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Testing Human Sera for Antibodies against Avian Influenza

Viruses:

Horse RBC Hemagglutination Inhibition vs. Microneutralization Assays

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Abstract

Background—The hemagglutination inhibition (HI) assay is a frequently used method to screen human sera for antibodies against influenza A viruses. Because HI has relatively poor sensitivity in detecting antibodies against avian influenza A strains, a more complicated microneutralization (MN) assay is often preferred. Recent research suggests that the sensitivity of the HI assay can be improved by switching from the traditionally used turkey, guinea pig, human, or chicken RBCs to horse RBCs.

Objective—To evaluate the performance of the Horse RBC HI when screening for human antibodies against avian influenza types H3, H4, H5, H6, H7, H9, H11, and H12.

Study design—We evaluated the reproducibility of horse RBC HI and its agreement with MN results using sera from people exposed or not exposed to wild and domestic birds.

Results—The horse RBC HI assay had high reliability (90-100%) and good agreement with MN assay results (52-100%).

Conclusion—The horse RBC HI assay is reliable, less expensive, less complex, and faster than the MN assay. While MN will likely remain the gold standard serologic assay for avian viruses, the horse RBC HI assay may be very useful as a screening assay in large scale epidemiologic studies.

Keywords

antibody specificity; epidemiology; influenza, human; influenza in birds; serology

Introduction

Since the first well-documented human case of avian influenza infection in man occurred in 1997 (Ungchusak et al., 2005), several other instances of bird-to-human transmission have been reported. These cases have been caused by different types of avian influenza virus and have occurred in numerous geographical regions (Liu, 2006; Stone, 2006; Wong et al., 2006). Most recently, strains of H5N1 virus emerged in Southeast Asia in 2004 and spread to large

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geographic regions of Asia, Europe, and Africa. Many agree that these H5N1 strains have the potential to cause an influenza pandemic (Osterholm, 2005; Bartlett et al., 2005).

In response to the threat of pandemic influenza, public health officials have highlighted epidemiologic surveillance as an important tool for detection and prevention of widespread epidemics (Stephenson et al., 2004; Osterholm, 2005). The hemagglutination inhibition (HI) assay using turkey, guinea pig, human, or chicken red blood cells (RBCs) is traditionally the preferred method for detecting antibodies against human influenza A viruses; however, similar HI assays were found to be less sensitive in detecting antibodies against avian strains (Profeta et al., 1986; Rowe et al., 1999). This reduced sensitivity may be explained by the fact that many avian influenza A viruses preferentially bind to sialic acid $\alpha 2,3$ Gal receptors which are less prevalent on turkey RBCs, compared to mammalian species RBCs (Stephenson et al., 2003). Thus, laboratories have shifted to using a microneutralization (MN) assay that is reported to be 80% or 88% sensitive and 93% or 100% specific in detecting anti-H5 antibody among adults or children who were confirmed by virus culture to be infected with avian H5N1 virus (Rowe et al., 1999). In 2004, a HI assay based on horse RBCs was shown to be more sensitive in detecting human antibodies against an avian H5N3 strain than an assay based on turkey RBCs (Stephenson et al., 2004). Subsequently, several researchers have used this horse RBC HI assay to test for human antibodies against avian H5N1, H7N3, H7N7, and H1N9 viruses (Gill et al., 2006; Meijer et al., 2006; Treanor et al., 2006; Puzelli et al., 2005).

In this report, we examine the reproducibility of the horse RBC HI and describe its agreement with MN assays for human antibodies against H3, H4, H5, H6, H7, H9, H11, and H12 avian influenza A strains. We explore potential antibody cross-reactivity between human and avian influenza viruses to learn whether antibodies against human influenza may confound results produced by the horse RBC HI.

Materials and Methods

Sera

A random-number generated sample of 75 human sera from individuals exposed (n=38) and not exposed (n=37) to domestic or wild birds were utilized to compare the two assays. Sera were obtained through informed consent during institutional review board-approved research (Gill et al., 2006; Myers et al., 2006).

Viruses, Antisera, and Cells

Avian influenza viruses and specific post immunization chicken, rabbit and goat antisera were kindly provided by Dr. Richard J. Webby, St. Jude Children's Research Hospital, Memphis, TN; Dr. Alexander I. Klimov, Influenza Branch, the Centers for Disease Control and Prevention (CDC), Atlanta, GA; the Biodefense and Emerging Infections Research Resources Repository, Manassas, VA; and Dennis A. Senne, MS, of the US Department of Agriculture, APHIS, National Veterinary Services Laboratories, Ames, IA (Table 1). When two specific antisera were available, both were studied. Nonimmunized sheep serum was obtained from a WHO 2005-2006 influenza reagent kit. Viruses were grown in the allantoic cavity of 10-day old embryonated chicken eggs for 72 hours at 37°C. Eggs were chilled at 4°C overnight, and then the allantoic and amniotic fluids were harvested. MDCK cells used for the microneutralization assays were maintained in DMEM containing 5% fetal bovine serum.

Horse RBC HI Assay

Horse blood was shipped in acid citrate dextrose (ACD) and received at the lab the day after extraction. Upon receipt, the blood was washed three times with PBS by mixing 20 ml of blood and 30 ml of PBS in a 50 ml centrifuge tube and centrifuging at 4°C, 1000 $\times$ g for 5 minutes.

A solution of 1% horse blood was prepared by adding pelleted horse RBCs to PBS containing 0.5% bovine serum albumin fraction V.

Sera were treated with receptor destroying enzyme and hemadsorbed on horse RBCs according to the sera preparation procedures of the CDC HI protocol for human influenza. Sera were further diluted with PBS to a final 1:10 dilution. Two-fold serial dilutions in 25 µl PBS was performed in rows B-H of 96-well V bottom microtiter plates. Next 25 µl of PBS containing 4 hemagglutination units (HAU) of virus were added to rows B through H. Row A served as control and received 25 µl of uninfected allantoic fluid. The sera and virus mixture was left to incubate at room temperature for 30 minutes after which 50 µl of 1% horse blood was added to all wells. Plates were incubated at room temperature and read after 1 hour. The serum titer result was expressed as the reciprocal of the highest dilution of serum where hemagglutination was inhibited. Sera that tested negative at a dilution of 1:10 were assigned a titer of 1:5. All assays were conducted twice (2 different days). Each assay included specific positive antisera (Table 1) and nonimmunized sheep serum controls. The back titer was run in duplicate and was only accepted when both replicates yielded matching results.

Guinea Pig RBC Hemagglutination Inhibition Assay

The CDC HI protocol for human influenza was followed. The same serum samples were tested against three human influenza strains: A/New Caledonia/20/99 (H1N1), A/Panama/2007/99 (H3N2), and A/Nanchang/933/95 (H3N2) using a 0.5% guinea pig blood solution. Results from this assay were compared with results from the horse RBC HI and MN assays to check for potential cross-reactivity between antibodies against human and avian influenza viruses.

Microneutralization Assay

The assay was modified from a previously described procedure (Rowe et al., 1999) and described elsewhere (Gill et al., 2006).

Statistical Analyses

Assays were considered to agree when they differed by no more than one dilution. The proportion of agreement was calculated by dividing the number of sera with agreeing results by the total number of sera tested. Exact or asymptotic 95% binomial confidence intervals were calculated around the proportion of agreement. A test for correlation between antibodies against human and avian influenza was performed using the Wilcoxon rank sum test, adjusting for multiple discrete comparisons with the modified Bonferroni method (Tarone, 1990). Analyses were performed with SAS 9.1 (SAS Institute, Cary, NC).

Results

The distributions of antibody titers obtained by the horse RBC HI, guinea pig RBC HI, and MN assays are shown in Table 2. With the exception of the avian H7 virus, horse RBC and MN agreement was high ranging between 81.3% (H3) and 100% (H12) (Table 3). In general, horse RBC HI test reliability when repeated on two different days was very good, ranging between 90.7% (H5) and 100% (H3, H9, and H12). We estimated our materials and supplies cost of conducting the horse RBC HI assay to be \$0.80/serum/virus, half of that required for running a MN assay. The horse RBC HI assay person-time cost is one third that for the MN assay. Furthermore, horse RBC HI results can be obtained 2 hours after hemadsorption while MN results take at least 30 hours after heat-inactivating the sera. The dilution factors needed to dilute the viruses to 4 HAU/25 µl for use in the horse RBC HI assays were lower than those needed to dilute the viruses to 100 TCID₅₀/50 µl for use in the MN assays, thus a larger volume of virus is needed to conduct the horse RBC HI assay (Table 4).

There was a statistically significant association between antibody titers against the human influenza H1N1 virus detected by traditional guinea pig RBC HI assays and antibody titers detected by horse RBC HI against avian influenza viruses H6 and H7 (Table 5). Similar significant associations were noted between antibody titers against human H1N1 and antibodies against avian H3, H6, H9, and H12 detected by MN. No significant associations were noted between antibodies against human H3N2 viruses and avian types.

Discussion

Previous studies have demonstrated the utility of screening human sera with horse RBC HI assays for antibodies against avian H5, H7, and H11 viruses (Gill et al., 2006; Meijer et al., 2006; Treanor et al., 2006; Puzelli et al., 2005). Our data support these findings and further demonstrate the procedure's usefulness in detecting antibodies to other avian influenza subtypes. In our hands, the horse RBC HI assay agreed well with MN testing for all viruses tested except H7. Regarding this disparity, MN showed little variability with a maximum antibody titer of 1:10 while the horse RBC HI assay detected a maximum titer of 1:160 in the first run and 1:80 on the repeat assay. We speculate that this increased horse RBC HI reactivity may be due to cross-reactivity between antibodies against human influenza viruses and this avian H7 strain (Table 5). A report by a World Health Organization working group raised a caution against using the horse RBC HI when detecting human infections with H7 influenza virus subtype (WHO, 2006). However, it was encouraging to note that all sera with elevated H7 antibody titers by MN assay also had elevated horse RBC HI titers ($\geq 1:10$). We were not able to quantify the sensitivity and specificity of our horse RBC HI because we did not have access to human sera confirmed positive for avian influenza virus infection through culture and/or real-time RT-PCR. Meijer et al. reported 85% sensitivity and 100% specificity for a horse RBC HI assay used in detecting human antibodies against avian H7N7 in truly infected individuals (Meijer et al., 2006). However, these figures were calculated using lower cut-off levels for positivity ($\geq 1:10$) and used only 2 HAU. Hence, the true sensitivity and specificity of the horse RBC HI assay remains to be established.

Significant associations between antibodies against human and avian influenza viruses are suggestive of antibody cross-reactivity. However, this cross-reactivity differs by the type of assay used possibly due to a difference in the sensitivity of each assay. Hence, it is important to adjust for the potential confounding effects that exposure to human influenza viruses or vaccines may influence when testing for antibodies against avian viruses regardless.

We developed our horse RBC HI protocol after experimenting with different shape plates, incubation periods and temperatures, blood anticoagulants, hemagglutination units, and sera hemadsorption. More consistent results were obtained by using V-bottom rather than U-bottom shaped plates, adding 4 HAU/25 μ l, incubating for 1 hour rather than 45 minutes, incubating at room temperature rather than 4°C, obtaining horse blood in ACD rather than in Alsever's solution, and hemadsorbing the sera.

Stephenson et al. reported that horse RBCs require more viral particles to agglutinate than do turkey RBCs (Stephenson et al., 2003). Similarly, our horse RBC HI assays required more virus than our MN assays. Thus, it is important to use horse RBCs rather than other types of RBCs when conducting HA titration on avian influenza viruses to be used for horse RBC HI assays.

Compared to MN, horse RBC HI assays are less expensive, faster, and less labor intensive. Our results suggest that the horse RBC HI assay is a useful screening tool for the detection of human antibodies against avian influenza viruses. This assay is particularly useful as a screening tool in large epidemiologic studies. However, prior to adapting the horse RBC HI

assay, laboratories should carefully evaluate the assay's performance for each avian subtype under study.

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Table 1**Influenza viruses and antisera**

Avian influenza virus subtype	Antigen used to produce antisera (host)
A/Duck/Ukraine/1/63 (H3N2)	A/Duck/Shantou/1283/01 (C)
A/Guinea Fowl/New Jersey/14190-23/96 (H4N8)	A/Duck/Shantou/461/00 (C)
A/Chucker/Minnesota/14591-6/95 (H5N2)	A/Goose/Hong Kong/437/99 (C) A/Tern/South Africa/61 (G)
A/Turkey/Massachusetts/65 (H6N2)	A/Turkey/Massachusetts/65 (G)
A/Turkey/Virginia/4529/02 (H7N2)	A/FPV/Rostock/34 (G) A/Turkey/Utah/24721-10/95 (C)
A/Turkey/Minnesota/38391-6/95 (H9N2)	A/Turkey/Minnesota/38391-6/95 (C) A/Gull/Maryland/4435/80 (C)
A/Duck/Memphis/546/76 (H11N9)	A/Duck/Hong Kong/M603/98 (C)
A/Duck/Alberta/60/76 (H12N5)	A/Duck/Alberta/60/76 (R)

C= chicken, G= goat, R= rabbit

Table 2

Distribution of antibodies titers against avian influenza viruses

Subtype	Microneutralization n (%)	HI*	
		Day 1 n (%)	Day 2 n (%)
Human H1N1			
<1:40	---	54 (81.8)	---
1:40	---	4 (6.1)	---
1:80	---	2 (3)	---
1:160	---	5 (7.6)	---
1:320	---	1 (1.5)	---
Human H3N2 Panama			
<1:40	---	12 (19.7)	---
1:40	---	16 (26.2)	---
1:80	---	14 (23)	---
1:160	---	15 (24.6)	---
1:320	---	2 (3.3)	---
1:640	---	2 (3.3)	---
Human H3N2 Nanchang			
<1:40	---	9 (12.7)	---
1:40	---	16 (22.5)	---
1:80	---	15 (21.1)	---
1:160	---	20 (28.2)	---
1:320	---	10 (14.1)	---
1:640	---	1 (1.4)	---
Avian H3			
<1:10	39 (52)	69 (92)	68 (90.7)
1:10	17 (22.7)	4 (5.3)	2 (2.7)
1:20	15 (20)	2 (2.7)	3 (4)
1:40	3 (4)	---	1 (1.3)
1:80	1 (1.3)	---	1 (1.3)
Avian H4			
<1:10	54 (72)	73 (97.3)	---
1:10	20 (26.7)	2 (2.7)	---
1:20	1 (1.3)	---	---
Avian H5			
<1:10	43 (57.3)	41 (54.7)	53 (70.7)
1:10	24 (32)	6 (8)	3 (4)
1:20	7 (9.3)	14 (18.7)	11 (14.7)
1:40	1 (1.3)	14 (18.7)	8 (10.7)
Avian H6			
<1:10	38 (50.7)	24 (39.3)	33 (44)
1:10	26 (34.7)	6 (9.8)	12 (16)
1:20	8 (10.7)	23 (37.7)	25 (33.3)
1:40	3 (4)	8 (13.1)	5 (6.7)
Avian H7			
<1:10	55 (73.3)	38 (50.7)	38 (50.7)
1:10	20 (26.7)	1 (1.3)	5 (6.7)
1:20	---	6 (8)	23 (30.7)
1:40	---	16 (21.3)	9 (12)
1:80	---	13 (17.3)	---
1:160	---	1 (1.3)	---
Avian H9			
<1:10	41 (54.7)	70 (93.3)	70 (93.3)
1:10	19 (25.3)	4 (5.3)	4 (5.3)
1:20	13 (17.3)	1 (1.3)	1 (1.3)
1:40	2 (2.7)	---	---
Avian H11			
<1:10	49 (65.3)	69 (92)	74 (98.7)
1:10	23 (30.7)	5 (6.7)	1 (1.3)
1:20	2 (2.7)	1 (1.3)	---
1:40	1 (1.3)	---	---
Avian H12			
<1:10	56 (74.7)	75 (100)	75 (100)
1:10	19 (25.3)	---	---

Due to missing values, the sum of the frequencies will not be the same for all the strains.

* Horse RBC HI for avian influenza subtypes and guinea pig RBC for human influenza subtypes.

Table 3
Percent agreement between horse RBC hemagglutination inhibition (HI) assay and microneutralization assay results, with horse RBC HI assay reliability. Horse RBC HI assays were run twice (2 different days)

Avian influenza subtype	Horse RBC HI vs. Microneutralization Assay				Horse HI Day 1 vs. Day 2			
	Day 1		Day 2					
	n	% agreement (95% CI)	n	% agreement (95% CI)	n	% agreement (95% CI)	n	% agreement (95% CI)
H3	75	81.3 (72.5-90.2)	75	84 (75.7-92.3)	75	100 (95.2-100)	75	100 (95.2-100)
H4	75	98.7 (96.1-100)	75	94.7 (89.6-99.8)	75	90.7 (84.1-97.3)	75	95.1 (89.7-100)
H5	75	88 (80.7-95.4)	75	92 (85.9-98.1)	61	97.3 (93.7-100)	75	97.3 (93.7-100)
H6	61	90.2 (82.7-97.6)	75	52 (40.7-63.3)	75	100 (95.2-100)	75	98.7 (96.1-100)
H7	75	53.3 (42-64.6)	75	85.3 (77.3-93.3)	75	98.7 (96.1-100)	75	100 (95.2-100)
H9	75	81.3 (72.5-90.2)	75	96 (91.6-100)	75	98.7 (96.1-100)	75	100 (95.2-100)
H11	75	94.7 (89.6-99.8)	75	100 (95.2-100)	75	100 (95.2-100)	75	100 (95.2-100)
H12	75	100 (95.2-100)	75	100 (95.2-100)	75	100 (95.2-100)	75	100 (95.2-100)

Horse RBC HI assay for antibodies against H4 strain was not conducted on Day 2

Due to missing values, the sum of the frequencies will not be the same for all the strains.

Table 4

Virus dilutions required by horse RBC hemagglutination inhibition (HI) and microneutralization (MN) testing

Avian influenza subtype	MN dilution [*]	Horse RBC HI dilution [†]
H3	1:300	1:4
H4	1:50	1:4.5
H5	1:20	1:6
H6	1:60	1:6
H7	1:1000	1:40
H9	1:108	1:8
H11	1:140	1:5
H12	1:140	1:7

* Dilution necessary to yield the virus' TCID₅₀ as determined by the Reed and Muench method (Reed et al., 1938)

[†] Determined by establishing the virus titer by the hemagglutination assay and adjusted to yield 4 HAU/25 µl

Table 5

P-values of the Wilcoxon rank sum test for correlation between antibodies titers against human influenza antibodies and antibodies titers against avian influenza, using horse erythrocyte hemagglutination inhibition (HI) and microneutralization (MN) assays

Avian influenza subtype	A/New Caledonia/20/99 (H1N1)						Human Influenza A/Panama/2007/99 (H3N2)						A/Panama/2007/99 (H3N2)					
	Horse HI			MN			Horse HI			MN			Horse HI			MN		
	Day 1		Day 2	Day 1		Day 2	Day 1		Day 2	Day 1		Day 2	Day 1		Day 2			
	n	p-value [*]	n	p-value [*]	n	p-value [*]	n	p-value [*]	n	p-value [*]	n	p-value [*]	n	p-value [*]	n	p-value [*]		
H3	66	0.03	66	0.05	66	0.003 [†]	61	0.19	61	0.44	61	0.59	71	0.26	71	0.62	71	0.03
H4	66	0.19	na [‡]	na [‡]	66	0.51	61	0.82	na [‡]	na [‡]	61	0.1	71	0.23	na [‡]	na [‡]	71	0.01
H5	66	0.007	66	0.03	66	0.04	61	0.04	61	0.05	61	0.03	71	0.01	71	0.03	71	0.0063
H6	54	0.002 [†]	66	0.003 [†]	66	0.003 [†]	50	0.55	61	0.79	61	0.04	59	0.34	71	0.18	71	0.01
H7	66	0.004 [†]	66	0.002 [†]	66	0.02	61	0.17	61	0.11	61	0.09	71	0.01	71	0.03	71	0.04
H9	66	0.34	66	0.28	66	0.003 [†]	61	0.71	61	0.01	61	0.09	71	0.59	71	0.02	71	0.02
H11	66	0.43	66	0.64	66	0.04	61	0.07	61	na [§]	61	0.16	71	0.02	71	0.35	71	0.02
H12	66	na	66	na	66	0.002 [†]	61	na	61	na	61	0.73	71	na	71	na	71	0.2

Antibody titers against human influenza viruses were determined using a guinea pig HI assay. Serologic assay procedures against avian influenza viruses were conducted using a horse RBC HI assay (repeated on 2 different days) and a MN assay.

Due to missing values, the sum of the frequencies will not be the same for all the strains.

* P-values of the Wilcoxon rank sum test. Horse HI first run = horse HI assay results at day one, when tested for the first time, Horse HI day2 = horse HI assay results at day two, when tested was repeated.

† Significant with Wilcoxon rank sum at a modified Bonferroni adjusted alpha of 0.00625 (Tarone, 1990).

‡ na = not available. Horse HI for antibodies against avian H4 influenza was not repeated due to sera depletion.

§ na = not available. Due to missing values, all remaining avian H11 influenza results available were <1:10.

|| na = not available. All avian H12 influenza results available were <1:10.