A Systematic Review and Meta-Analysis of the Seroprevalence of Influenza A(H9N2) Infection Among Humans

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Introduction. Given that influenza A(H9N2) is recognized as a pandemic threat, we evaluated the overall burden of influenza A(H9N2) infections among avian-exposed human populations.

Methods. We performed a systematic search of PubMed, AGRICOLA, and CAB Abstracts databases for literature published during 1997–2013. Studies reporting serological evidence of human influenza A(H9N2) infection among avian-exposed populations were included. We used a World Health Organization (WHO)–recommended case definition for serological evidence of infection based on results of hemagglutination inhibition (HI) and microneutralization (MN) assays. We calculated overall seroprevalence through a random effects meta-analysis model.

Results. Seroprevalence data reported by the studies ranged from 1% to 43% (median, 9%) by HI, which was not significantly different from the seroprevalence estimated through the WHO-recommended case definition (median, 1.3%; range, 0.5%–42.6%). Reported seroprevalence by MN ranged from 0.6% to 9% (median, 2.7%), which was greater than the seroprevalence estimated through the WHO-recommended case definition (median, 0.3%; range, 0.1%–1.4%).

Conclusions. A small proportion of avian-exposed humans had evidence of influenza A(H9N2) infection. As the virus has a near global distribution in poultry, it seems likely that present surveillance efforts are missing mild or asymptomatic infections among avian-exposed persons. It seems prudent to closely monitor avian-exposed populations for influenza A(H9N2) infection to provide prepandemic warnings.

Keywords. influenza A virus; H9N2 subtype; seroprevalence; systematic review; meta-analysis; hemagglutination inhibition test; microneutralization test.

The first known infection by influenza A(H9N2) occurred in 1966 among turkeys in Wisconsin. Since the 1990s, the virus has readily circulated among domestic poultry populations in several Asian countries and is now considered to have a near global, albeit sporadic distribution among poultry. Influenza A(H9N2) has also been identified in wild birds, domestic mammals, and, occasionally, in humans [1, 2]. Zoonotic transmission of influenza A(H9N2) was not considered a concern until 1998–1999, when the virus was first isolated from pig samples [2]; seropositive human cases were detected in Guangdong Province, China [3]; and the virus was isolated from hospitalized patients in Hong Kong [1]. Although human influenza A(H9N2) cases have only been reported in Hong Kong and mainland China, and more recently in Bangladesh [1, 4, 5], serological evidence of human infection has been reported in Asia, the Middle East, Africa, and parts of North America (Figure 1).

Human infection with influenza A(H9N2) generally results in mild or asymptomatic illness and often goes unnoticed [6]. However, influenza A(H9N2) is a potential pandemic threat because of its rapid evolution, ability to acquire and transfer genetic materials from other pathogenic subtypes, and efficiency in poultry-to-human...
transmission [7, 8]. Currently, influenza A(H9N2) infections in poultry have led to multiple reassortments, resulting in many novel genotypes of influenza A(H9N2) with gene segments from various lineages and serving as a constant reminder of its ability to acquire and transfer pandemic potential among the circulating influenza strains [9, 10]. Molecular analyses of the recently emerged influenza A(H7N9) and influenza A(H10N8) suggest that they have acquired gene segments from influenza A(H9N2) [11, 12], which probably enabled them to survive and be repeatedly transmitted among poultry before adapting to humans. Unlike other avian influenza viruses (eg, influenza A[H5N1]) that chiefly bind to human receptors in the lower respiratory tract, influenza A(H9N2) binds to α-2,6 sialic acid receptors that are abundant in the human upper respiratory tract [13], allowing for much greater infection efficiency.

Given the pandemic potential of influenza A(H9N2), it is important to know the burden of human infections. However, the variation attributed to serological assays makes it difficult to accurately estimate the overall burden of influenza A(H9N2) infections. Hence, for this systematic review we sought to evaluate the overall burden of influenza A(H9N2) infection among avian-exposed human populations by summarizing serological data identified in the published literature. In addition, we compared the disease burden revealed by the 2 assays with that yielded by a standardized case definition for serological evidence of influenza A(H9N2) infection.

**METHODS**

**Search Strategy**

We performed a systematic, Internet-based search using PubMed, AGRICOLA, and CAB Abstracts databases. The following keywords were used: “influenza H9N2” AND “serological surveys” OR “seroprevalence” OR “sero-prevalence” OR “sero-epidemiology” OR “sero-epidemiology”. To minimize publication bias, we retrieved the reference lists and manually searched for relevant studies that met our inclusion criteria. Since the initial serological and virological evidences of first human influenza A(H9N2) infections were reported between 1997 and 1999 [1, 3, 14], our temporal limit for the search was set from January 1997 to December 2013. If data were missing, we contacted the corresponding authors of the studies. We followed PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines for this systematic review and used meta-analysis to aggregate the data [15]. Our protocol for the systematic review will be made available upon request.

**Inclusion and Exclusion Criteria**

After receiving training, 2 individuals (each with a Masters in Public Health degree) independently reviewed the abstracts and identified articles for detailed assessment. We considered studies regardless of their language, and abstracts that were not written in English were translated. Articles were selected only if an abstract contained data on serological assessment of human samples for evidence of influenza A(H9N2) infection and if the study involved a poultry and wild bird–exposed population. We included surveillance reports, cross-sectional studies, and prospective studies in our data extractions. Studies that were not done in avian-exposed populations, diagnostic evaluations, pathogenesis models, animal models, reviews, comments to editors, perspectives, and personal opinions were excluded because they did not provide primary data for a population-
based estimate of influenza A(H9N2) seroprevalence in populations occupationally exposed to poultry and wild birds.

**Data Abstraction and Study Outcome**

We used a standardized method for extraction of data from the articles. For each of the studies, we first determined the type of serological test they used and the total population tested. Later, we extracted the number of end point–titer positives for each of the dilution cutoffs (1:10 to 1:640). For our analysis, we aggregated hemagglutination inhibition (HI) assay and microneutralization (MN) test positivity data at different dilution cutoffs, as well as data regarding seroconversion (Supplementary Table 1 and 2). For cohort studies, we counted the number of individuals followed up for a specific year and the number of individuals who tested positive by means of the assays or seroconverted during the follow-up period. Results from the cohort studies were broken down by the number of years studied.

**Study Quality and Risk-of-Bias Assessment**

We assigned 2 reviewers to critically review and assess the quality of the studies and reduced the risk of bias through a structured approach described elsewhere [15, 16]. We assessed each study for 11 assessment criterion: whether it was a population-based study; whether the sample size was ≥100; whether HI was performed; whether MN was performed; whether it used horse red blood cells (RBCs) for the HI assay; whether the World Health Organization (WHO) standard cutoffs for HI assays (an HI titer of ≥1:160 titer or a 4-fold rise in titer over time) were used to define seropositivity [17]; whether the WHO standard cutoffs for MN tests (an MN of ≥1:80 or a 4-fold rise in titer over time) were used to define seropositivity [17]; whether the study population had avian exposures; whether study participants’ age distribution was mentioned; whether end point titers of the HI assay were provided in the results; and whether end point titers of the MN assay were provided in the results.

**Statistical Analysis**

We calculated nonadjusted seroprevalence rates for the range of end point titers used for each diagnostic test. The rates were modeled by applying a random effects meta-analysis model that assumed heterogeneity across the aggregated study population. For each of the titer cutoffs for a test (either HI or MN), we generated a forest plot that showed individual prevalence estimates for each study, by study size.

We assessed heterogeneity by the Pearson χ² test, which used the I² index statistic to estimate the proportion of total variation due to heterogeneity [18]. An I² score of <25% indicated low heterogeneity, and a score of ≥75% suggested a very high degree of heterogeneity.

We considered an HI titer of ≥1:160, an MN titer of ≥1:80, or seroconversion (ie, a 4-fold rise in HI or MN titer over time) as a standardized case definition for influenza A(H9N2) seropositivity, because it is generally recognized as such by the WHO and others [17]. We performed the meta-analysis of the seropositive results through this standard case definition and compared results with the reported influenza A(H9N2)–seropositive results from the selected studies. Because the mean values were markedly affected by outliers, we used median estimates and performed the Wilcoxon rank sum test to assess whether there was a significant difference in the influenza A(H9N2) seroprevalences calculated through the WHO standard case definition and the studies’ individual case definitions.

**Mapping Influenza A(H9N2) in Humans and Animals**

We performed a database search on PubMed and CAB Abstracts to identify reports of human and animal cases of influenza A(H9N2) infection. We also reviewed reports from the WHO (available at: http://www.who.int), the World Organization for Animal Health (available at: http://www.oie.int), and the Food and Agriculture Organization of the United Nations (EMPRES-i data sets; available at: http://empres-i.fao.org/eipws3g/#h=1) to identify reported animal and human influenza A(H9N2) cases and mapped them to show their global distribution based on the serological and virological assessments.

**RESULTS**

**Search Results and Study Selection**

Our literature search identified 193 studies. Sixteen were duplicates and were removed. With the previously stated inclusion and exclusion criteria, we screened the remaining study abstracts and identified 31 articles for full review. We received replies from 5 of 6 authors of the selected articles. Details of the article-screening procedure and reasons for exclusion are summarized in Figure 2.

**Study Characteristics**

Through screening, we identified data from 25 articles that were suitable for meta-analysis [3, 19–43]. The articles were published between 1999 and 2013 from 10 countries of Asia, Africa, the Middle East, and North America. In the majority (78%) of the studies reporting participants’ age, all participants were aged ≥18 years, and all of the reports included participants who had varying degrees of avian exposure (Supplementary Table 3). Avian exposure ranged from being in daily contact with poultry in the live bird markets to having occasional contact with poultry in farms and rural villages and with wild birds. The characteristics of the individual studies are detailed in Supplementary Table 4. Of the 25 articles, 14 (56%) reported results of HI assays, with influenza A(H9N2) seroprevalences ranging from 1% to 43%. MN assay data were available from 14 studies (56%),...
reported influenza A(H9N2) seroprevalences ranged from 0.0% to 9.1% (Supplementary Table 1 and 2).

Quality and Heterogeneity of Selected Studies
We detailed the quality assessments of the individual studies in Supplementary Table 3. Of the 25 studies included for final analysis, 20 were cross-sectional surveys, 4 were prospective cohort studies, and 1 involved surveillance. Of the 14 articles that reported performing HI assays, 8 (57%) considered a titer cutoff of ≥1:20 for influenza A(H9N2) seropositivity, 4 (29%) had a titer cutoff of ≥1:40, and 2 (14%) had a titer cutoff of ≥1:160 (Supplementary Table 1). Of the 14 studies that reported performing MN assays, 9 (64%) considered a ≥1:10 titer cutoff for influenza A(H9N2) seropositivity, 2 (14%) had a titer cutoff of ≥1:20, and 2 (14%) had a titer cutoff of ≥1:40 (Supplementary Table 2).

We identified a high degree of heterogeneity of the selected studies that performed HI assays (I² = 0.98; P < .001) and MN assays (I² = 0.88; P < .001) with their individually defined cutoffs for influenza A(H9N2) seropositivity (Figure 3).

**Influenza A(H9N2) Seroprevalence**

The meta-analysis demonstrated that the overall median HI seroprevalence calculated using the antibody cutoffs reported by the studies was 4.9% (range, 0.6%–42.6%; I² = 0.99), which was not significantly different from the standardized case definition (≥1:160) for seropositivity (median seroprevalence, 1.3%; range, 0.5%–42.6%; I² = 0.99; Figure 3A and 3B). For the MN meta-analysis, the overall reported median seroprevalence was 2.7% (range, 0.5%–9%; I² = 0.88), which was significantly greater (P < .05) than that yielded by the standardized case definition (≥1:80) for seropositivity (median seroprevalence, 0.3%; range, 0.1%–1.4%; I² = 0.29; Figure 3C and 3D).

The HI and MN assay titers at individual cutoffs were analyzed for meta-seroprevalence and plotted with their 95% confidence intervals in Supplementary Figure 1. Data presented in Supplementary Figure 1A demonstrated that the seroprevalence at an HI dilution of ≥1:10 was about 9%, which gradually decreased to 4% at a dilution of ≥1:160 and peaked to 8% at a dilution of ≥1:320 before diminishing at a ≥1:640 dilution. Supplementary Figure 1B demonstrated a gradual decrease in
MN titer positivity, from 1.7% to 0.05%, from dilution 1:10 to dilution 1:320.

**Global Distribution of Influenza A(H9N2) in Humans and Animals**

Figure 1 shows the global distribution of reported influenza A(H9N2) infections in humans, poultry, pet birds, and other domestic animals. Influenza A(H9N2) was detected in humans chiefly in China, Hong Kong, and Bangladesh. However, serological evidence of human influenza A(H9N2) infection has been found in Asia, the Middle East, Africa, and North America. Thus far, influenza A(H9N2) has been detected in wild waterfowl, pigs, dogs, horses, and a companion bird in China, Hong Kong, and Japan. However, the virus had a near global distribution in domestic poultry populations.

**DISCUSSION**

We performed a systematic review and meta-analysis with the goal of estimating the global prevalence of influenza A(H9N2) infection among avian-exposed populations. Studies reporting seroconversion on the basis of HI and MN assay results identified that a relatively small proportion of the at-risk population had evidence of previous infection with influenza A(H9N2). Compared with the seroprevalence of influenza A(H9N2) reported by the studies, the WHO-recommended standard cutoffs resulted in an even lower seroprevalence through MN assays.

Although the prevalence of human infection is low, it may still pose a global pandemic threat because of the following factors. First, the virus has a near global distribution. Should these viruses become highly transmissible and virulent, they may...
cause marked worldwide morbidity. Although reports on influenza A(H9N2) antibody kinetics are scarce, kinetic data from influenza A(H5N1) infections suggest that individuals who had mild influenza A(H5N1) infection experienced reduced antibody titers, dropping from 4-fold to 32-fold 11 months after infection [44]. Second, there is considerable probability of the immune system failing to produce an anti-H9 neutralizing antibody response [44, 45]. Humans in general have poor immune responses to infections with avian viruses. For instance, studies have shown that individuals with mild or asymptomatic influenza A(H5N1) infection had a lower initial antibody titer than individuals who developed severe illness [44]. Third, because influenza A(H9N2) commonly causes mild or asymptomatic human infections, it seems reasonable to assume that the infection will trigger a weaker immune response and lower detectable antibody titers than other highly pathogenic influenza viruses. However, there is currently no universally agreed upon antibody titer cutoff for these assays, which explains why different antibody titer cutoffs were used in the included studies. Selecting a lower titer cutoff can often lead to overestimation of the seroprevalence of influenza A virus infections, particularly due to cross-reactivity with commonly circulating seasonal influenza viruses, resulting in a low sensitivity for assays such as the HI assay [45–47]. To overcome such limitations of traditional MN and HI assays, newer diagnostic tests, such as the virus-specific T-cell response assay and the subtype-specific pseudotype particle-based MN assay, have been developed [44, 45]. However, one of the major limitations of these new assays is their time dependence. The assay results vary depending on when a case was sampled after the hosts’ infection. This is particularly striking for the virus-specific T-cell response assay because T-cell responses are only detectable within a limited interval after an infection [48]. Overcoming these limitations may require the development of standardized sampling protocols specific to the assays or development of a modified assay that minimizes these constraints.

Low rates of influenza A(H9N2) infection in poultry-exposed individuals may cause an epidemic with a longer duration or a greater magnitude than if the virus was introduced to a completely unexposed population [49]. The virus also demonstrates its potential to efficiently transmit itself from avian hosts and share genetic materials with other highly pathogenic influenza A virus subtypes of public health significance (eg, H5N1, H7N9, and H10N8) [11, 12, 50]. These phenomena raise the possibility that influenza A(H9N2) may acquire genes from highly pathogenic influenza virus subtypes and cause large epidemics. It also seems possible that influenza A(H9N2) may transfer genes to other low-pathogenic influenza viruses to cause sporadic human infections, as has occurred with influenza A(H7N9) and influenza A(H10N8).

Our study has multiple limitations. First, we had to calculate the nonadjusted seroprevalence of influenza A(H9N2) infection in avian-exposed populations, because not all studies reported demographic characteristics (eg, age distribution) for their sampled populations (Supplementary Table 3). Second, only a handful of the studies included in our analysis used horse RBCs for their HI assay; not doing so is thought to reduce the sensitivity of the outcome. Third, we generally expected to identify fewer individuals to have higher antibody titers, because titers wane over time. Our analysis identified a nonlinear decrease in seroprevalence over increasing titer cutoffs through HI assays (Supplementary Figure 1A). This is not surprising, because many of the studies included for meta-analysis were cross-sectional in nature, and the individuals were expected to have elevated titers of antibodies against influenza A(H9N2). This may also be the result of lower sensitivity and specificity of the HI assay, which is supported by Supplementary Figure 1B, which shows a linear decreasing trend of influenza A(H9N2) antibody titers measured by the MN assay. Finally, a few of the studies used noncirculating influenza A(H9N2) strains in performing the MN assay, which might have reduced the sensitivity for detecting cases. However, because the virus has a near global distribution in poultry and efficient surveillance tools are not always in place, the studies might have identified evidence of infections that were missed by routine surveillance efforts.

Findings from the meta-analysis suggest that few avian-exposed humans are infected with influenza A(H9N2). Because many of the influenza virus subtypes, including influenza A(H9N2), produces mild or asymptomatic infections in humans, the current serological assays have a limited sensitivity and specificity to identify evidence of infections. Developing standardized sampling protocols specific to the assays or development of a modified assay that could detect serological evidence of mild or asymptomatic infections could minimize the current constraints. However, should influenza A(H9N2) further adapt to human hosts and develop more-virulent characteristics, they may spread quite rapidly among humans, causing significant morbidity and mortality. Efforts should be increased to conduct more-aggressive surveillance for influenza A(H9N2) strains, such that genetic changes might be identified in time to provide prepandemic warnings.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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**References**


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