "U" or "V" bottom microtiter plates were used. In this set-up the demarcation between presence and absence of hemagglutination was often not sharply defined in one or two serum dilution steps. Changing the buffer system to phosphate buffered saline improved reading of the results. The transition from complete inhibition of agglutination to complete agglutination occurred most often in neighbouring wells. Occasionally, a maximum of one well with partial inhibition was seen in between the wells with complete inhibition of agglutination and complete agglutination.

To determine the enhancement in sensitivity when using horse erythrocytes instead of turkey erythrocytes, a selection of 20 sera with high titers with 1% horse erythrocytes and 2 HAU of A(H7N7) virus were tested with 0.5% turkey erythrocytes and 2 HAU of virus. Twelve sera with a titer of 40 and seven with a titer of 80 with horse erythrocytes, had a titer of <10 with turkey erythrocytes. A single serum with a titer of 160 with horse erythrocytes had a titer of 10 with turkey erythrocytes, suggesting a 16-fold enhancement.

Since antibodies against A(H3) and A(H1) viruses have been reported to cross-react with avian influenza virus A(H9N2) that infected humans (Eick et al., 2000 and Peiris et al., 1999), the specificity of the assay was further confirmed by cross HI between the human A(H1N1) and A(H3N2) viruses, the A(H7N7) virus and ferret antisera raised against the human viruses and a chicken antiserum raised against an avian A(H7N1) influenza virus with similar H7 specificity as the A(H7N7) virus. This showed only HI reactivities in the homologous combinations. In addition, the titers of the human sera against the human influenza viruses and against the A(H7N7) avian influenza virus were compared for possible correlations, which would suggest cross-reactivity. As expected, antibody titers against the A(H1N1) virus were significantly correlated with those against the A(H3N2) virus in most groups because of infection and vaccination history with both viruses (not shown). No clear significant correlation was found between antibody titers against the human influenza viruses and titers against the A(H7N7) virus (not shown), except possibly for A(H1N1) versus A(H7N7) in persons exposed to an A(H7) infected person, which was close to significance (Spearman $r = 0.35$; 95% CI = -0.02 to 0.60 ; p [two-tailed] = 0.0604).

3.2. HI results of serum banks

Using the final assay format with a cut-off titer of ≥ 10 , A(H7) specific HI antibodies were detected in 29 (85%) of 34 A(H7) infected persons, in 251 (49%) of 508 poultry-exposed persons (239 [51%] when restricted to the 469 with direct exposure to infected poultry), and in 40 (64%) of 63 persons exposed to an A(H7) infected person (33 [59%] when restricted to the 56 persons without any self-reported contact with [possibly] infected poultry) (Fig. 1). When increasing the cut-off to ≥ 40 , as this would represent a four-fold rise over the detectable limit, 2 (6%) of A(H7) infected persons, 36 (7%) of 508 poultry-exposed persons and 4 (6%) of 63 persons exposed to A(H7) infected persons showed A(H7) specific antibodies (Fig. 1). However, this would have lowered the sensitivity to 6% (Table 2). No A(H7) specific antibodies were detected in the vaccine trial sera (Fig. 1). No correlation of the height of the titers with age of the persons was found (Fig. 1). Among the group of poultry-exposed persons that did not take

antiviral prophylaxis (oseltamivir) ($n = 108$), occurrence of any eye symptoms was more frequent in persons with detectable antibodies (25 of 57; 44%) than in persons without antibodies (13 of 51; 26%) (RR = 1.72; 95% CI = 0.99–2.99; $p = 0.046$). In addition, prophylactic use of oseltamivir was associated with a lower risk of conjunctivitis (OR = 0.14; 95% CI = 0.08–0.27; $p = 0.00$) and a lower likelihood of developing of anti-H7 antibodies (OR = 0.48; 95% CI = 0.25–0.89; $p = 0.02$).

Fig. 1. Age-stratified A(H7)-specific HI data of three groups of persons with different types of exposure to the A(H7N7) virus of the epizootic in 2003 in The Netherlands and of a control group of persons vaccinated with the seasonal human influenza vaccine prior to the epizootic and therefore no exposure to the A(H7N7) virus.

3.3. Virus neutralization

A subset of the sera with detectable HI antibodies was tested by microneutralization for confirmation. A chicken antiserum raised against an A(H7N1) virus had a titer of 160 by HI and a titer of 320 by microneutralization with the A(H7N7) virus. In addition, high titers of neutralizing antibodies to the human influenza viruses were detected which were also higher than in the HI-assay. Both findings show the usually found higher sensitivity of the microneutralization assay as compared with routine HI-test. However, none of the human sera tested showed neutralization of the A(H7N7) virus in this assay.

3.4. Receptor specificity

The receptor specificity of the viruses was determined using erythrocytes from different animal species (Fig. 2). The human viruses had sialic acid (SA) α (2,6)Gal specificity as they did agglutinate turkey and not horse erythrocytes. The A(H7N7) virus had SA α (2,3)Gal specificity as it agglutinated horse in addition to turkey and pig erythrocytes. In addition, the A(H1N1) and A(H7N7) virus bound to both NeuGc and NeuAc sialic acids, although to NeuGc with lower affinity than to NeuAc as shown by lower titers with pig or horse erythrocytes than with turkey erythrocytes. The A(H3N2) virus bound only to NeuAc.

Receptor type	Receptor distribution on erythrocytes from:		
	Horse	Turkey	Pig
Sialic acid $\alpha(2,3)$ Gal	+	+	+
Sialic acid $\alpha(2,6)$ Gal	-	++	++
NeuAc	-	+	(-)
NeuGc	+	-	+

(A)

Virus	Hemagglutination pattern and titer with erythrocytes from:		
	Horse	Turkey	Pig
A/New Caledonia/20/99 (H1N1)
	<2	64	4
A/Panama/2007/99 (H3N2)
	<2	32	<2
A/Netherlands/33/03 (H7N7)
	16	64	16
Buffer control

(B)

Fig. 2. (A) Relative receptor distribution on horse, turkey, and pig erythrocytes as described by Ito et al. (1997) and Thompson et al. (2004). Most of the sialic acid on pig erythrocytes is NeuGc (Ito et al., 1997). (B) Hemagglutination patterns and titer of the same stock of viruses with the different erythrocytes. Percentage erythrocytes used in the assays: horse 1%, turkey 0.5%, and pig 1%.

4. Discussion

This report describes the application and evaluation of an adapted HI-test using horse erythrocytes for the specific detection of human antibodies against the Dutch A(H7N7) influenza virus that caused an epizootic among poultry and transmission to humans in 2003. Using this assay, a measurable antibody response was detected in a high proportion of sera from persons exposed to the A(H7N7) virus. This finding was somewhat surprising, as in a similar study in Italy among persons known to be exposed to A(H7N1) infected poultry no antibody responses were found using a microneutralization assay (Capua et al., 2002). However, using microneutralization assay also no antibody responses against the Dutch A(H7N7) virus were found. If the infecting virus had been a human influenza virus, this lack of confirmation would be regarded as evidence for false reactivity in the HI-assay. In this case, however, the situation is probably different. The A(H7N7) virus uses a different receptor on erythrocytes than the human viruses, as shown by the data in Fig. 2. Changing to the use in the HI-assay of erythrocytes from a different animal species that has better receptors for the avian influenza viruses enhanced significantly the sensitivity of detection. The same may apply for the microneutralization assay, which employs animal cells, as there is a direct correlation between neutralization of virus infectivity and inhibition of virus binding to cells or agglutination of erythrocytes (Benne et al., 1994, De Jong et al., 2003 and Knossow et al., 2002). Alternative tests like single radial haemolysis and Western blot may be used to confirm the HI results, however, these tests are either laborious or less sensitive compared to the microneutralization assay. Therefore, a more promising approach that is currently being investigated is the use of cell lines with enhanced sensitivity for avian influenza viruses, e.g. by overexpression of $\alpha(2,3)$ -sialyltransferase, in the microneutralization assay.

The use of a cut-off titer of ≥ 10 may be controversial as a four-fold rise in titer from negative is generally used for seroconversion, and a titer of ≥ 40 for being protective (De Jong et al., 2003). However, evidence in favour of regarding the results of the HI-test specific, even if titers were quite low, are the absolute absence of reactivity in persons who had been sampled prior to the A(H7N7) outbreak and had been vaccinated recently with the regular human influenza virus vaccine that has also been used to prevent circulation of human influenza virus among poultry workers and farmers. This is a stringent control; any cross-reactivity between antibodies against avian and human influenza virus would have been detected in this study population. Nevertheless, HI reactivity was not seen, rendering the specificity of the assay 100%. A correlation close to significance was found between the titers against A(H7N7) and those against A(H1N1), but only in the A(H7N7) infected persons. A possible explanation could be age-related cross-reactivity. However, if this would explain the observed possible correlation it should have been observed also in the age-matched control group, which was not the case (Fig. 1). A final indication for the specificity of the HI-test came from the finding that seropositivity correlated with the presence of ocular symptoms known to be associated with A(H7N7) infection (Koopmans et al., 2004), and the finding that prophylactic use of oseltamivir was associated with a lower risk of developing A(H7) antibodies. Although it has been demonstrated that oseltamivir does not prevent the individual from being infected and hence from developing an antibody response (Ward et al., 2005), these findings were for human viruses and respiratory infection. Nothing is known about the effect of oseltamivir on the antibody response following the infection of the conjunctiva with an avian influenza virus. The known differences between the immune response in the eye compartment and a systemic immune response (Meek et al., 2003) could have accounted for the observed effect of oseltamivir on the lower likelihood of developing of serum anti-H7 antibodies. Taken all

results together, the reactivities in HI using A(H7) virus were considered true evidence for A(H7) virus exposure and infection.

Routinely, chicken or turkey erythrocytes are used for HI. In accordance with previous observations (Stephenson et al., 2003a), the results show that human HI antibody titers against avian influenza viruses could be measured reproducibly and specifically with enhanced sensitivity when using horse erythrocytes instead of turkey erythrocytes. The increased sensitivity of the HI-test using horse erythrocytes as compared with the HI-test using turkey erythrocytes and microneutralization cannot be explained simply as a consequence of fewer viruses used in the horse erythrocyte based assay. The amount of virus in the HI-test using horse erythrocytes was in reality higher than in the HI-test using turkey erythrocytes as more virus was needed to agglutinate horse erythrocytes compared with turkey erythrocytes, partly because of the two-fold higher amount of horse erythrocytes (1%) used in the assay compared to turkey erythrocytes (0.5%) (see Fig. 2). In addition, the amount of virus used in the HI-test using horse erythrocytes was similar to that used in the microneutralization assay. Therefore, the most plausible explanation is that the virus receptors on horse erythrocytes offer a better substrate for HI of avian influenza virus by human antisera.

The receptor specificities of the A(H1N1) and A(H3N2) viruses as determined with horse, turkey, and pig erythrocytes were consistent with previously reported specificities of similar viruses (Ito et al., 1997 and Matrosovich et al., 2000), showing the validity of the used method. Using the same approach the A/Netherlands/33/03 (H7N7) virus was shown to have NeuAc/NeuGc(2,3)Gal receptor specificity. This specificity is as could expected from the amino acids 190E, 225G, 226Q, and 228G (based on A(H3) numbering) present in the A/Netherlands/33/03 (H7N7) hemagglutinin receptor binding pocket (GenBank accession no. AY338457). These amino acids determine SA(2,3)Gal and NeuGc preference in A(H1), A(H2), and A(H3) viruses (Ito et al., 2000 and Matrosovich et al., 2000).

Horse erythrocyte agglutination requires recognition of NeuGc(2,3)Gal (Ito et al., 1997), and sera from A(H7) infected persons could inhibit this agglutination efficiently. The A(H7N7) virus does agglutinate turkey erythrocytes by binding to NeuAc(2,3)Gal, but only one serum with a high HI-titer as measured using horse erythrocytes could inhibit this agglutination. This may be explained by the higher affinity of the A(H7) virus for NeuAc(2,3)Gal than for NeuGc(2,3)Gal as evidenced by the four-fold higher hemagglutination titer with erythrocytes containing NeuAc as compared with erythrocytes containing NeuGc (Fig. 2). From these findings the conclusion is drawn that human antibodies against the A(H7N7) virus could be measured with highest sensitivity in HI when the lower affinity binding of the virus to NeuGc(2,3)Gal on horse erythrocytes was exploited.

The presence of antibodies that preferably inhibit binding of the A(H7N7) virus to only one of the four possible receptor configurations of the influenza virus receptor, may also explain why we could not detect neutralizing activity of the human sera in the microneutralization assay. MDCK cells contain both SA(2,3)Gal and SA(2,6)Gal (Meijer et al., 2004 and Verkoelen et al., 2000) but the amount of NeuAc is approximately 100-fold higher than the amount of NeuGc (Hughes et al., 2001). Therefore, and since virus neutralization is a dynamic process in which higher affinity bindings favour over lower affinity bindings (Knossow et al., 2002), binding of the A(H7N7) virus to NeuAc(2,3)Gal on MDCK cells and subsequent virus entry and replication may mask the inhibiting activity of human sera on virus binding to NeuGc(2,3)Gal.

The results suggest that humans undergo more subclinical infection with avian influenza viruses resulting from poultry-to-human or human-to-human transmission than is generally thought. Although, serological evidence of subclinical infection with avian influenza viruses has been demonstrated previously (Eick et al., 2000, Guo et al., 1999, Shortridge, 1992, Stephenson et al., 2003b and Zhou et al., 1996). The recognition that the reported (worst) clinical cases of human infection with avian influenza viruses are possibly only the tip of the iceberg, as is also evidenced from the repeated re-emergence of the A(H5N1) virus since 1997, has important implications for pandemic preparedness planning activities, like availability of vaccines and antiviral agents. In addition, widespread subclinical infection increases the risk for the emergence of reassortant viruses when avian influenza viruses circulate during seasonal human influenza epidemics. The re-emergence of the A(H5N1) virus in 2003 (Peiris et al., 2004) and 2004 (Tran et al., 2004) in Asia (for up to date information see http://www.who.int/csr/disease/avian_influenza/en/) is of particular concern, since only clinical, mainly hospitalised, cases are reported and currently no information is available on subclinical spread among people in the areas in which poultry has been affected badly.

In conclusion, the results show that antibodies to avian influenza virus A(H7N7) in human serum can be detected by a modified HI-test, in which turkey erythrocytes have been replaced by horse erythrocytes that contain a better receptor for avian influenza viruses. The absence of "classical" confirmation of antibodies against influenza virus by neutralization assay may result from the same selective receptor usage, resulting in a sub-optimal assay format. The data from the validation experiments and the additional epidemiological evidence strongly suggest that the HI titers are real. Therefore, the results suggest a far greater proportion of immune responders to (subclinical) avian influenza virus infection than has been recognized previously.

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