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Detection of Antibody to Avian Influenza A (H5N1) Virus in Human Serum by Using a Combination of Serologic Assays

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From May to December 1997, 18 cases of mild to severe respiratory illness caused by avian influenza A (H5N1) viruses were identified in Hong Kong. The emergence of an avian virus in the human population prompted an epidemiological investigation to determine the extent of human-to-human transmission of the virus and risk factors associated with infection. The hemagglutination inhibition (HI) assay, the standard method for serologic detection of influenza virus infection in humans, has been shown to be less sensitive for the detection of antibodies induced by avian influenza viruses. Therefore, we developed a more sensitive microneutralization assay to detect antibodies to avian influenza in humans. Direct comparison of an HI assay and the microneutralization assay demonstrated that the latter was substantially more sensitive in detecting human antibodies to H5N1 virus in infected individuals. An H5-specific indirect enzyme-linked immunosorbent assay (ELISA) was also established to test children's sera. The sensitivity and specificity of the microneutralization assay were compared with those of an H5-specific indirect ELISA. When combined with a confirmatory H5-specific Western blot test, the specificities of both assays were improved. Maximum sensitivity (80%) and specificity (96%) for the detection of anti-H5 antibody in adults aged 18 to 59 years were achieved by using the microneutralization assay combined with Western blotting. Maximum sensitivity (100%) and specificity (100%) in detecting anti-H5 antibody in sera obtained from children less than 15 years of age were achieved by using ELISA combined with Western blotting. This new test algorithm is being used for the seroepidemiologic investigations of the avian H5N1 influenza outbreak.

In May 1997, an avian influenza A H5N1 virus infection resulted in the death of a 3-year-old child in Hong Kong. The child died from complications of influenza-associated pneumonia, including acute respiratory distress syndrome, Reye's syndrome, and multiorgan failure. Although serologic evidence for infection of humans with H5N1 influenza virus had previously been reported (26), this incident resulted in the first isolation of an avian virus from a human with severe respiratory disease. In November and December 1997, 17 additional cases, 5 of them fatal, were associated with avian H5N1 influenza virus infections (6, 7, 27).

The emergence of avian H5N1 virus in humans prompted a series of seroepidemiological studies to determine the mode of transmission of the virus and the risk factors associated with infection. However, a sensitive and specific serologic assay for the detection of human antibodies to avian viruses was not available. Detection of antibodies to avian influenza viruses in mammalian species, including humans, using hemagglutination inhibition (HI) assays has generally failed even in cases where experimental infection was confirmed by virus isolation (1, 12, 21). Lu et al. (17) showed that HI testing with subunit hemagglutinin (HA), but not intact virus, could detect antibodies to an avian H2N2 virus. However, neutralizing antibodies were readily detected with whole infectious virus. A single radial hemolysis test has been used to detect human antibody to avian viruses (26), but this assay may detect antibody to internal antigens in addition to those antibodies directed against surface glycoproteins and, as a result, may lack specificity for the detection of antibodies to HA. An HA-specific enzyme-linked immunosorbent assay (ELISA) requires highly purified antigen, which was not available early in the investigation, and in some cases, the ELISA may detect cross-reactivity among HAs of different subtypes (4, 23).

Because of the limitations of these assays, we first explored the usefulness of the virus neutralization assay, which required only a stock of infectious virus as the antigen and could be streamlined to process 100 to 150 serum samples per assay. The neutralization assay, like the HI assay, has the advantage of identifying functional, strain-specific antibodies in human serum. When purified recombinant H5 (rH5) HA became available, an H5-specific ELISA and Western blot assay were developed. We report here the relative sensitivities and specificities of the microneutralization assay and Western blotting or ELISA and Western blotting combinations for the detection of antibody to avian influenza A (H5N1) virus in humans.

MATERIALS AND METHODS

Cells and viruses. All microneutralization assays were performed with Madin-Darby canine kidney (MDCK) cells. The particular sublineage used was originally derived by David Tyrrell (The Common Cold Laboratory, Porton Down, Salisbury, United Kingdom) and was obtained from John Wood (National Institute for Biological Standards and Control, Potters Bar, United Kingdom). The cells were used for a maximum of 25 passages and maintained in Dulbecco's modified Eagle's medium (Gibco/BRL, Gaithersburg, Md.) containing 6% fetal bovine serum (Hyclone Laboratories Inc., Logan, Utah), 2 mM L-glutamine, and the antibiotics penicillin and streptomycin (Gibco/BRL). The cultures were incubated at 37°C in a 5% CO_2 humidified atmosphere. The influenza viruses used in this study were as follows: the H5N1 viruses A/Hong Kong/156/97 (HK/156), A/Hong Kong/483/97 (HK/483), A/Hong Kong/485/97 (HK/485), A/Hong Kong/486/97 (HK/486), and A/Hong Kong/488/97 (HK/488); the H5N3 virus A/Duck/Singapore-Q/F119-3/97 (Dk/Sing; provided by Alan Hay, World Health Organization, Mill Hill, London, United Kingdom); the H5N9 virus A/Turkey/

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Wisconsin/68 (Tk/Wisc; provided by Michael Perdue, Southeastern Regional Poultry Laboratory, U.S. Department of Agriculture, Athens, Ga.); and the H3N2 virus A/South Africa/1147/95 (A/SA). Virus stocks used as challenge antigens were propagated in the allantoic cavities of 10-day-old embryonated hen's eggs. The allantoic fluid was harvested either 17 to 20 (H5N1) or 48 (H5N9, H5N3, and H3N2) h postinoculation and clarified by centrifugation (500 × *g* for 20 min). Virus concentrations were determined by HA titration as previously described (16). The virus stocks were aliquoted and stored at −70°C until used. The 50% tissue culture infectious dose ($TCID_{50}$) of each virus was determined by titration in MDCK cells. Briefly, 1/2-log dilutions of virus were carried out in 100 μl of Dulbecco's modified Eagle's medium containing 1% bovine serum albumin and antibiotics (V diluent) in high-binding 96-well styrene immunoassay plates (Dynex Technologies, Inc., Chantilly, Va.). Freshly trypsinized MDCK cells were adjusted to 1.5×10^5/ml in V diluent, and 100 μl was added to each well. The plates were covered and incubated for 18 h at 37°C and 5% CO_2. The monolayers were washed with phosphate-buffered saline (PBS) and fixed in cold 80% acetone in PBS for 10 min. The presence of viral nucleoprotein (NP) was detected by ELISA as described below. Wells having an absorbance reading greater than 3 standard deviations above the mean absorbance of wells containing only MDCK cells were scored positive for virus growth. The $TCID_{50}$ of each stock virus was calculated by the method of Reed and Muench (22). Virus titrations of H5 viruses were performed with and without the addition of exogenous L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (TPCK-trypsin; Sigma Immunochemical Co., St. Louis, Mo.). Trypsin was not required for infection of MDCK cells with H5N1 viruses, which is characteristic of avian pathogenic viruses (15, 29). Because overnight infection of MDCK cells with a human H3N2 virus (A/SA) was only marginally improved by the addition of TPCK-trypsin, trypsin was not used in the neutralization assay for any of the viruses.

Laboratory facilities. Because of the potential risk to humans and poultry, all experiments with live pathogenic avian H5 viruses were conducted using appropriate biosafety level 3-plus (BSL3+) containment procedures (24). To further minimize the risk for human exposure, all investigators were required to wear appropriate HEPA-filtered masks or respirators (RACAL Health and Safety, Inc., Frederick, Md.). U.S. Department of Agriculture permits were obtained before working with avian influenza viruses. Work with Dk/Sing, which is nonpathogenic for chickens, was conducted under BSL2 laboratory conditions.

Serum samples. Human serum samples were obtained in November and December 1997 from 16 individuals confirmed or suspected to be infected with H5N1 virus. An attempt was made to collect serum samples from suspect individuals as close to diagnosis as possible. Sera collected ≤7 days post-symptom onset were referred to as S1 samples. Follow-up serum samples were collected when possible. For patients with milder disease it was possible to collect S2 samples 14 or more days after symptom onset. For other patients, the collection of an S2 serum was compromised due to a severe or fatal clinical outcome, and sera were collected ≤14 days after symptom onset. Single serum samples collected 11 or more days after symptom onset were referred to as S2 samples, since it was considered likely that they contained H5-specific antibody. Paired (S1 and S2) samples were obtained from eight individuals. S2 samples only were obtained from six individuals. S1 samples only were obtained from two individuals and were not included in this study. Patients designated "adult" were aged 19 to 59 years (median age, 29.5 years), and patients designated "children" were ≤14 years of age (median age, 3 years). Sera from five adults and eight children were tested by all three serologic assays. The volumes of sera from the remaining patients were insufficient for complete testing. Control sera were obtained from non-H5N1 virus-exposed adult Hong Kong Red Cross blood donors (21 to 55 years of age) and children enrolled in a hepatitis B virus vaccine study (5 to 11 years of age) in Hong Kong. Additional control sera were obtained from non-H5N1 virus-exposed U.S. children (3 years of age) and adults (18 to 59 years of age). A total of 24 control sera from children and 85 control sera from adults were tested by all three serologic assays. A larger number of adult control sera were tested because of the reduced specificity observed in some assays. Ferret antisera directed against H5N1 virus strains were obtained by intranasally inoculating ferrets with 0.5 ml of HK/156 virus allantoic fluid. Two weeks later, the ferrets were given an intranasal booster dose of 0.5 ml of tk/Wisc. The ferrets were euthanized and exsanguinated 2 weeks after being boosted. The sera were separated and treated with receptor-destroying enzyme (RDE) (Denka Seiken Co. Ltd., Tokyo, Japan) according to a previously described procedure (16). Goat antiserum to H5N2 A/Tern/South Africa/61 virus (GαTSA) was obtained from the National Institutes of Health reagent repository (Bethesda, Md.) and was also RDE treated.

Microneutralization assay. The microneutralization assay was modified from a previously described procedure (11). Human sera were heat inactivated for 30 min at 56°C, and twofold serial dilutions were performed in a 50-μl volume of V diluent in immunoassay plates. The diluted sera were mixed with an equal volume of V diluent containing influenza virus at 2×10^3 $TCID_{50}$/ml. Four control wells of virus plus V diluent (VC) or V diluent alone (CC) were included on each plate. After a 2-h incubation at 37°C in a 5% CO_2 humidified atmosphere, 100 μl of MDCK cells at 1.5×10^5/ml was added to each well. The plates were incubated for 18 h at 37°C and 5% CO_2. The monolayers were washed with PBS and fixed in cold 80% acetone for 10 min. The presence of viral protein was detected by ELISA with a monoclonal antibody (A-3) to the influenza A NP (28).

The ELISA was performed at room temperature. The fixed plates were washed three times with PBS containing 0.05% Tween 20 (wash buffer). The anti-NP antibody diluted 1/4,000 in PBS containing 1% bovine serum albumin and 0.1% Tween 20 (E diluent) was added to each well. The plates were incubated at room temperature for 1 h. The plates were washed four times in wash buffer, and 100 μl of horseradish peroxidase-labeled goat anti-mouse immunoglobulin G (IgG) (Kirkegaard & Perry, Gaithersburg, Md.) diluted 1/2,000 in E diluent was added to each well. The plates were incubated for 1 h at room temperature and then washed six times with wash buffer. One hundred microliters of freshly prepared substrate (10 mg of *o*-phenylenediamine dihydrochloride per 20 ml of 0.05 M phosphate citrate buffer, pH 5.0, containing 0.03% sodium perborate) was added to each well, and the plates were incubated at room temperature for approximately 5 min. The reaction was stopped with an equal volume of 1 N sulfuric acid. The absorbance was measured at 490 nm (A_{490}) with an MRX automated plate spectrophotometer and analyzed with Revelation software (Dynex Technologies). The average A_{490} was determined for quadruplicate wells of virus-infected (VC) and -uninfected (CC) control wells, and a neutralizing endpoint was determined by using a 50% specific signal calculation. The endpoint titer was expressed as the reciprocal of the highest dilution of serum with A_{490} value less than X, where X = [(average A_{490} of VC wells) − (average A_{490} of CC wells)]/2 + (average A_{490} of CC wells). Sera which tested negative at a dilution of 1/20 were assigned a titer of 10. Sera were considered positive for antibody to H5 viruses if titers of ≥80 were obtained in at least two independent assays. Sera that gave equivocal results in two assays were retested in a third or fourth assay.

H5N1 Western blotting. The sera were analyzed by Western immunoblotting with a purified baculovirus-expressed recombinant HA (rHA) protein (rH5) derived from A/Hong Kong/156/97 virus. The initial antigen for testing was provided by Bethanie Wilkinson (Protein Sciences Corporation, Meriden, Conn.). The rH5 was generated in insect cells and purified by a previously described method (20). The rH5 was loaded (10 to 30 μg/cm^2) onto a 10% discontinuous polyacrylamide gel and run overnight at ~40 to 60 V. The gel was transferred to a nylon membrane (Immobilon-P; Millipore Corporation, Bedford, Mass.) with a semidry transfer apparatus (Bio-Rad Laboratories, Hercules, Calif.) at 0.7 to 0.8 A for 45 min. The blot was blocked overnight at 4°C in PBS containing 5% dry nonfat milk, 0.1% Tween 20, and 0.01% (wt/vol) thimerosal (blocking buffer). The blot was transferred to a miniblotting apparatus (Immunetics, Cambridge, Mass.), and a 1/100 dilution of human serum or 1/500 dilution of animal control serum was added to each lane of the miniblotting apparatus (250 μl/lane) and incubated at room temperature for 2 h. Positive human controls were sera from culture-confirmed H5N1-positive individuals, which were also positive by microneutralization assay with a titer of 1/1,280 or greater. Negative adult controls were selected from the sera described in the previous section. Animal control sera were obtained from ferrets infected with HK/156 virus or noninfected ferrets. The wells were washed three times with PBS containing 0.1% Tween 20 (wash buffer) for 5 min per wash. Horseradish peroxidase-conjugated goat anti-human IgG, IgM, or IgG-IgA-IgM (Kirkegaard & Perry Laboratories, Inc.) was added to each well at a dilution of 1/2,000 in blocking buffer. The blot was incubated for an additional hour at room temperature, washed three times in wash buffer and once in PBS, and developed with LumiGLO chemiluminescent substrate (Kirkegaard & Perry Laboratories, Inc.).

ELISA with HA. rHA for A/Hong Kong/156/97 (H5N1) virus (Protein Sciences) was adjusted to a concentration of 1 μg per ml in PBS, and 100 μl was added to each well of 96-well immunoassay plates (Dynex Technologies, Inc.). The antigen was incubated overnight at 4°C. The serum samples were diluted 1/25 in PBS containing 0.5% (wt/vol) gelatin, 0.15% Tween 20, and 4% goat serum (ELISA diluent) and incubated for approximately 1 h at 37°C. Antigen-coated immunoassay plates were washed three times with wash buffer, and 100 μl of ELISA diluent was added to each well. Next, 33 μl of the diluted serum was added to the first row of the immunoassay plates (1/100 final dilution) and the serum was diluted fourfold from 1/100 to 1/409,600. Following a 1.5-h incubation at 37°C, the plates were washed four times in wash buffer. Then, 100 μl of horseradish peroxidase-labeled goat anti-human IgG or IgM (Kirkegaard & Perry), diluted 1/4,000 or 1/1,000, respectively, in ELISA diluent, was added to each well. The plates were incubated for an additional hour at room temperature and washed six times with wash buffer. One hundred microliters of freshly prepared substrate (10 mg of *o*-phenylenediamine dihydrochloride per 20 ml of 0.05 M phosphate citrate buffer, pH 5.0, containing 0.03% sodium perborate) was added to each well and stopped with an equal volume of 1 N sulfuric acid after color development. The absorbance was measured at 490 nm with an MRX automated plate spectrophotometer and analyzed with Revelation software. Age group-matched control human sera (three to six) were tested in each ELISA to establish endpoint cutoffs. The ELISA titer for test sera was calculated as the reciprocal of the highest dilution of test sera that gave an A_{490} value greater than the mean A_{490} plus 3 standard deviations of three to six negative controls at an equivalent dilution of sera. A titer of ≥1,600 was considered positive for the ELISA.

HI assay. The sera were treated with RDE by diluting one part serum with three parts enzyme and were incubated overnight in a 37°C water bath. The enzyme was inactivated by a 30-min incubation at 56°C followed by addition of six parts 0.85% physiological saline for a final dilution of 1/10. HI assays were

TABLE 1. HI and neutralization assay responses of H5N1 virus-infected individuals

Serum	Sample	Response[a] (antibody titer)					
		HK/156 (H5N1)		A/SA (H3N2)		Dk/Sing (H5N3)	
		HI	Neutralization	HI	Neutralization	HI	Neutralization
Human[b]							
A	S1	<20	20	<20	80	<20	20
	S2	<20	160	<20	80	<20	160
B	S1	<20	10	160	640	<20	10
	S2	<20	80	80	1,280	<20	40
C	S2	80	1,280	40	2,560	80	1,280
D	S2	<20	320	<20	80	<20	160
E	S2	80	1,280	80	1,280	80	640
H3 control-1	S1	<20	10	40	160	<20	10
	S2	<20	10	160	2,560	<20	10
H3 control-2	S1	ND	ND	<20	40	ND	ND
	S2	ND	ND	80	640	ND	ND
Animal[c]							
FαHK/156		**160**	1,600	<10	100	640	3,200
FαA/SA		<10	100	**640**	1,600	<10	100
FαDk/Sing		80	400	<10	100	**160**	800
N. Ferret		<10	100	<10	100	<10	100
GαTSA		1,280	25,600	<10	100	2,560	25,600
N. Goat		<10	100	<10	100	<10	100

[a] ND, not determined. Boldface indicates titer to homologous virus.
[b] Serum samples from H5N1 or H3N2 virus-infected patients.
[c] RDE-treated reference ferret (F) antisera generated against antigens A/Hong Kong/156/97 (H5N1), A/duck/Singapore-Q/F119-3/97 (H5N3), and A/South Africa/1147/95 (H3N2) and goat (G) antiserum to A/Tern/South Africa/61 (H5N2). Normal ferret and normal goat sera are represented by N. Ferret and N. Goat, respectively.

performed in V-bottom 96-well microtiter plates (Corning Costar Co., Cambridge, Mass.) with 0.5% turkey erythrocytes, as previously described (16).

RESULTS

Comparison of HI and virus microneutralization assays to detect antibody to H5N1 virus in human sera. Although the HI assay is considered the "gold standard" for serologic diagnosis of infection with human influenza viruses, the assay has been reported to be less sensitive for detecting antibody responses to avian viruses in mammalian sera (12, 21). During the serologic analysis associated with the investigation of the first case of H5N1 virus in a 3-year-old boy (5, 27), we performed a preliminary comparison of the HI and microneutralization assays for the detection of antibody to H5N1 virus. Anti-H5 antibody was detected by the microneutralization assay but not by the HI assay in individuals with a history of exposure to poultry and in the first patient (data not shown). This result suggested that the microneutralization assay might be more sensitive than the HI assay, and we initiated further refinement of the microneutralization assay. However, the initial comparison lacked a positive control serum from a culture-confirmed H5N1 virus-infected individual, since no detectable neutralizing antibody was found in serum collected from the index case 10 days after symptom onset. Once serum from additional patients with culture-confirmed H5N1 virus infections became available, it was possible to again compare the microneutralization and HI assays for the ability to detect anti-H5 antibody. Table 1 shows a comparison of sera from five patients with culture-confirmed H5N1 virus infections. Comparison of all 14 convalescent sera was not possible due to insufficient quantities of serum.

Paired S1 and S2 sera from two individuals (A and B) failed to show any rise in titer by the HI assay, whereas the microneutralization assay with HK/156 virus demonstrated at least an eightfold rise between S1 and S2 serum samples. In contrast, significant rises between S1 and S2 sera from two influenza A H3N2 virus-infected individuals (H3 controls) were detected by both assays. Similarly, differences in titers between nonimmune and H5- or H3-specific animal sera were detected by both assays, although a greater fold difference between titers obtained in the two assays was observed for the H5 response. The HI assay detected H5-specific antibody (titer, 80) in single S2 serum samples from two of three patients with culture-confirmed H5N1 virus infections with high neutralizing-antibody titers (1,280). The remaining serum from a patient with a culture-confirmed H5N1 virus infection (D) had a neutralizing-antibody titer of 320 but no detectable HI titer. Based on these results, the HI assay appeared less sensitive than the microneutralization assay for detecting seroconversion to H5N1 virus, perhaps due to an inability to detect the lower levels of antibody that may be present in S2 sera from H5N1 virus-infected individuals. A comparison of the H5 viruses used in the microneutralization assay indicated that titers detected by the nonpathogenic Dk/Sing (H5N3) virus were within twofold of those obtained with HK/156 virus.

As stated previously, other investigators have shown that the HI assay sensitivity for avian influenza viruses can be improved through the use of subunit HA rather than intact virus. We also compared ether-disrupted H5 virus or purified baculovirus-expressed rH5 HA to intact virus for detecting antibody by the HI assay. HI titers obtained with rHA or ether-disrupted virus were similar to those obtained with intact virus (data not shown). Therefore, the use of disrupted or isolated HA failed to improve the sensitivity of the HI assay for detecting anti-H5 antibody in human sera.

Comparison of the microneutralization assay with an H5-specific indirect ELISA. The sensitivity of the microneutralization assay with HK/156 virus was also compared to those of a

TABLE 2. Sensitivities and specificities of serologic assays for detection of antibodies to H5N1 virus

Age group[a]	Parameter[b]	Values (%)				
		Individual serologic tests[c]			Combination of tests[d]	
		N	E	W	N-W	E-W
Child						
	Sensitivity (n = 8)	88	100	100	88	100
	Specificity (n = 24)	100	92	83	100	100
Adult						
	Sensitivity (n = 85)	80	80	80	80	80
	Specificity (n = 85)	93	62[e,f]	85	96	84[e]

[a] Serum samples from individuals from 1 to 14 years of age (Child) or from individuals 18 to 59 years of age (Adult).
[b] Sensitivity, number of H5N1 virus-infected patients testing positive for antibody divided by the total number of patients with confirmed H5N1 infections tested. Specificity, number of control age-matched sera tested minus the number of control sera testing positive for antibody divided by total number of control sera.
[c] Tests for determination of H5N1 virus positivity (N, microneutralization test; W, Western blotting; E, ELISA).
[d] Combination of tests. Microneutralization test with A/Hong Kong/156/97 virus followed by Western blot confirmation with rHA of A/Hong Kong/156/97 virus (N-W) and ELISA with rHA of A/Hong Kong/156/97 virus followed by Western blot confirmation with rHA of A/Hong Kong/156/97 virus (E-W).
[e] Number of samples, 50.
[f] Statistical analysis for positive association between test result and known status of samples was not significant (Fisher exact test; P = 0.067). All other assays were significant (P = 0.003).

TABLE 3. Evaluation of three serologic assays for the detection of H5-specific antibody in confirmed and suspected H5N1 virus-infected individuals and nonexposed controls

Age group	Serum	Sample	H5-specific antibody response			
			N[a]	ELISA[b]		WB[a]
				IgG	IgM	
Children						
	B	S1	10	5.6	5.6	−
		S2	80	16.6	5.6	+[d]
	F	S2	14	14.6	16.6	+[d]
	G	S1	10	5.6	8.6	−
		S2	320	14.6	14.6	+[d]
	H	S1	10	5.6	5.6	NT[e]
		S2	450	18.6	16.6	+
	Control (n = 24)		11	6.0	5.6	
Adults						
	A	S1	20	18.6	5.6	+
		S2	160	18.6	5.6	+
	D	S1	10	6.6	6.6	−
		S2	320	18.6	18.6	+[d]
	I	S1	10	5.6	5.6	−
		S2	1,280	18.6	18.6	+
	J	S2	10	5.6	5.6	−
	Control (n = 46)		20	11.0	6.0	

[a] N, microneutralization assay; GMT to A/Hong Kong/156/97 virus.
[b] GMT ($\log_2$).
[c] W, Western blotting. +, positive; −, negative.
[d] Serum also positive with IgM.
[e] NT, not tested.

standard indirect ELISA, which used H5 rHA as the coating antigen, and a Western blot test, which used the same purified H5 HA. Sera from individuals not exposed to influenza A H5N1 virus were tested with both ELISA and the microneutralization assay to establish baseline reactivity. When negative control sera from non-H5N1 virus-exposed individuals from Hong Kong and the United States were tested by ELISA, it became apparent that the specificity of the ELISA for adult sera was low (Table 2). Therefore, the evaluation of the tests for children (aged ≤14 years) and adults (aged 18 to 59) were done separately.

A representative pattern of antibody responses detected by the assays is shown for four children and four adults in Table 3. Patients A, B, D, and G with culture-confirmed H5N1 virus infections for whom paired sera were available, had ≥8-fold rises in neutralizing antibody, with S2 titers of 80 or greater. Although virus was not isolated from patients H and I, initial diagnosis of H5N1 virus infection was made following the demonstration of seroconversion by the HI assay. A 32-fold rise in H5N1-specific neutralizing antibody confirmed that these individuals had been infected with the avian virus. For patients for whom paired sera were available, the H5-specific IgG ELISA also demonstrated a substantial rise in titer. An H5-specific IgM response was detected in four of six paired sera. The single serum sample from patient F taken 11 days after symptom onset had a $\log_2$ neutralizing geometric mean antibody titer (GMT) of only 14, which was similar to the neutralizing GMTs of nonexposed controls. However, by ELISA, this individual had high titers of H5-specific IgG and IgM antibody that were well above the GMTs of controls. In contrast, both the microneutralization assay and ELISA failed to detect a substantial antibody titer in a single convalescent serum sample from patient J with a culture-confirmed infection. This individual had an immunocompromising illness unrelated to the H5N1 virus infection. For all patients, when the microneutralization assay and/or ELISA detected anti-H5 antibody, Western blotting positively confirmed these results.

A baseline response for H5N1 virus from nonexposed controls was established so the assay could be used in several serosurveys. Neutralizing GMTs of 11 and 20 were obtained for 24 child and 46 adult controls, respectively. Using this baseline response and the result that showed that patients with culture-confirmed infections had neutralizing-antibody titers of 80 or greater in S2 sera (14), a titer of 80 or greater in at least two independent assays was considered positive for neutralizing antibody.

In children, ELISA IgG and IgM $\log_2$ GMTs of 6.0 (64) and 5.6 (50), respectively, were obtained. An eightfold increase relative to the baseline IgG response (titer, 512) was established to improve the specificity of the assay. Since sera were titrated serially fourfold, beginning with a 1/100 dilution, a titer of 1,600 was the minimum that was considered to be positive for anti-H5 antibody. A similar positive cut-off was assigned for the H5-specific IgM response. Limitations in the specificity of the ELISA for adults were observed, as evidenced by the high GMT for IgG in adult controls ($\log_2$ 11.0 = 2,048) by the specificity analysis described below.

Sensitivities and specificities of H5-specific serologic tests. To determine whether the microneutralization assay and/or ELISA could be used to detect H5-specific antibody in single serum samples, thereby providing evidence of infection of humans with H5N1 virus, the relative sensitivities and specificities of the assays were compared (Table 2). Since the Western blot test is too labor intensive to be considered a diagnostic test for screening several thousand sera, it was used in this study as a secondary serologic test to confirm either the microneutralization assay or ELISA. Sensitivity was defined as the proportion of assays that correctly identified H5-specific antibody in con-

firmed cases. Specificity was established with sera from non-H5N1 virus-exposed individuals that were not expected to react in either assay.

For children, the microneutralization assay had a sensitivity of 88% compared with 100% for ELISA. The difference was based on the single serum from a patient with a culture-confirmed infection (F) that failed to react to a positive titer in the neutralization assay with HK/156 virus (Table 3). When each assay was evaluated for specificity, the microneutralization assay was more specific than ELISA (100 versus 92%). However, combined with the confirmatory Western blot test, ELISA showed improved specificity and retained improved sensitivity compared with the microneutralization assay and Western blotting combination. Based on this analysis, sera from children were considered positive for antibody to H5N1 virus if they tested positive by ELISA for H5-specific IgG and/or IgM (titer of ≥1,600) and tested positive by Western blotting for antibody of the same class (IgG and/or IgM).

For adults, the microneutralization assay and ELISA had equivalent sensitivities (80%). However, the specificity of the microneutralization assay was notably superior to that of ELISA (93 and 62%, respectively). When combined with Western blotting, each test improved in specificity (96 versus 84%); however, maximum sensitivity and specificity were still achieved by a combination of the microneutralization assay and Western blotting. Based on this analysis, sera from adults were considered positive for antibody to H5N1 virus if they tested positive by microneutralization assay (titers of ≥80 in at least two independent assays) and tested positive for anti-H5 IgG by Western blotting.

All serologic assays showed reduced specificity for sera collected from non-H5N1 virus-exposed adults aged 60 years and over. Thirty-two percent of the microneutralization tests (23 of 73) and 33% of the Western blot IgG tests (24 of 73) were identified as false positives from the sample of known controls. Therefore, the serologic tests were not considered valid for adults aged 60 years and older.

Antigenic cross-reactivity of human H5-specific antibody. The 16 viruses isolated from 18 patients fell into two genetically distinct groups. These groups could also be distinguished antigenically in the HI assay with reference ferret antisera raised to representative viruses from each group (2). To determine whether human postinfection sera also detected antigenic differences between the two groups of viruses, representative group A and B viruses were used to detect neutralizing-antibody responses in human and ferret postinfection sera (Table 4). In general, the neutralizing-antibody response in humans infected with either group A or B viruses was cross-reactive for the heterologous virus group. The neutralizing-antibody titer of serum from patient F was consistently lower than 80 when HK/156 virus was used as the test antigen. However, a positive titer of 80 was achieved with another group A virus (HK/486) and a group B virus (HK/485). Serum from patient K gave a fourfold-lower titer on the group B virus used in the test, although this patient was infected with a group B virus. When tested in the microneutralization assay, reference ferret antisera raised to group A or B viruses also cross-reacted with heterologous H5N1 viruses. These results indicated that, with one exception, the group A virus HK/156 could detect neutralizing antibody in humans infected with either group A or group B viruses. The lower titer against HK/156 virus repeatedly obtained with serum from patient F may, in part, be due to the early sampling time of this serum.

TABLE 4. Cross-reactivity of neutralizing-antibody responses for H5N1 group A and B viruses

Serum	Subgroup of virus isolate	Neutralizing-antibody titer in serum		
		HK/156 (A)	HK/486 (A)	HK/485 (B)
Human[a]				
A	A	160	160	160
E	A	640	1,280	320
L	A	640	1,280	640
C	B	640	2,560	1,280
D	B	1,280	1,280	640
F	B	40	80	80
K	B	320	320	80
Ferret reference				
Anti-HK/156	A	3,200	12,800	3,200
Anti-HK/488	A	6,400	6,400	3,200
Anti-HK/483	B	12,800	12,800	12,800
Anti-HK/485	B	3,200	6,400	3,200

[a] Human S2 serum samples.

DISCUSSION

The microneutralization assay with HK/156 virus was a sensitive and specific assay for detecting antibody to avian influenza A H5N1 virus in adult human sera. In comparison, the traditional HI assay detected only H5-specific antibody in S2 sera with high neutralizing-antibody titers and did not detect the relatively low serum antibody titers that developed in many individuals following primary infection with avian H5N1 virus. Although the HI assay is routinely used for the detection of rises in serum of antibody to human influenza A and B viruses, some studies have demonstrated that microneutralization assays may also be more sensitive either in detecting a higher rate of antibody rises than that detected by the HI assay for these viruses (3, 8, 10) or in detecting antibody in individuals seronegative by HI (11).

The sensitivity of the microneutralization assay in both adults and children was less than 100%. For adults, the reduced sensitivity (80%) was the result of a neutralizing-antibody-negative serum from a patient with a culture-confirmed case (J) with a prior immunocompromising disorder. This serum sample also tested negative for H5-specific IgG and IgM by ELISA. For children, the reduced sensitivity (88%) was based on serum from one patient (F) collected only 11 days after symptom onset that was negative for neutralizing antibody when HK/156 virus was used in the assay. However, a positive titer (80) was obtained for this serum when another H5N1 group A virus (HK/486) or a group B virus (HK/485) was used in subsequent assays. The kinetics of the neutralizing-antibody response to HK/156 virus has been described elsewhere (14) and is similar to the primary response to human influenza A viruses (18). In general, anti-H5 neutralizing-antibody titers of 80 or greater were detected only in serum collected a minimum of 14 days after symptom onset, suggesting that the early time of collection from patient F limited the detection of neutralizing antibodies, at least by HK/156 virus. That ELISA and the Western blot test detected H5-specific IgG and IgM in this sample, suggests that these assays with purified HK/156 virus HA may detect antibody of lower avidity and/or quantity than that required for detection by the microneutralization assay. Although these results suggest that ELISA may have a greater sensitivity than the microneutralization assay for the screening of children's sera, particularly if only one serum sample is available, the estimates of assay sensitiv-

ities are based on a relatively small number of sera available from patients with confirmed H5N1 infections ($n = 8$).

Although ELISA was both sensitive and specific for antibody in children, the assay, in particular the IgG ELISA, lacked specificity for adult sera (62%). Since the baculovirus-expressed HA is highly purified, it is unlikely that the antigen was detecting antibodies in human sera that cross-react with insect proteins. More likely, the apparent nonspecific reactivity of the adult human sera resulted from cross-reactive epitopes common to HAs of different influenza A subtypes that may become exposed with the partial denaturation of antigen bound to a solid surface, such as an ELISA plate. Such cross-reactivities have previously been observed in ELISA with human sera and H8 HA (4). Furthermore, HAs of the H1, H2, H5, and H6 subtypes have been shown to contain a cross-reactive epitope recognized by a mouse monoclonal antibody (9, 19). Surprisingly, all of the serologic assays showed diminished specificity for sera from adults aged 60 years and older. Many studies have reported a high proportion of false positives for serologic assays, primarily ELISA, in this age group. Rheumatoid factor (25) and autoantibodies (13) contribute to some false positives observed in other systems. The reasons for the decreased specificity of the microneutralization assay for this older age group are under investigation.

The integrity of the MDCK cells is most critical for maximizing the appropriate use of the microneutralization assay. We observed that MDCK cells of different sublineages (laboratory origins) supported different levels of replication of influenza viruses. The line used for the microneutralization assay was derived from a sublineage obtained from the Common Cold Laboratory in Salisbury, England. Initial optimization of the assay should include the testing of several sublineages of MDCK cells. A low passage number was also desirable for optimal replication of the test viruses. MDCK cells were routinely used for no more than 25 passages before fresh cells were obtained from liquid nitrogen storage.

The microneutralization assay in our study was performed primarily with A/Hong Kong/156/97, a highly pathogenic avian virus that was isolated from a 3-year-old boy who died as a result of the infection. Consequently, the assay was performed under BSL3+ conditions. So that other laboratories lacking such containment facilities may also use the assay to detect antibody to the pathogenic H5N1 viruses, a surrogate virus was sought that cross-reacted with the H5N1 viruses isolated from humans in Hong Kong. The nonpathogenic A/Duck/Singapore-Q/F119/97 (H5N3) was found to be an appropriate surrogate with reasonable cross-reactivity for detecting antibody to the H5N1 viruses, and it has been used successfully to screen several thousand human serum samples by the microneutralization assay in BSL2 conditions at the Department of Health Government Virus Unit in Hong Kong.

The microneutralization assay has several additional advantages for detecting antibody to novel influenza viruses. First, the test detects functional anti-HA antibody which is highly specific for the subtype in question. Second, since infectious virus is used, the assay can be developed quickly upon recognition of a novel virus and is available before suitable recombinant or purified viral proteins become available for use in other assays. In this situation, the microneutralization assay could also be used to detect H5 antibody in sera from children, provided that paired acute and convalescent serum samples collected at optimal times were available. Finally, the microneutralization assay described here is relatively rapid and can accommodate the testing of over 100 serum samples per assay. Automation would further streamline the process.

Based on the sensitivity and specificity analysis described here, the microneutralization assay is now being used to detect antibodies to H5 virus in sera from several thousand adults evaluated as part of a seroepidemiological investigation of the 1997 H5N1 outbreak in Hong Kong. The investigation is comprised of a number of cohort studies that will provide information about the extent of poultry-to-human and human-to-human transmission of the H5N1 viruses. The microneutralization assay may also be applied to serosurveys to detect evidence of human infection with avian influenza A viruses of other subtypes. These avian viruses may also induce low levels of serum antibody and may not have been detected in previous surveys that relied on the HI assay (21). Development of such assays will be an important further step in preparation for the next influenza pandemic.

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