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Supporting Online Material for

Seroevidence for H5N1 Influenza Infections in Humans: Meta-Analysis

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Supplementary Online Materials

Table *S*1. WHO Criteria for Confirmation of H5N1 Infection in Humans (*3*).

Suspected H5N1 infection		
Body temperature > 38°C (100.3° F) with acute lower respiratory illness and dyspnea.	**AND** ≥ 1 of the following within the 7 days preceding symptom onset:	**A)** Close contact with person who likely has H5N1 infection. **B)** Exposure to live or dead birds or bird feces in area with likely H5N1 circulation. **C)** Consumption of raw/undercooked poultry in area with likely H5N1 circulation. **D)** Close contact with non-bird animal with confirmed H5N1 infection. **E)** Handling samples suspected of containing H5N1 virus.
Probable H5N1 infection (Definition 1)		
Person meets criteria for suspected case.	**AND** 1 of the following additional criteria:	**A)** Infiltrates or evidence of acute pneumonia on chest x-ray with evidence of respiratory failure (hypoxemia, severe tachypnea). **B)** Lab confirmation of influenza A infection without specific evidence of H5N1 infection.
Probable H5N1 infection (Definition 2)		
Person dying of an unexplained acute respiratory illness who has been exposed to a probable or confirmed H5N1 case.		
Confirmed H5N1		
Person meets criteria for suspected or probable case.	**AND** 1 of following positive results conducted in influenza laboratory whose H5N1 test results are accepted by WHO:	**A)** Isolation of H5N1 virus. **B)** H5N1 PCR amplification of 2 different virus targets (ex: HA and NA genes). **C)** ≥ 4-fold rise in neutralizing antibody titer for H5N1 based on paired-serum samples (one acute, one convalescent specimen). Convalescent neutralizing titer must be ≥ 1:80. **D)** Microneutralization titer for H5N1 ≥ 1:80 in a singe serum sample collected ≥ 14 days post symptom onset with positive result using a separate serological assay (ex: HI, WB).

HA: Hemagglutinin, NA: Neuraminidase, HI: Hemagglutination Inhibition, WB: Western Blot

Table S2. Compiled Data Describing Seroprevelance of H5N1 virus infection.

STUDY POPULATION	WHERE/WHEN	POSITIVE (%)	CRITERIA USED	INCLUDED IN PRIMARY ANALYSIS?	REF
217 Exp HCWs ◆	Hong Kong/1997	8/217 (3.7%)	WHO	YES	(*7*)
309 Unexp HCWs ◆	Hong Kong/1997	2/309 (0.7%)	WHO	YES	(*7*)
51 House contacts ◆	Hong Kong/1997	6/51 (11.7%)	WHO	YES	(*5*)
73 Mixed exp ◆	Hong Kong/1997	1/73 (1.3%)	WHO	YES	(*5*)
1525 Poultry wks ◆	Hong Kong/1997-8	81/1525 (5.3%)**	WHO	YES	(*4*)
293 Gov wks ◆	Hong Kong/1997-8	9/293 (3%)	WHO	YES	(*4*)
231 Poultry wks	China/2004	7/231 (3.0%)	OTHER	NO	(*15*)
983 Mixed exp	China/2004	23/983 (2.34%)	OTHER	NO	(*15*)
322 Poultry wks ◆◆	Thailand/2004	0/322	WHO	YES	(*20*)
25 Exp HCWs ◆	Thailand/2004	0/25	WHO	YES	(*23*)
24 Unexp HCWs ◆	Thailand/2004	0/24	WHO	YES	(*23*)
351 Mixed exp	Cambodia/2005	0/351	WHO	YES	(*24*)
500 Poultry wks	Vietnam/2005	1/500 (0.2%) / 3/500 (0.6%)	WHO/OTHER	YES*	(*17*)
901 Mixed exp	Thailand/2005	0/901 / 13/901 (1.4%)	WHO/OTHER	YES*	(*13*)
841 Mixed exp	Indonesia/2005	0/841	WHO	YES	(*22*)
87 Poultry wks	Indonesia/2005	0/87	WHO	YES	(*22*)
295 Poultry wks ◆◆◆	Nigeria/2006	0/295	WHO	YES	(*21*)
25 Lab wks ◆◆◆	Nigeria/2006	0/25	WHO	YES	(*21*)
674 Mixed exp	Cambodia/2006	7/674 (1%)	WHO	YES	(*25*)
376 Mixed exp	Turkey/2006	27/376 (7.1%)	OTHER	NO	(*12*)
91 Mixed exp	China/2007	0/91	WHO	YES	(*26*)
495 Poultry wks ϕ	Indonesia/2007	0/495	OTHER	NO	(*16*)
700 Mixed exp	Cambodia/2007	18/700 (2.6%)	WHO	YES	(*8*)
97 Mixed exp ϕ	Germany/2007	0/97	OTHER	NO	(*11*)
2191 Mixed exp	China/2007-08	4/2191 (0.2%)	OTHER	NO	(*18*)
800 Mixed exp	Thailand/2008	45/800 (5.6) or*** 28/800 (3.5%)	OTHER	NO	(*14*)
200 Blood donors ϕ	China/2009	0/200	OTHER	NO	(*19*)

Exp: Exposed, Unexp: Unexposed, HCWs: Heath care workers, Wks: workers, Gov: government
Mixed exposure populations: Persons living in an area where H5N1 infections were confirmed in animals or humans or persons with possible exposure to H5N1 for reasons other than location of residence (possible exposure during work activities, travel, etc).
WHO criteria: see methods. OTHER criteria: other than that used by WHO (ex: Titer ≥1:20 or ≥1:40 or ≥1:160 was considered a positive result), using a commercial kit or the authors did not specify the criteria that was used.
* Only samples confirmed using WHO criteria were counted in the primary analysis. ** The authors tested only 52% of their samples that were positive in an initial assay using a second assay. In accordance with WHO criteria, we have only counted the samples that were tested and scored positive in two independent assays. *** It was unclear from this report whether the 5.6% and 3.5% overlapped at all - in our analysis, we assumed that all positive samples overlapped and used 45/800 as the overall rate of seropositivity. ϕ Adult subjects specified. ◆Persons ≤ 18 and/or ≥ 60 years specifically excluded from analysis. ◆◆Persons ≥ 50 years excluded from analysis. ◆◆◆ Persons ≤ 12 or ≥ 60 years excluded from analysis.

Materials and Methods

Case definition: A confirmed case of H5N1 infection under WHO guidelines is defined (at a minimum) as a person who is documented to have an acute febrile illness with respiratory symptoms, have a known exposure to H5N1 virus during the 7 days preceding presentation, and have laboratory-based molecular confirmation of H5 virus infection (SOM Table 1) (*3*).

Where any ambiguity existed for the primary meta-analysis (using WHO criteria, see methods), we were conservative in our interpretation of data so as not to overestimate seroprevalence. This applies, for example, to one of the largest studies of 1,525 poultry workers by Bridges *et al.* (*4*), in which only 52% of samples positive in a primary screen were examined by a secondary screen; the authors extrapolate, from the rate of secondary screen confirmation, an overall positive rate of 10% within their sample population. To meet WHO criteria, we only scored as positive samples that were confirmed in two different assays, resulting in an artificially low seroprevalence.

Of note, many of the studies in this analysis exclude persons over the age of 60 and/or under the age of 18, while the majority of confirmed H5N1 infections have occurred in people under the age of 18 (SOM Table 2) (*27*). Furthermore, there is a relatively narrow window of detection for prior H5N1 infection by serology, because peak titers are found during weeks 4 - 6 post infection and a reduction by 4 - 32-fold in H5N1

neutralizing antibody titer is observed by 10-11 months post infection (*6*). Maximum sensitivity for detection of H5N1 serum antibody can be achieved by using a microneutralization assay with confirmation by Western blot to a sensitivity of 80% and specificity of 96% (*28*). This 80% sensitivity has been approximated from data describing seropositivity, using WHO criteria, in serum from persons with confirmed H5N1 infection (*6, 12, 29*). Together, these observations suggest that the true rate of H5N1 infection may be underestimated in this analysis.

The study population of this meta-analysis is highly heterogeneous. Data were obtained using non-standardized methods in different countries over a period of twelve years. It is also likely that distinct H5N1 virus strains were circulating in different study populations, with some strains infecting humans more efficiently than others. We have attempted to standardize the interpretation of the combined data by using the WHO criteria for H5N1 seropositivity, where possible. The majority of study subjects lived in rural regions with documented human or avian H5N1 infections. While some study participants lived in urban areas, sufficient data from non-specific exposure groups did not exist in order to generate a meaningful sub-analysis of urban versus rural H5N1 seroprevalence.

Meta-analysis

Meta-analysis was used to combine the individual estimates of seroprevalence from each study into an overall seroprevalence estimate. The meta-analysis employed a random effects approach as described by DerSimonian and Laird (*9*), to account for the clear variability present between the individual study estimates. Two meta-analyses were performed, one using the modified WHO criteria for seropositivity, and the other using the criteria reported by each respective author. Forest plots summarize the results from each study, and overall, in terms of estimated seroprevalence with associated 95% confidence intervals (*30*). The confidence intervals for individual studies are based on the "exact" approach of Clopper and Pearson (*31*).

Data Evaluation

Data in studies were evaluated by WHO criteria for serological diagnosis of human infection with H5N1 influenza virus. The WHO protocol for serological identification of antibodies against avian influenza A (H5N1) is either i) the detection of a 4-fold or greater increase in neutralizing antibody titer in paired acute and convalescent sera, with the convalescent serum having a titer of ≥1:80, or ii) antibody titre of ≥1:80 in a single serum collected at day 14 or later after onset of symptoms and a positive result using a different serological assay (e.g., H5-specific Western blot, hemagglutination inhibition assay). To allow for the detection of asymptomatic infections, we modified the WHO criteria for this analysis by eliminating the requirement that study participants be symptomatic. Of note, these WHO criteria for identification of H5N1 antibodies are not

specific for the identification of viruses expressing a neuraminidase subtype 1 or for viruses expressing a hemagglutinin with a multi-basic cleavage site. However, it is more likely that human infection is due to highly pathogenic avian influenza viruses (with a multi-basic cleavage site) since they replicate to high titers in infected poultry.

Search Strategy and Inclusion/Exclusion Criteria

We searched PubMed for articles published between 1999-2011 that describe antibody-based evaluation of human serum for evidence of H5N1 virus infection. Search terms included: human, influenza A, H5N1, seropositivity, seroprevelance, serology, subclinical, infections. Studies were included in the primary analysis if they clearly state methods that are interpretable by WHO criteria for serological diagnosis of human infection, the study population was >10 people, whether or not the participants had known exposure to H5N1-infected animals or humans, wore personal protective equipment during known exposure, or received prophylactic medications following known exposure. Studies/portions of studies were excluded if they describe patients with confirmed H5N1 infection or if they analyzed populations already taken into account in a separate study. These criteria eliminated some published reports (*6, 32-37*). After completion of the analysis, three papers came to our attention which had been excluded (*38-40*); seropositivity in these studies were were 0/60 ((*40*), not WHO criteria) 0/83 ((*38*), not WHO criteria) and 1/110 ((*39*), WHO criteria).

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