Assessment of Serosurveys for H5N1

Eric S. Toner,[1] Amesh A. Adalja,[1] Jennifer B. Nuzzo,[1] Thomas V. Inglesby,[1] Donald A. Henderson,[1] and Donald S. Burke[2]

[1]Center for Biosecurity, University of Pittsburgh Medical Center, Baltimore, Maryland; and [2]Graduate School of Public Health, University of Pittsburgh, Pennsylvania

(See the Editorial Commentary by Morens on pages 1213–5.)

Background. It has been suggested that the true case-fatality rate of human H5N1 influenza infection is appreciably less than the figure of approximately 60% that is based on official World Health Organization (WHO)–confirmed case reports because asymptomatic cases may have been missed. A number of seroepidemiologic studies have been conducted in an attempt to identify such missed cases.

Methods. We conducted a comprehensive literature review of all English-language H5N1 human serology surveys with detailed attention to laboratory methodology used (including whether investigators used criteria set by the WHO to define positive cases), laboratory controls used, and the clades/genotypes involved.

Results. Twenty-nine studies were included in the analysis. Few reported using unexposed control groups and one-third did not apply WHO criteria. Of studies that used WHO criteria, only 4 found any seropositive results to clades/genotypes of H5N1 that are currently circulating. No studies reported seropositive results to the clade 2/genotype Z viruses that have spread throughout Eurasia and Africa.

Conclusions. This review suggests that the frequency of positive H5 serology results is likely to be low; therefore, it is essential that future studies adhere to WHO criteria and include unexposed controls in their laboratory assays to limit the likelihood of false-positive results.

Keywords. H5N1; influenza; seroprevalence; seroepidemiology; case-fatality rate.

Recent debate about research on modified highly pathogenic avian influenza H5N1 viruses has included consideration of the true case-fatality rate of human H5N1 infection. It has been suggested that the case-fatality rate of approximately 60% that is based on official World Health Organization (WHO) case reports is too high because many mild or asymptomatic cases may have been missed [1]. In an attempt to identify such missed cases, there have been several studies of mild and moderate respiratory infections in endemic countries in which patients were tested for H5N1. These have detected few mild or moderate H5N1 infections [2–6]. In addition, a number of seroepidemiologic studies have been conducted over the last 15 years. These studies have recently been reviewed by several groups, including by 2 of the authors of this paper [1, 7, 8]. Although there was considerable overlap, the studies included in these reviews were not identical; however, all 3 recent reviews and prior reviews [9] have found that most serosurveys reported no specimens showing antibody evidence of H5N1 infection and that of those serosurveys that did have positive findings and followed WHO criteria for serologic testing of patients with prior infection, the rates of positives were <3%. However, the conclusions drawn by the various reviews were quite different. One analysis concluded that asymptomatic human H5N1 infection is likely widespread; others concluded just the opposite. These differing conclusions have created considerable debate [10, 11]. To better understand these differing analyses, we conducted a new, more detailed review of all published English-language H5N1 serosurveys.

METHODS

We conducted a keyword literature search in PubMed using the terms *H5N1*, *serology*, *seroprevalence*,

serosurvey, and *seroepidemiology* from 1997 through 1 November 2012 identifying studies published in English that involved serologic assays for H5N1 antibodies from asymptomatic human subjects This search identified 30 unique studies. We reviewed each of these studies in detail to determine (1) laboratory methodology used (including whether investigators used laboratory criteria set by the WHO to define positive serology results for prior H5N1 infection); (2) laboratory controls used; and (3) the geographic areas and specific viral clade and genotype involved.

Although the WHO criteria specifically apply to people with prior symptomatic infection (≥14 days after the onset of symptoms), we applied these criteria because, in our judgment, they set a high enough threshold that it is likely that positive results would be "true positives" and not due to cross-reaction with other antigens. The WHO criteria were used by a majority of the studies we reviewed and have been applied by other researchers who have reviewed these serosurveys [1, 7]. There are no established criteria for serosurveys of asymptomatic H5 infections.

We paid particular attention to whether unexposed control groups were included. We defined an optimal unexposed testing control, as inserting blinded samples from individuals with essentially no chance of H5N1 infection (eg, people from urban areas of the Americas with no history of travel to endemic areas) into the serology runs. Cross-reactivity between H5N1 and other strains of influenza is well documented, and, as shown in H5N1 vaccine trials [12], a surprisingly high rate of elevated titers that react to H5 occurs even in populations never known to have been exposed to the virus. Therefore, the frequency of seropositivity among a study population must be compared to a control group to be meaningful. An alternative would be to compare the serology results to well-defined standard sera panels such as those that have been created for use in H5N1 vaccine trials [13].

We also noted when and where each study was conducted and the specific viral clade and genotype that were involved in each study. Most of the studies included sufficient information about the viral strain against which the specimens were tested to determine the specific clade involved. Where this was not reported, the clade was inferred from other sources that reported the H5N1 viruses found in that location at that time.

RESULTS

Of the 30 unique studies that were identified and reviewed, 1 study [14] was excluded because all the subjects had been provided oseltamivir prophylaxis—leaving 29 studies that were included in this analysis. See Tables 1 and 2.

Many of the studies did not describe the laboratory methodology used in sufficient detail for us to judge the validity of the results. Few of the studies stated whether or not unexposed controls were used. None of the studies we reviewed reported having used controls that fully met our definition of optimal unexposed controls.

Of the 29 studies analyzed, 9 did not use the WHO criteria. The WHO criteria state that

"paired sera, collected first during the acute phase of illness and then 14 days or later after the onset of illness, should be tested simultaneously. Retrospectively, infection with H5N1 is confirmed when one the following criteria are met:

- Fourfold or greater rise in antibody titre against A(H5N1) in paired sera (acute and convalescent) with the convalescent serum having a titre of 1:80 or higher.
- Antibody titre of 1:80 or more in a single serum collected at day 14 or later after onset of symptoms and a positive result using different serological assay (eg titre of 1:160 or greater in HI using horse red blood cell or an H5–specific western blot)." [15]

Studies that use a lower cutoff, or are not confirmed by a separate test, would be expected to show higher positivity rates due to cross-reaction to other influenza viruses. Thus, low titer reactivity to H5 antigen in such studies cannot be confidently ascribed to prior H5N1 infection.

Seven of the 29 studies involved clades and genotypes of H5N1 that have not been detected in many years and are different from strains that are currently circulating. Four were conducted immediately following the original outbreak of human cases in Hong Kong in 1997 [16–18, 36]. One of these studies did not use WHO criteria [36]. The remaining 3 Hong Kong studies did use WHO criteria and reported seropositivity rates of 3%–10%. Another of the 7 studies was conducted in South Korea following the poultry outbreak there in 2003–2004 and used WHO criteria [20]. This study reported 0.4% seropositivity among poultry cullers. The sixth study was conducted in Vietnam in 2001 and found seropositivity rates to clade 0 viruses of 1.0–2.5% among poultry workers using WHO criteria [19]. The seventh study was conducted in China in 2004, did not use WHO criteria, and found a seropositivity rate of 2.5% [38]. In general, these 7 studies, involving early genotypes of H5N1, reported the highest seropositivity rates among the 29 studies included in this analysis.

Among the studies that involved H5N1 genotypes that are currently circulating, 7 did not use WHO criteria. Of the 16 studies that tested for antibodies against currently circulating H5N1 viruses and used WHO criteria, 12 reported no positives. Four studies found at least 1 positive result by WHO criteria [19, 29, 30, 32] (Figure 1).

Table 1. Summary of H5N1 Serosurveys Meeting World Health Organization Criteria (N = 20)

Reference	Location	Year of Study[a]	H5N1 Antigen(s) Used	Clade(s)/Genotype	Survey Type[b]	WHO Criteria [15]	Positives (%)
[16]	Hong Kong	1997–1998	A/duck/Singapore/-Q/F119–3/97	0/GsGd	FSP	Yes	3%–10%[b]
[17]	Hong Kong	1997–1998	A/Hong Kong/156/97 A/duck/Singapore/-Q/F119–3/97	0/GsGD	CT	Yes	8/217 (3.7%)[c]
[18]	Hong Kong	1999	A/Hong Kong/156/97 A/duck/Singapore/-Q/F119–3/97	0/GsGD	CT	Yes	7/124 (5.6%)
[19]	Vietnam	2001	A/goose/Vietnam/113/01 A/Hong Kong/156/97 A/Hong Kong/213/03	0/? 0/GsGD 1/Z	FSP	Yes	2/200 (1%) 5/200 (2.5%)[d] 6/200 (3%)[d]
[20]	South Korea	2003–2004	A/chicken/Korea/ES/03	2.5/ V	FSP	Yes	9/2512 (0.4%)
[21]	Vietnam	2004	Not reported	1/ Z[e]	CT	Yes	0
[22]	Thailand	2004	A/Thailand/16/04	1/Z	CT	Yes	0
[23]	Thailand	2004	Not reported	1/ Z[e]	FSP	Yes	0
[24]	Thailand	2005	A/Thailand/1(KAN-1)/04	1/Z	FSP	Yes	0
[25]	Indonesia	2005	A/chicken/Bangli Bali/BBPV6/04	2.1/Z	FSP	Yes	0
[26]	Vietnam	2005	A/Vietnam/1194/04 A/Vietnam/30850/05	1/Z 2.3/Z	FSP	Yes	0
[27]	Cambodia	2005	Not reported	1/Z[e]	FSP	Yes	0
[28]	Nigeria	2006	A/chicken/Nigeria/246/06 A/chicken/Nigeria/42/06	2.2/Z	FSP	Yes	0
[29]	China	2006	A/Hong Kong/486/97 A/Vietnam/1194/04	0/GsGd 1/Z	FSP	Yes	1/110 (0.9%)
[30]	Cambodia	2006	A/Vietnam/JP/14/05	1/Z	FSP	Yes	7/674 (1%)
[31]	China	2007	A/Jiangsu/1/07	2.3/Z	CT	Yes	0
[32]	Cambodia	2007	A/Cambodia/R0405050/07	1/Z	FSP	Yes	18/700 (2.6%)
[33]	Indonesia	2007	A/Ck/Banten/05-1116/05 A/H5N1/Indo/05/IBCDC-RG	2.1/Z 2.1/Z	FSP	Yes	0
[34]	Bangladesh	2008–2009	A/Bangladesh/207095/08	2.2/Z	FSP	Yes	0
[35]	China	2009	H5N1 vaccine seed virus, PR8/vn1194 6:2 reassortant	1/Z	FSP	Yes	0

Abbreviations: CT, contact tracing; FSP, focused seroprevalence (ie, seroprevalence among high-risk population); WHO, World Health Organization.

[a] Year in which serum was obtained.

[b] Numerator and denominator not reported.

[c] Two of 309 (1%) of unexposed healthcare workers were also positive.

[d] One percent of controls were also positive.

[e] Clade/genotype inferred from strains reported to be circulating at the time and place of the study.

One of these 4 studies that found a positive result was conducted in Cambodia in 2006 and involved 2 villages where there had been recent outbreaks of clade 1 H5N1 in poultry and humans. This study found that 7 of 674 subjects (1%) were seropositive [30]. Another study, conducted in 2007 in a neighboring province of Cambodia where there had also been an outbreak of clade 1 H5N1, showed a seropositivity rate of 2.6% (18/700) [32]. In both studies, seropositivity was most strongly associated with bathing or swimming in outdoor ponds that may have been contaminated with feces from infected ducks. The third study was of poultry purveyors in Guangzhou, China. One of 110 subjects was positive and he

reported slaughtering >100 birds per day for 5 years. He was positive at high titer to both clade 0 and clade 1 antigens [29]. The previously mentioned study from Vietnam in 2001 tested serum of poultry workers for antibodies to a clade 1 virus as well as clade 0 viruses mentioned above—6 of 200 (3%) were positive [19].

DISCUSSION

Of the 29 studies included in this analysis, nearly a third did not use WHO criteria for establishing seropositivity. None of

Table 2. Summary of H5N1 Serosurveys Not Meeting World Health Organization Criteria (n = 9)

Reference	Location	Year of Study	H5N1 Antigen Used	Clade(s)/ Genotype	Survey Type	WHO Criteria [15]	Positives (%)
[36]	Hong Kong	1997	Not reported	0/GsGd[d]	CT, FSP	[a]	2%[b]
[37]	Vietnam	2004	A/Vietnam/1194/04 A/Vietnam/3212/04	1/Z 1/Z	CT	No	0
[38]	China	2004	A/goose/Guangdong/1/96	0/GsGd	FSP	No (1:20)	2.5% by HI[c]
[39]	Germany	2006	A/whooper swan/ R65-2/Germany/06 A/bar-headed goose/ Qinghai/1A/05 A/whooper swan/ Mongolia/244/05	2.2/Z 2.2/Z 2.2/Z	FSP	No	0
[40]	Turkey	2006	A/Turkey/13/06	2.2/Z	CT, FSP	No	1/381 (0.3%)
[41]	Guangdong, China	2007–2008	A/Hong Kong/486/97 A/ Vietnam/1194/04	0/GsGd 1/Z	FSP	[a]	4/2191 (0.2%)
[42]	Thailand	2008	A/Thailand/676/05 A/Thailand/384/06	1/Z 1/Z	FSP	No (1:10)	9.1%[b]
[43]	Vietnam	2008–2009	Not reported other than as clades 1 and 2.3.4	1, 2.3/Z	FSP	No	5%[b]
[44]	Jiangsu, China	2010	A/Anhui/1/05 A/Hubei/1/10	2.3/Z 2.3/Z	FSP	No	8/306 (2.6%)

Abbreviations: CT, contact tracing; FSP, focused seroprevalence; HI, hemagglutination inhibition; WHO, World Health Organization.

[a] Insufficient information to determine.
[b] Numerator and denominator not reported.
[c] Nine of 10 were negative when tested by microneutralization.
[d] Clade/genotype inferred from strains reported to be circulating at the time and place of the study.

the 29 serostudies included what we would consider to be optimal, blinded unexposed controls in their published methodologies, that is, including in the serology runs blinded samples from individuals with essentially no chance of H5N1 infection. Serologic assays can easily produce misleading results, especially when paired sera are not available. Because antibody titers may wane over time, a low titer on a single specimen does not preclude prior infection. This is especially
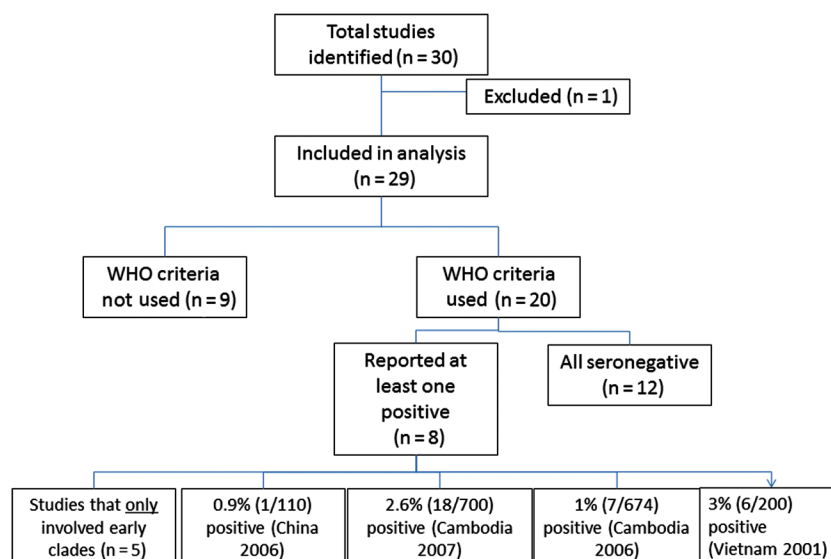

Figure 1. H5N1 serosurvey analysis flowchart. Abbreviation: WHO, World Health Organization.

true if the serum is obtained long after the exposure. In some of the serosurveys included in this review that involved tracing of contacts of known human cases or surveys of people exposed in discrete poultry outbreaks, the approximate time of exposure was known and reported. For the most part, the investigators in these studies obtained serum within months of the time of exposure. In other surveys, particularly those of poultry workers in which repeated exposure over a prolonged time was likely, the time of exposure was not known.

Conversely, because antibodies may cross-react with related antigens, some elevation of H5 antibody titers (especially in low titer) or infrequent "positive" results are likely to be "false positives," that is, a cross-reaction to another antigen. Using unexposed controls could reduce the chance of misinterpreting such false-positives. This is especially important when low positivity rates are reported, because when a true positive is very rare in a study population, even if cross-reactivity is relatively uncommon, it is more likely that a positive result will be the result of cross-reactivity than be a true positive (ie, low positive predictive value). Therefore, because few of the studies included unexposed controls, the low rates of positive results reported by these studies must be interpreted with caution.

Several of the studies included in this analysis involved early H5N1 genotypes and clades that are no longer circulating. The 1997 Hong Kong strain is designated clade 0/genotype GsGd. The clade designation refers to mutations in the hemagglutinin and the genotype refers to the constellation of the individual gene segments. The 1997 Hong Kong virus descended from a GsGd genotype virus that was isolated in 1996 in China. Though this ancestral virus is thought to be the progenitor of all subsequent highly pathogenic avian influenza H5N1 strains, it has since reassorted multiple times with other avian influenza viruses to create many new genotypes. One of these reassortants, genotype V (clade 2.5), caused the 2003–2004 poultry outbreak in South Korea [45]. Neither the 1996 ancestral virus nor the clade 0/genotype GsGd virus that caused the 1997 Hong Kong outbreak have been detected since that outbreak was controlled [46].

All current human and most current poultry infections are related to genotype Z viruses, which first emerged in 2002 [47]. Thus, all currently circulating H5N1 viruses are reassortants of viruses that preceded the Hong Kong and Korea viruses and belong to separate evolutionary branches. They have different hemagglutinin antigens and different constellations of internal genes. It seems reasonable, therefore, to question whether they would have the same infectivity and transmissibility characteristics. Consequently, the results from the serostudies involving these early viruses cannot be extrapolated with confidence to the subsequent genotypes and clades that have caused human and poultry outbreaks elsewhere.

There were only 4 studies using WHO criteria that show seropositive results to the genotype Z viruses that have spread throughout Asia, Europe, and Africa, and all 4 involved clade 1 viruses prior to 2007. Clade 1 was only found in Indochina and southern China and is has now largely been replaced by clade 2.3 viruses [48].

None of the studies in this review were designed to be population-based seroprevalence studies (ie, looked for H5 antibodies in a random sample of a general population); rather, all were either seroepidemiologic prevalence studies of contacts or focused seroprevalence studies of particular populations thought to be at especially high risk of infection (eg, poultry workers or residents of villages with known outbreaks). Therefore, no conclusion can be made from the results about the general populations of the countries involved; however, it is unlikely that the seropositivity rates in the general population would begin to approximate that in high-risk groups.

Thus, with the exception of 1 poultry seller in China, the only convincing serologic evidence we can find of unrecognized human infections to currently circulating H5N1 viruses is in Cambodia and Vietnam with clade 1 viruses. In the 2 Cambodian studies, there seems to be an association with environmental exposure to water potentially contaminated with bird feces. We can find no unequivocal serologic evidence of unrecognized infection with any of the clade 2/genotype Z viruses that are now circulating in most affected countries. Although it is possible that an unknown number of other mildly symptomatic or asymptomatic cases of H5N1 infection have occurred, there is currently no unequivocal serologic evidence to support this speculation.

As noted previously, antibody titers tend to wane over time and thus serologic studies can miss prior infections, especially if performed years after exposure. It is essential that more specific tests for prior H5N1 infection be developed. Other testing approaches, such as measuring T-cell responses, may provide evidence for prior asymptomatic H5N1 infections that have been missed by serostudies [43].

CONCLUSIONS

It is critical that well-executed and well-controlled studies be conducted to improve our understanding of the true prevalence and, therefore, the true severity of H5N1 infections. Judgments about the true prevalence and severity of H5N1 infections have been central to ongoing policy discussions. The results of serologic studies have been an important component in the deliberations and these can be expected drive policy decisions in the future. The results of H5 serologic studies that have been conducted to date and which have been used as evidence in recent policy debates have been useful but are not capable of establishing the true prevalence or severity of

H5N1 human infections. None were designed to determine the prevalence of H5N1 infections in the general population, most did not include unexposed control groups, and many used titer thresholds or methodologies that increase the likelihood of false-positive results due to cross-reaction with other viruses. More specific tests for prior H5N1 infection are desperately needed.

Despite the difficulties in undertaking effective population studies throughout the diverse and underresourced areas where cases have been occurring and with the limitations in the laboratory resources available, important information has accrued. Observations and conclusions from the serologic studies are broadly in agreement: Surprisingly few mild cases of H5N1 illness appear to have occurred or been able to be detected. Because of this low frequency, it is essential that future serologic studies adhere to WHO criteria and include unexposed control groups in their laboratory assays to limit the likelihood of misinterpreting false-positive results.

Note

References

1. Wang TT, Parides MK, Palese P. Seroevidence for H5N1 influenza infections in humans: meta-analysis. Science **2012**; 335:1463.
2. Horby P, Farrar J, Hien TT. Online comment to seroevidence for H5N1 influenza infections in humans: meta-analysis. Science **2012**; 335:1463. Available at: http://comments.sciencemag.org/content/10.1126/science.1218888. Acessed 11 August 2012
3. Shinde V, Hanshaoworakul W, Simmerman J, et al. A comparison of clinical and epidemiological characteristics of fatal human infections with H5N1 and human influenza viruses in Thailand, 2004–2006. PLoS One **2011**; 6:e14809.
4. Wallensten A, Salter M, Bennett S, et al. No evidence of transmission of H5N1 highly pathogenic avian influenza to humans after unprotected contact with infected wild swans. Epidemiol Infect **2010**; 138:199–209.
5. Zaman R, Alamgir ASM, Rahman M, et al. Influenza in outpatient ILI case-patients in national hospital-based surveillance, Bangladesh, 2007–2008. PLoS One **2009**; 4:e8452.
6. Nguyen H, Dharan N, Le M, et al. National influenza surveillance in Vietnam, 2006–2007. Vaccine **2009**; 28:398–402.
7. Osterholm MT, Kelley NS. Mammalian-transmissible H5N1 influenza: facts and perspective. mBio **2012**; 3:e00045-12.
8. Toner E, Adalja A. Is H5N1 really highly lethal? Biosecur Bioterror **2012**; 10:237–40.
9. Van Kerkhove M, Mumford E, Mounts A, et al. Highly pathogenic avian influenza (H5N1): pathways of exposure at the animal-human interface, a systematic review. PLoS One 6:e14582.
10. Van Kerkhove M, Riley S, Lipsitch M, et al. Comment on "Seroevidence for H5N1 influenza infections in humans: meta-analysis." Science **2012**; 336:1506.
11. Wang TT, Palese P. Response to comment on "Seroevidence for H5N1 influenza infections in humans: meta-analysis." Science **2012**; 336:1506.
12. Heijmans S, De Meulemeester M, Paul Reynders P, et al. Immunogenicity profile of a 3.75-μg hemagglutinin pandemic rH5N1 split virion AS03A-adjuvanted vaccine in elderly persons: a randomized trial. J Infect Dis **2011**; 203:1054–62.
13. Stephenson I, Heath A, Major D, et al. Reproducibility of serologic assays for influenza virus A (H5N1). Emerg Infect Dis **2009**; 15:1252–9.
14. Belmaker I, Lyandres M, Bilenko N, et al. Adherence with oseltamivir chemoprophylaxis among workers exposed to poultry during avian influenza outbreaks in southern Israel. Int J Infect Dis **2009**; 13: 261–5.
15. World Health Organization. Recommendations and laboratory procedures for detection of avian influenza A(H5N1) virus in specimens from suspected human cases. **2007**. Available at: http://www.who.int/influenza/resources/documents/h5n1_laboratory_procedures/en/index.html. Acessed 11 August 2012.
16. Bridges CB, Lim W, Hu-Primmer J, et al. Risk of influenza A (H5N1) infection among poultry workers, Hong Kong, 1997–1998. J Infect Dis **2002**; 185:1005–10.
17. Buxton Bridges C, Katz JM, Seto WH, et al. Risk of influenza A (H5N1) infection among health care workers exposed to patients with influenza A (H5N1), Hong Kong. J Infect Dis **2000**; 181:344–8.
18. Katz JM, Lim W, Bridges CB, et al. Antibody response in individuals infected with avian influenza a (H5N1) viruses and detection of anti-H5 antibody among household and social contacts. J Infect Dis **1999**; 180:1763–70.
19. Uyeki T, Nguyen D, Rowe T, et al. Seroprevalence of antibodies to avian influenza a (H5) and a (H9) virus among market poultry workers in Hanoi, Vietnam, 2001. PLoS One **2012**; 7:e43948.
20. Kwon D, Lee JY, Choi JH, et al. Avian influenza A (H5N1) virus antibodies in poultry cullers, South Korea, 2003–2004. Emerg Infect Dis **2012**; 18:986–88.
21. Schultz C, Bong VC, Chau NVV, et al. Avian influenza H5N1 and healthcare workers. Emerg Infect Dis **2005**; 11:1158.
22. Apisarnthanarak A, Erb S, Stephenson I, et al. Sero-prevalence of anti-H5 antibody among Thai health care workers after exposure to avian influenza (H5N1) in a tertiary care center. Clin Infect Dis **2005**; 40:e16–8.
23. Hinjoy S, Puthavathana P, Laosiritaworn Y, et al. Low frequency of infection with avian influenza virus (H5N1) among poultry farmers, Thailand, 2004. Emerg Infect Dis **2008**; 14:499–501.
24. Dejpichai R, Laosiritaworn Y, Phuthavathana P, et al. Sero-prevalence of antibodies to avian influenza virus A (H5N1) among residents of villages with human cases, Thailand, 2005. Emerg Infect Dis **2005**; 15:756–60.
25. Santhia K, Ramy A, Jayaninqsih , et al. Avian influenza A H5N1 infections in Bali province, Indonesia: a behavioral, virological and seroepidemiological study. Influenza Other Respi Viruses **2009**; 3:81–9.
26. Schultsz C, Nquyen VD, Hai le T, et al. Prevalence of antibodies against avian influenza a (H5N1) virus among cullers and poultry workers in Ho Chi Minh City, 2005. PLoS One **2009**; 4:e7948.
27. Vong S, Coghlan B, Mardy S. Low frequency of poultry-to-human H5N1 transmission, southern Cambodia, 2005. Emerg Infect Dis **2006**; 12:1542–7.
28. Ortiz JR, Katz MA, Mahmoud MN. Lack of evidence of avian-to-human transmission of avian influenza A (H5N1) virus among poultry workers, Kano, Nigeria, 2006. J Infect Dis **2007**; 196: 1685–91.
29. Wang M, Di B, Zhou DH, et al. Food markets with live birds as source of avian influenza. Emerg Infect Dis **2006**; 12:1773–5.
30. Vong S, Ly S, Van Kerkhove MD, et al. Risk factors associated with subclinical human infection with avian influenza a (H5N1) virus—Cambodia, 2006. J Infect Dis **2009**; 199:1744–52.
31. Wang H, Feng Z, Shu Y. Probable limited person-to-person transmission of highly pathogenic avian influenza A (H5N1) virus in China. Lancet **2008**; 371:1427–34.

32. Cavailler P, Chu, Ly S, et al. Sero-prevalence of anti-H5 antibody in rural Cambodia, 2007. J Clin Virol **2010**; 48:123–6.

33. Robert M, Holle du Rv, Setiawaty V, Pangesti KN, Sedyaninqsih ER. Sero-prevalence of avian influenza A/H5N1 among poultry farmers in rural Indonesia, 2007. Southeast Asian J Trop Med Public Health **2010**; 41:1095–103.

34. Nasreen S, Khan SU, Azziz-Baumgartner E, et al. Sero-prevalence of H5N1 antibodies among poultry workers in Bangladesh, 2008–09. Abstract presented at International Conference on Emerging and Infectious Diseases, Atlanta, GA, 11–14 March **2012**. http://www.iceid.org/images/iceid_2012_finalprogram_final.pdf. Accessed 12/05/12.

35. Zhang R, Rong X, Pan W, Peng T. Determination of serum neutralization antibodies against seasonal influenza A strain H3N2 and the emerging strains 2009 H1N1 and avian H5N1. Scand J Infect Dis **2011**; 43:216. –20.

36. Update: isolation of avian influenza A(H5N1) viruses from humans—Hong Kong, 1997–1998. MMWR Morb Mortal Wkly Rep **1998**; 46:1245–7.

37. Nguyen TL, World Health Organization International Avian Influenza Investigation Team, Vietnam, Wilina L. Lack of H5N1 avian influenza transmission to hospital employees, Hanoi, 2004. Emerg Infect Dis **2005**; 11:210–5.

38. Lu CY, Lu JH, Chen WQ, et al. Potential infections of H5N1 and H9N2 avian influenza do exist in Guangdong populations of China. Chin Med J (Engl) **2008**; 121:2050–3.

39. Cai W, Schweiger B, Buchholz U, et al. Protective measures and H5N1-sero-prevalence among personnel tasked with bird collection during an outbreak of avian influenza A/H5N1 in wild birds, Ruegen, Germany, 2006. BMC Infect Dis **2009**; 9:170.

40. Ceyhan M, Yildirim I, Ferraris O. Serosurveillance on transmission of H5N1 virus during a 2006 avian influenza epidemic. Epidemiol Infect **2010**; 138:1274–80.

41. Wang M, Fu CX, Zheng BJ. Antibodies against H5 and H9 avian influenza among poultry workers in China. N Engl J Med **2009**; 360:2583–4.

42. Khuntirat BP, Yoon IK, Blair PJ, et al. Evidence for subclinical avian influenza virus infections among rural Thai villagers. Clin Infect Dis **2011**; 53:e107–16.

43. Powell TJ, Fox A, Peng Y, et al. Identification of H5N1-specific T-cell responses in a high-risk cohort in Vietnam indicates the existence of potential asymptomatic infections. J Infect Dis **2011**; 205:20–7.

44. Huo X, Zu R, Qi X, et al. Sero-prevalence of avian influenza A (H5N1) virus among poultry workers in Jiangsu Province, China: an observational study. BMC Infect Dis **2012**; 12:93.

45. Neumann G, Chen H, Gao GF, Shu Y, Kawaoka Y. H5N1 influenza viruses: outbreaks and biological properties. Cell Res **2010**; 20:51–61.

46. Guan Y, Peiris JS, Lipatov AS, et al. Emergence of multiple genotypes of H5N1 avian influenza viruses in Hong Kong SAR. Proc Natl Acad Sci U S A **2002**; 99:8950–55.

47. Li KS, Wang J, Smith GJ, et al. Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern China. Nature **2004**; 430:209–13.

48. WHO/OIE/FAO H5N1 Evolution Working Group. Continued evolution of highly pathogenic avian influenza A (H5N1): updated nomenclature. Influenza Other Respi Viruses **2012**; 6:1–5.