Short Report: Pre-Existing Cross-Reactive Antibodies to Avian Influenza H5N1 and 2009 Pandemic H1N1 in US Military Personnel

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Abstract. We studied cross-reactive antibodies against avian influenza H5N1 and 2009 pandemic (p) H1N1 in 200 serum samples from US military personnel collected before the H1N1 pandemic. Assays used to measure antibodies against viral proteins involved in protection included a hemagglutination inhibition (HI) assay and a neuraminidase inhibition (NI) assay. Viral neutralization by antibodies against avian influenza H5N1 and 2009 pH1N1 was assessed by influenza (H5) pseudotyped lentiviral particle-based and H1N1 microneutralization assays. Some US military personnel had cross-neutralizing antibodies against H5N1 (14%) and 2009 pH1N1 (16.5%). The odds of having cross-neutralizing antibodies against 2009 pH1N1 were 4.4 times higher in subjects receiving more than five inactivated whole influenza virus vaccinations than those subjects with no record of vaccination. Although unclear if the result of prior vaccination or disease exposure, these pre-existing antibodies may prevent or reduce disease severity.

Outbreaks of 1997 avian influenza H5N1 and 2009 pandemic (p) H1N1 in humans have provided an opportunity to gain insight into cross-reactive immunity. The US military periodically collects and stores serum samples from service members linked to medical records.¹ We measured cross-reactive antibodies in stored serum to avian influenza H5N1 and 2009 pH1N1 from US military personnel and identified factors associated with presence of neutralizing antibodies.

Two hundred archived serum samples were obtained from the US Department of Defense Serum Repository. They were representative of a wide cross-section of active military personnel at the times of collection, whereas specific geographic information was not available on the individual selected; the cohort represents the general US military population, which is deployed throughout the United States and globally. Fifty samples each were selected from four birth cohorts: (1) < 1949, (2) 1960–1965, (3) 1966–1971, and (4) 1972–1977. Within each cohort, 25 samples were collected in the year 2000 (before the introduction of intranasal live attenuated influenza vaccine [LAIV]), and 25 samples were collected in 2008 (where 51% of donors had received LAIV). It has been suggested that LAIV elicits cross-reactive immunity.^{2,3} The samples were all collected before the outbreak of 2009 pH1N1, and there have not been any reported outbreaks of H5N1 in US military personnel.

Assays used to measure antibodies included a hemagglutination inhibition (HI) assay and a neuraminidase inhibition (NI) assay.⁴ Viral neutralization by antibodies against H5N1 and 2009 pH1N1 was assessed by influenza (H5) pseudotyped lentiviral particle-based (H5pp)⁵ and microneutralization assays, respectively. Electronic medical and vaccination records from the Defense Medical Surveillance System (DMSS), which captured records before the serum sample date, were linked to samples and compared with the *in vitro* results.¹

The odds ratios (ORs) and 95% confidence intervals (95% CIs) of univariate and multivariate binary logistic regression

analyses were used to determine the association between donor characteristics and positive antibody responses. A multiple logistic regression model was constructed, and it included independent variables with a *P* value of < 0.05 in univariate logistic regression. A *P* value of < 0.05 was considered to indicate statistical significance. SPSS 12.0 for Windows (SPSS Inc., Chicago, IL) was used to perform all statistical analysis.

Cross-reactivity is summarized in Table 1. Although HI assay titers to H5N1 were uniformly low (0.5%), neutralizing antibodies were considerably higher: 14% for the more sensitive H5pp assay⁵ and 22.5% for the NI assay. H5pp and NI antibody titers to H5N1 were evenly distributed among birth cohorts and did not differ substantially based on history of vaccination or prior respiratory infections. Of those individuals with neutralizing antibodies to H5N1 (*N* = 28), 32.1% also had neutralizing antibodies to pH1N1, whereas 19.3% of those individuals with any H5N1-specific antibody response also had neutralizing antibodies to pH1N1 (Table 1).

As with H5N1, samples with positive HI titers were low for 2009 pH1N1 at 5.5%, whereas neutralizing antibody titers were higher, with 16.5% positive in the microneutralization assay but only 9% positive in the NI assay. Positive neutralization titers were less evenly distributed among birth cohorts, with only 4% positive in the 1972–1977 birth cohort, whereas 30% were positive in the 1960–1965 cohort. Like H5N1, positive antibody titers to 2009 pH1N1 did not differ substantially based on history of vaccination or prior respiratory infections. Of those individuals with neutralizing antibodies to pH1N1 (*N* = 33), 27.3% also had neutralizing antibodies to H5N1, whereas 28.9% of those individuals with any pH1N1-specific antibody response also had neutralizing antibodies to H5N1.

Univariate associations between the prevalence of cross-reactive antibodies to H5N1 and 2009 pH1N1 and independent variables, including year of birth, serum collection year, sex, and seasonal influenza vaccination history, are shown in Table 2. The odds of having cross-neutralizing antibodies against 2009 pH1N1 were threefold higher in those donors who received inactivated whole influenza virus vaccine more than five times than those donors with no record of vaccination (95% CI = 1.1–8.9). In relation to subjects born between

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TABLE 1

Serum cross-reactivity to avian influenza H5N1 and 2009 pH1N1 indicating the percentage of subjects considered to have positive titers and the geometric mean titers for each assay

Characteristics (n)	H5N1			2009 pH1N1§		
	HI assay* % positive (GM titer)	H5pp† % positive (GM titer)	NI assay‡ % positive (GM titer)	HI assay % positive (GM titer)	Neutralization % positive (GM titer)	NI assay % positive (GM titer)
Total						
200	0.5 (5.1)	14.0 (21.4)	22.5 (121.6)	5.5 (7.1)	16.5 (20.4)	9.0 (92.8)
Birth cohort						
1936–1949 (50)	2.0 (5.3)	18.0 (22.0)	24.0 (126.0)	6.0 (7.3)	16.0 (19.5)	12.0 (97.6)
1960–1965 (50)	0.0 (5.0)	16.0 (20.3)	26.0 (129.6)	6.0 (7.7)	30.0 (27.5)	6.0 (90.3)
1966–1971 (50)	0.0 (5.0)	12.0 (23.3)	20.0 (117.9)	10.0 (8.0)	16.0 (23.6)	10.0 (92.2)
1972–1977 (50)	0.0 (5.3)	10.0 (20.0)	20.0 (113.7)	0.0 (5.7)	4.0 (13.6)	8.0 (91.5)
Serum collection year						
Y2000 (100)	0.0 (5.1)	15.0 (21.7)	21.0 (120.3)	7.0 (7.3)	16.0 (20.6)	11.0 (94.5)
Y2008 (100)	1.0 (5.2)	13.0 (21.1)	24.0 (123.0)	4.0 (7.0)	17.0 (20.1)	7.0 (91.2)
Sex						
Female (32)	3.1 (5.7)	21.9 (26.3)	12.5 (102.4)	3.1 (6.9)	12.5 (19.2)	6.3 (96.7)
Male (168)	0.0 (5.0)	12.5 (20.5)	24.4 (125.7)	6.0 (7.2)	17.3 (20.6)	9.5 (92.1)
Any cross-reactive antibody to H5N1 (57)				8.8 (8.9)	19.3 (25.2)	22.8 (119.9)
pH1N1 (45)	2.2 (5.3)	28.9 (31.2)	37.8 (165.2)			
Neutralizing antibodies to H5N1 H5pp (28)				10.7 (9.5)	32.1 (33.6)	25.0 (116.9)
2009 pH1N1 neutralization (33)	3.0 (5.4)	27.3 (28.9)	30.3 (140.3)			
Lifetime seasonal vaccinations						
No record (66)	0.0 (5.1)	10.6 (20.2)	27.7 (128.1)	7.6 (7.4)	15.2 (20.6)	12.1 (96.5)
1–5 vaccinations (88)	1.1 (5.2)	15.9 (21.5)	17.0 (109.2)	5.7 (7.1)	17.0 (20.5)	6.8 (89.1)
> 5 vaccinations (46)	0.0 (5.1)	15.2 (22.2)	32.6 (138.8)	2.2 (6.8)	17.4 (19.7)	8.7 (95.0)
Time since last vaccine						
No record (66)	0.0 (5.1)	10.6 (20.2)	22.7 (128.1)	7.6 (7.4)	15.2 (20.6)	12.1 (96.5)
≤ 1 year (96)	0.0 (5.1)	15.6 (21.5)	24.0 (120.7)	4.2 (7.1)	19.8 (21.0)	8.3 (92.0)
> 1 year (38)	2.6 (5.3)	15.8 (22.4)	18.4 (113.4)	5.2 (6.8)	10.5 (18.3)	5.3 (90.6)
Vaccination history lifetime (at least one dose)						
No record of vaccination (66)	0.0 (5.1)	10.6 (20.2)	22.7 (128.1)	7.6 (7.4)	15.2 (20.6)	12.1 (96.5)
Inactivated whole virus (71)	0.0 (5.0)	14.1 (20.4)	22.5 (115.7)	2.8 (6.4)	15.5 (19.6)	5.6 (87.1)
Split type (102)	1.0 (5.0)	15.7 (20.4)	21.6 (115.7)	4.9 (6.4)	19.6 (19.6)	6.9 (87.1)
Influenza vaccine not otherwise specified (16)	0.0 (5.2)	12.5 (27.9)	37.5 (166.4)	0.0 (6.2)	6.3 (16.1)	12.5 (102.3)
Live attenuated intranasal (50)	0.0 (5.1)	10.0 (18.8)	20.0 (112.2)	4.0 (7.0)	18.0 (20.3)	4.0 (85.2)
History of respiratory illness						
No record of illness (119)	0.0 (5.0)	10.1 (18.5)	18.5 (112.6)	4.2 (7.0)	15.1 (20.5)	8.4 (90.7)
Influenza-like illness (4)	0.0 (5.0)	25.0 (20.7)	0.0 (80.0)	0.0 (8.4)	25.0 (28.3)	25.0 (100.2)
Upper respiratory infection (65)	1.5 (5.4)	23.1 (29.3)	27.7 (135.0)	7.7 (7.3)	18.5 (20.7)	9.2 (93.1)
Lower respiratory infection (37)	2.7 (5.6)	18.9 (30.2)	35.1 (157.6)	8.1 (8.1)	21.6 (22.4)	13.5 (108.4)
Respiratory illness past year (28)	0 (5.1)	25.0 (25.1)	32.1 (154.9)	7.1 (8.0)	28.6 (24.4)	3.6 (86.3)

Titers with a value of zero (below the detection limit) were assigned a value of five for calculation of geometric means (GMs).

*H5N1, A/Vietnam/1203/2004; positive titer ≥ 40.

†H5 hemagglutinin (A/Cambodia/408008/05) pseudotyped lentiviral particle; positive titer ≥ 160.

‡Reassortant H1N1 (HA, PB1, PB2, PA, NP, and M from H1N1 [A/PR/8/34]; N1 from H5N1 [A/Vietnam/DT-036/2005]); positive titer ≥ 160.

§2009 H1N1, A/California/04/2009; same positive titer cutoffs as for H5N1.

1972 and 1979, the odds of having cross-reactive neutralizing antibodies against 2009 pH1N1 were significantly higher in subjects born from 1960 to 1965 (OR = 10.3; 95% CI = 2.2–47.9). In a multiple logistic regression model adjusted for serum collection year, birth cohort, and frequency of receiving inactivated whole virus vaccine, birth cohorts, including 1960–1965 (adjusted OR = 11; 95% CI = 2.3–52.9), and a history of more than five inactivated whole influenza virus vaccinations (adjusted OR = 4.4; 95% CI = 1.3–15.6) were statistically significant covariates associated with higher positivity rates for pH1N1 neutralizing antibodies. Within these groups, we found no convincing data to suggest that the specific viral compositions in influenza vaccines have an effect on cross-reactive neutralizing antibodies to pH1N1 (data not shown). No statistically significant associations were observed between the prevalence of cross-reactive antibodies to H5N1 and independent variables in univariate or multivariate analyses.

To the best of our knowledge, the present study is the first report of cross-reactive antibodies to both H5N1 and 2009

pH1N1 in a US military population. Cross-reactive antibodies to both influenza viruses were common in this population. Most serum samples (86%) positive in the H5N1 neutralization assay had no detectable HI activity (titer ≥ 10), whereas 94% of samples that neutralized 2009 pH1N1 also had detectable HI activity (titer ≥ 10; data not shown). In addition, cross-reactive antibodies to avian influenza H5N1 were not necessarily accompanied by cross-reactive antibodies to 2009 pH1N1. Taken together, these findings suggest that the observed cross-reactive neutralization against the two influenza viruses was caused by different antibodies in serum samples.

This report is also the first report to associate history of receiving more than five doses of inactivated whole influenza virus vaccine with neutralizing antibodies against 2009 pH1N1. This finding suggests a protective advantage of repeated vaccination with seasonal whole virus vaccine, generating cross-reactive antibodies against previously unencountered strains. It has been suggested that the high immunogenicity of the inactivated whole virus vaccine is partly caused by the adjuvant

TABLE 2
Analysis of characteristics associated with cross-reactive neutralizing antibodies against 2009 pH1N1 and H5N1

Characteristic (n)	2009 pH1N1			H5N1	
	Prevalence	OR (95% CI)	Adjusted OR (95% CI)	Prevalence	OR (95% CI)
Positive neutralizing antibody	33 (16.5%)			28 (14.0%)	
Serum collection year					
2000 (100)	16 (16.0%)	Reference	Reference	15 (15.0%)	Reference
2008 (100)	17 (17.0%)	1.1 (0.5–2.3)	0.7 (0.3–1.8)	13 (13.0%)	0.9 (0.4–1.9)
Birth cohort					
1936–1949 (50)	8 (16.0%)	4.6 (0.9–22.7)	5.3 (1.0–27.0)	9 (18.0%)	2.0 (0.6–6.4)
1960–1965 (50)	15 (30.0%)	10.3 (2.2–47.9)	11.0 (2.3–52.9)	8 (16.0%)	1.7 (0.5–5.7)
1966–1971 (50)	8 (16.0%)	4.6 (0.9–22.7)	5.1 (1.0–26.2)	6 (12.0%)	1.2 (0.4–4.3)
1972–1977 (50)	2 (4.0%)	Reference	Reference	5 (10.0%)	Reference
Sex					
Female (32)	4 (12.5%)	Reference		7 (21.9%)	Reference
Male (168)	29 (17.3%)	1.5 (0.5–4.5)		21 (12.5%)	0.5 (0.2–1.3)
Positive neutralizing antibody titers					
H5pp (57)	11 (19.3%)	1.3 (0.6–2.9)			
pH1N1 (45)				13 (28.9%)	3.8 (1.6–8.7)
Vaccination record					
Number of seasonal influenza vaccinations					
No record (66)	10 (15.2%)	Reference		7 (10.6%)	Reference
1–5 vaccinations (88)	15 (17.0%)	1.2 (0.5–2.8)		14 (15.9%)	1.6 (0.6–4.2)
> 5 vaccinations (46)	8 (17.4%)	1.2 (0.4–3.3)		7 (15.2%)	1.5 (0.5–4.7)
Time since last vaccination					
No record (66)	10 (15.2%)	Reference		7 (10.61%)	Reference
≤ 1 year (96)	19 (19.8%)	1.4 (0.6–3.2)		15 (15.6%)	1.6 (0.6–4.1)
> 1 year (33)	4 (10.5%)	0.7 (0.2–2.3)		6 (15.8%)	1.6 (0.5–5.1)
Number of inactivated whole virus vaccinations					
No record (129)	22 (17.1%)	Reference	Reference	18 (14.0%)	Reference
1–5 vaccinations (53)	4 (7.5%)	0.4 (0.1–1.2)	0.4 (0.1–1.4)	7 (13.2%)	0.9 (0.4–2.4)
> 5 vaccinations (18)	7 (38.9%)	3.1 (1.1–8.9)	4.4 (1.3–15.6)	3 (16.7%)	1.2 (0.3–4.7)
Time since last inactivated whole virus vaccination					
No record (129)	22 (17.1%)	Reference		18 (14.0%)	Reference
≤ 1 year (19)	4 (21.1%)	1.3 (0.4–4.3)		3 (15.8%)	1.2 (0.3–4.4)
> 1 year (52)	7 (13.5%)	0.8 (0.3–1.9)		7 (13.5%)	1.0 (0.4–2.5)
Number of split type vaccinations					
No record (98)	13 (13.3%)	Reference		12 (12.2%)	Reference
1–5 vaccinations (94)	19 (20.2%)	1.7 (0.8–3.6)		14 (14.9%)	1.3 (0.6–2.9)
> 5 vaccinations (8)	1 (12.5%)	0.9 (0.1–8.2)		2 (25.0%)	2.4 (0.4–13.2)
Time since last split type vaccination					
No record (98)	13 (13.3%)	Reference		12 (12.2%)	Reference
≤ 1 year (44)	10 (22.7%)	1.9 (0.8–4.8)		10 (22.7%)	2.1 (0.8–5.3)
> 1 year (58)	10 (17.2%)	1.4 (0.6–3.3)		6 (10.3%)	0.8 (0.3–2.3)
Number of intranasal LAIV vaccinations					
No record (150)	24 (16.0%)	Reference		23 (15.3%)	Reference
1–5 vaccinations (50)	9 (18.0%)	1.2 (0.5–2.7)		5 (10%)	0.6 (0.2–1.7)
Time since last intranasal LAIV vaccination					
No record (150)	24 (16.0%)	Reference		23 (15.3%)	Reference
≤ 1 year (34)	7 (20.6%)	1.4 (0.5–3.5)		3 (8.8%)	0.5 (0.2–1.9)
> 1 year (16)	2 (12.5%)	0.8 (0.2–3.5)		2 (12.5%)	0.8 (0.2–3.7)

effect of the viral RNA presented, stimulating innate immunity through the Toll-like receptor (TLR) 7-dependent pathway.⁶ We hypothesize that the combined effect of adjuvant activity and the heterogenous mix of flu strains that an individual would be exposed to over the course of multiple seasonal vaccinations may enhance the breadth of antibody response and promote the generation of cross-reactive antibodies.

A retrospective case-control study conducted in US military personnel after the outbreak of 2009 pH1N1 showed that both 2008–2009 seasonal influenza vaccine and history of seasonal vaccine in the prior 4 years afforded some protection against pH1N1. Vaccine effectiveness (VE) was high in persons ≥ 40 (55%) or < 25 (50%) years of age but very low in persons 25–39 years of age (< 10%).⁷ These findings correlate with the high levels of cross-reactive 2009 pH1N1 antibodies reported here, with 30% in the 1960–1965 cohort (age range = 35–48) but only 4% in the 1972–1977 cohort (age range = 23–36). Our findings

are similar to the results found recently in an elderly population in the United States.⁸ The exception is in those individuals born before 1950, in whom antibody responses were much higher in this cohort. Both our study and the US study differ from two recent seroprevalence studies in Singapore and China, where cross-reactive antibodies were rare in various age groups.^{9,10} High seasonal influenza vaccination rates in US military personnel found here and prior studies¹¹ may explain the differences observed in these populations, although results from small retrospective seroprevalence studies should be interpreted cautiously. Possible alternative explanations include differences in laboratory assay methods, natural influenza exposure in the sampled populations, and/or use of convenience sampling methods.

Studies in humans suggest that the antibody to influenza neuraminidase is associated with resistance to influenza.¹² A recent serological study in a small number of human serum

samples showed that 24% had cross-reactive antibodies to avian N1,¹³ similar to our findings (22.5%). In addition, we observed that 9% of serum samples had cross-reactive antibodies to pH1N1.

Like pH1N1, persons < 40 years old seem to be most affected by H5N1 infection, with infection rarer in older individuals.¹⁴ However, we did not find a difference in cross-reactive antibody prevalence to either neuraminidase or neutralizing antibodies (H5pp) with year of birth or other immunologic markers of exposure, including vaccination history or prior respiratory illness.

A possible limitation of our study is that the DMSS may not have captured all relevant medical encounter and/or vaccination data, particularly for encounters that were not entered into the system electronically or coded accurately. Data in the DMSS are provider-dependent, and the DMSS captures data from various historical time periods, dating back to 1980 for immunization data, 1985 for Department of Defense Serum Repository specimens, 1990 for demographic data, and only 1996 for outpatient data. Interpretation of data presented on history of respiratory illness, which is entirely dependent on voluntary provider reporting and International Classification of Diseases (ICD-9) coding, is particularly limited by lack of virologic confirmation.

Cross-reactive immunity to pathogenic influenza strains was found in a subset of US military service members, and it may serve to prevent or reduce the severity of influenza. A better understanding of the mechanisms underlying the development of cross-reactive antibodies will aid in the development of more effective preventive and therapeutic measures.

Received March 22, 2013. Accepted for publication July 21, 2013.

Published online November 25, 2013.

Acknowledgments: The authors thank Dr. Angelia Eick for providing serum samples from the Department of Defense serum repository. We also thank Drs. Malik Peiris, Richard Jarman, and Gray Heppner for their support.

Financial support: This research was supported by National Institutes of Health, National Institute of Allergy and Infectious Diseases Grant Y1-AI-5026-01, the Thailand Research Fund, and Chulalongkorn University.

Disclaimer: The views of the authors do not purport to reflect official policy of the US Department of the Army or the Department of Defense.

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