*Pakistan J. Zool., pp 1-8, 2025.* **DOI**: https://dx.doi.org/10.17582/journal.pjz/20181127121128

# Prevalence of H9 Avian Influenza Virus among Poultry Workers in District Rawalpindi, Pakistan

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## ABSTRACT

Avian influenza virus (AIV) subtype H9N2 has become endemic in poultry in Pakistan and carries great zoonotic potential. The purpose of the present study was to report the seroprevalence of the H9 virus among the poultry workers. Overall 325 sera samples were obtained from poultry-exposed population and a general population of Rawalpindi city between January 2018 to September 2018. Haemagglutinin-inhibiting (HI) assay was performed to determine the seropositivity for serum samples. Overall seroprevalence rate of anti-H9 antibody titre was 12 % (39/325) in occupational individuals (>11%) and general population (<1%). Anti-H9 antibody titre was 8.8%, 8.3% and 34.1% in age groups of 16-39, 40-59 and >60 years, respectively among poultry-exposed workers. On the other hand, anti-H9 antibody titre of general population was 25% and 16.6% in age groups of 16-39 and 40-59, respectively. The highest percentage of seropositivity (13.7%) of antibody titer to H9N2 was found in the poultry butchers followed by poultry farm workers 11.4%. The lowest seropositivity (7.1%) was recorded in veterinarian. Serum samples from males revealed significantly higher anti-H9 antibody titre (12.4%) than females (8.5%) of occupational individuals. It was revealed that antibody titer of butcher, had significant ($P<0.05$) difference from antibody titer of poultry salesmen and veterinarians but non-significant difference from poultry farm workers. In general population, 17 individual (68%) had no detectable antibody titer against H9N2 and only 12% people had antibody titer of ≥ 1:40. Our findings revealed potential transmission AIV H9 subtype from avian to humans and highlights that poultry professional linked people are at great risk of AIV infections than general population.

**Article Information**
**Received 27 November 2018**
**Revised 20 December 2018**
**Accepted 31 December 2018**
**Available online 07 May 2025**
**(early access)**

**Authors' Contribution**
**SU, MEB and MF designed the study. SS performed the experimental work. MEB and MF supervised the work. MU, AK and MNA provided technical support and participated in draft and revision of the manuscript.**

**Key words**
**Seroprevalence, Serum, Avian influenza virus, Poultry workers, Rawalpindi, Pakistan.**



## INTRODUCTION

Influenza A virus belongs to the family *Orthomyxoviridae* and classified on surface glycoprotein which are designated as hemagglutinin (HA) and neuraminidase (NA). Till to date 18 HA and 9 NA subtypes have been identified in different species (Tong *et al*., 2013). Theoretically 144 possible combinations of HA-NA subtype are possible. At least 116 of subtypes have been isolated from birds. Aquatic avian species are the natural hosts of influenza A viruses, and occasionally transmit to mammalian species, including humans leading to zoonotic infections (Subbarao and Joseph, 2007; Perdue and Swayne, 2005). Avian influenza viruses (AIVs) play a key role in the emergence of pandemic strains. Mutations and/or reassortment between avian and human/swine viruses may result in potential human influenza infection or even a new pandemic strain.

Low pathogenicity avian influenza virus (LPAIV) subtype H9N2 is the most prevalent LPAIV in poultry in the world and have a great zoonotic potential (Pusch and Suarez, 2018). AIV of the H9N2 subtype first detected from turkeys in the United States in 1966 and since then H9N2 subtype infections have been reported in wild and domestic poultry of several countries of Asia, Middle East, Europe, Africa and North America (Liu *et al*., 2014; Horman *et al*., 2018; Pusch and Suarez, 2018). LPAİV of subtype H9N2 are classified into Eurasian and American lineages on the basis of HA gene sequences. The Eurasian lineage are further divided into three distinct sub-lineages: A/Duck/Hong Kong/Y280/1997 (BJ/94-like or Y280 like), A/quail/Hong Kong/G1/97-like (G1-like) and A/duck/Hong Kong/Y439/97 (Y439-like or Korean-like). A fourth

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0030-9923/2025/0001-0001 $ 9.00/0

poultry adapted lineage has been circulating in Europe since at least 2013 primarily in the turkey industry. Of the four defined H9N2 poultry lineages, only two lineages, the G1 and the Y280 lineages, are associated with human infections (Pusch and Suarez, 2018).

The poultry-adapted H9N2 viruses have not only become a major concern for poultry health in the last 20 years, but also a threat to public health. Some amino acid substitution (L226Q) in the HA gene enables these viruses to bind to analogs sialic acid receptors in humans (SAα2, 6Gal) thus enhancing zoonotic potential of these viruses. Recent experimental studies have revealed that LPAİV of subtype H9N2 in Guinea pigs could efficiently transmit to ferrets *via* respiratory droplets. Moreover, serial passages of LPAIV of subtype H9N2 thought to induce amino acid substitution leading to efficient contact transmission among mammalian models. In mainland China, LPAİV of subtype H9N2 was first isolated from Guangdong province in 1994. It has subsequently spread and become the most prevalent subtype of influenza viruses in poultry (Xu *et al*., 2007; Lee and Song, 2013; Sun and Liu, 2015; Umar *et al*., 2016). The first outbreak LPAIV of subtype H9N2 in Pakistani and İranian poultry was reported in 1998; isolates showed a close relationship to subtype H9N2 AIVs circulating in Hong Kong, China during 1997 that were grouped within the G1 lineage. LPAİV of subtype H9N2 have become endemic in Pakistani poultry despite of control measures (Umar *et al*., 2016). These are becoming major threat to poultry industry and can contribute in reassortment events leading to the generation of novel AIV that can infect humans. In recent years, LPAİV of subtype H9N2 genes have reasserted extensively, generating novel genotypes on the Indian subcontinent. Widespread co-circulation of H9N2 with other AIVs (*e.g.*, highly pathogenic AIVs H5N1 and H7N3) could instigate the generation of novel variant and reasserted viruses, possibly with increased zoonotic potential (Chaudhry *et al*., 2015; Horman *et al*., 2018; Pusch and Suarez, 2018). Recently, a novel AIV of H7N9 subtype emerged in China and was found to infect humans (Gao *et al*., 2013). All the genes from the H7N9 virus in China were of avian origin, with six internal genes from AIV H9N2 subtype (Gao *et al*., 2013; WHO, 2013).

After first isolation of H9N2 AİV in China from chicken in 1994, domestic pigs were confirmed with H9N2 infection in 1998 in Hong Kong. A year later, H9N2 viruses were isolated for the first time from humans in Hong Kong in 1999 and further human infections were reported in 2003. H9N2 AIV isolated from diseased humans showed close association with avian H9N2 viruses isolated from chicken. World Health Organization (WHO) in 2015 has reported new cases in Egypt and Bangladesh (Haideri *et al*., 2016). Although, H9N2 human infections usually show no clinical signs or only mild clinical signs. However, their wide circulation raises public health concerns on their potential role as candidates for the next influenza pandemic. Nevertheless, seropositivity against H9 subtype in humans have been reported particularly in occupationally exposed workers in China, India, Iran, Veitnam, Thailand, Cambodia, Romania, Egypt and Pakistan suggesting infection is commonly occurring (Pusch and Suarez, 2018; Ahad *et al*., 2014Anvar *et al*., 2013; Haidari *et al*., 2016; Gomaa *et al*., 2015; Wang *et al*., 2015).

Serological studies have provided strong evidence of H9 avian viral infections in humans. In 2006, 1.7% of the serum specimens from farmers in Xinjiang (north-western China) were H9 positive (Jia *et al*., 2009). Another serologic surveillance study showed that in Guangzhou from 2007 to 2008, the prevalence of anti-H9 antibodies among all participants was much higher than anti-H5 antibodies (Wang *et al*., 2009). Similarly, a higher seroprevalence has been reported from Pakistan recently in poultry exposed people (Ahad *et al*., 2013). All these data indicate the existence of asymptomatic AIV H9N2 infections among humans, although the H9N2 seropositivity has not yet been associated with any clinical disease (Ahad *et al*., 2013, 2014; Śmietanka, 2014; Al-Garib *et al*., 2016).

It has been demonstrated that humans acquired AIV primarily through close contact with infected birds or contaminated poultry products. Therefore, possible acquisition of H9N2 asymptomatic infection may be through routine contact to birds (*e.g.,* Purchasing live or fresh killed poultry such as chicken, duck, pigeon, quail from wet markets, touching unclean eggs with poultry feces, preparing poultry for cooking, *etc*.). The specific environmental exposure could be a risk factor for AIV infection. A previous survey report suggested that contaminated environmental exposure (*e.g.* contaminated water) as a risk factor for seropositivity (Wang *et al*., 2015). There is scarcity of data on serosurveillance of H9N2 infections in human popultion in Pakistan. To fill the literature gap, we, therefore designed the present study and investigated the seroprevalence of LPAİV of subtype H9N2 in human population to better understand the potential of the H9N2 infections to human in Pakistan.

## MATERIALS AND METHODS

*Sample collection*

The current study plan was approved by animal welfare, ethics and research committee of Virtual University of Lahore, Pakistan. After approval, a total of 325 blood samples were taken from occupational poultry-exposed workers and general population in January 2018 to September 2018 in Rawalpindi district, Pakistan. Poultry-exposed workers defined as butchers, retailors,

salesmen, poultry transporters, and veterinarians involved in vaccination and treatment of poultry flocks. The general population (control group) consisted of house wives, children and unemployed people who have no exposure to poultry. The samples were collected purely on the willingness of people. All people involved in this study showed no illness and were not vaccinated against avian influenza. Demographic characteristics (age and gender) and work-related exposure (poultry butchers, retailors, salesmen, poultry transporters, and veterinarians) related information were collected during the study (Table I). Blood samples were kept at room temperature for 1 h and then centrifuged to harvest serum samples. The sera samples were preserved at -20°C till processed.

*Haemagglutination inhibition (HI) assay*

Anti-influenza (H9) anti bodies were tested from serum samples using haemagglutination inhibition (HI) assays according to the protocols described by World Health Organization (WHO) for influenza viruses (WHO, 2011). To avoid non-specific HA agglutination and false positive results, sera samples treated with receptor-destroying enzyme (RDE) (Denka Seiken, Tokyo, Japan) prior to HI assays. Special V-bottom 96-well plates were used for HI assay as reported previously. Briefly, chicken red blood cells (RBCs 0.5%) suspension was prepared in Phosphate buffer saline (PBS). RDE treated sera were diluted in two fold serial dilutions with PBS in HI assay microtiter plates. After that, 4 HA units of virus antigen were mixed to the wells. Subsequently, microtiter plates incubated at room temperature (RT) for 30 min. After 30 min incubation, 0.5 % chicken RBCs were then mixed and the plates were again incubated at RT for 30 min. Along with unkonw tested sera samples, known positive and negative serum controls were also run for better comparison and evaluation. Reciprocal of the highest serum dilution that completely inhibiting haemagglutination of 4 HA units of the virus was used to calculate HI titre. HI titre showing a value of 40 or greater were considered positive as reported previously (de Jong *et al*., 2003; Killian *et al*., 2013).

*Statistical analysis*

Statistical analysis was performed to determine seropositivity percentage. Serum samples showing a value of > 1:40 for Hi titre were considered negative in this study. A value of P>0.05 was used for statistical significance.

**Table I.- Characteristics of participants involved in the study (*n* = 325).**

| Characteristics | Poultry exposed population | General population |
|---|---|---|
| Total | 300 | 25 |
| **Gender** | | |
| Male | 265 (88.3%) | 10 (40%) |
| Female | 35 (11.7%) | 15 (60%) |
| **Age (years)** | | |
| 0-15 | 25 (8.3%) | 3 (12%) |
| 16-39 | 113 (37.6%) | 4 (16%) |
| 40-59 | 131 (43.6%) | 12 (48%) |
| ≥60 | 41 (13.6%) | 6 (24%) |
| **Occupation** | | |
| Poultry butchers | 109 (36.3%) | - |
| Poultry farm workers | 122 (40.6%) | - |
| Poultry salesmen | 55 (18.3%) | - |
| Veterinarians | 14 (4.6%) | |



**Table II.- HI assay antibody titre against LPAİV (H9) in different age groups and genders of selected poultry-exposed and general population.**

| Age (years) / Gender | Samples tested (n=325) | HI titre | | | | | | | No (%) of samples with HI titre ≥ 1 : 40 |
|---|---|---|---|---|---|---|---|---|---|
| | | >1:10 | 1:10 | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | |
| **Poultry exposed population** | | | | | | | | | |
| 0-15 | 15 | 4 | 7 | 3 | 1 | 0 | 0 | 0 | 1 (4%) |
| 16-39 | 113 | 79 | 8 | 16 | 6 | 3 | 1 | 0 | 10 (8.8%) |
| 40-59 | 131 | 101 | 15 | 4 | 6 | 2 | 3 | 0 | 11 (8.3%) |
| ≥60 | 41 | 11 | 7 | 9 | 3 | 5 | 5 | 1 | 14 (34.1%) |
| Male | 265 | 185 | 24 | 23 | 15 | 8 | 9 | 1 | 33 (12.4%) |
| Female | 35 | 10 | 13 | 9 | 1 | 2 | 0 | 0 | 3 (8.5%) |
| **General population** | | | | | | | | | |
| 0-15 | 3 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0 (0%) |
| 16-39 | 4 | 1 | 0 | 2 | 1 | 0 | 0 | 0 | 1 (25%) |
| 40-59 | 12 | 3 | 4 | 3 | 0 | 0 | 2 | 0 | 2 (16.6%) |
| ≥60 | 6 | 4 | 2 | 0 | 0 | 0 | 0 | 0 | 0 (0%) |
| Male | 10 | 4 | 0 | 3 | 1 | 0 | 2 | 0 | 3 (3%) |
| Female | 15 | 6 | 7 | 2 | 0 | 0 | 0 | 0 | 0 (0%) |

**Table III.- HI assay antibody titre against LPAIV (H9) in selected poultry-exposed and general population (control subjects).**

| Occupation | n | Hi titre | | | | | | | No (%) of samples with HI titre ≥ 1 : 40 |
|---|---|---|---|---|---|---|---|---|---|
| | | >1:10 | 1:10 | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | |
| Poultry butchers | 109 (36.3%) | 75 | 12 | 7 | 9 | 2 | 3 | 1 | 15 (13.7%) |
| Poultry farm workers | 122 (40.6%) | 87 | 9 | 12 | 5 | 5 | 4 | 0 | 14 (11.4%) |
| Poultry salesmen | 55 (18.3%) | 27 | 14 | 8 | 1 | 3 | 2 | 0 | 6 (10.9%) |
| Veterinarians | 14 (4.6%) | 6 | 2 | 5 | 1 | 0 | 0 | 0 | 1 (7.1%) |
| Control | 25 (8.3%) | 10 | 7 | 5 | 1 | 0 | 2 | 0 | 3 (12%) |

## RESULTS

A total of 325 sera samples were taken from live poultry exposed people (n = 300) and the general population (n = 25). The majority of live poultry exposed people were in the age between 16 and 59 years (Table I). Aanti-H9 antibody titre were measured using HI assays and antibody titre ≥ 1 : 40 of serum samples were considered as positive.

HI titre for 325 serum specimens sampled between January 2018 to September 2018 are provided in Tables II and III. Overall seroprevalence rate of anti-H9 antibody titre was 12 % (39/325) in occupational individuals and general population (control group). The seropositivity for H9 AIV in occupational poultry-exposed workers was 8.8%, 8.% and 34.1% in age groups of 16-39, 40-59 and >60 years, respectively. On the other hand, seropositivity for H9 AIV in general population was 25% and 16.6% in age groups of 16-39 and 40-59, respectively.

The highest percentage of seropositivity (13.7%) of antibody titer to H9N2 was found in the poultry butchers followed by poultry farm workers 11.4%. The lowest seropositivity (7.1%) was recorded in veterinarian. It revealed that antibody titer of butcher, had significant ($P<0.05$) difference from antibody titer of poultry salesmen and veterinarians but non-significant difference from poultry farm workers (Table III). Antibody titer of butcher had differed significantly ($P<0.05$) as compared to group of general population. In the control group (general population group), 17 individual (68%) had no detectable antibody titer (Table II) against H9N2 and only 12% people had antibody titer of 1:40 or above (≥ 1 : 40).

## DISCUSSION

Avian influenza viruses (AIVs) are causing great havocs by continuous evolution, thus can play a key role in the occurrence of future pandemic. Recurrent interspecies spread of AIV from poultry to humans poses a great threat of a pandemic. Our findings revealed existence of avian-to-human transmission of AIV H9N2 subtype. Anti-influenza antibody titre was significantly higher in professional poultry-exposed workers than that in the general population in Rawalpindi. These findings are in line with previously reported studies from Pakistan, Iran and China (Heidari *et al*., 2016; Ahad *et al*., 2013; Jia *et al*., 2009; Wang *et al*., 2009, 2015; Huang *et al*., 2013, 2015; Li *et al*., 2017) indicating that that poultry workers are at risk of getting zoonotic AIV.

H9N2 is not endemic in poultry in USA, therefore, extremely low rate of seroprevalence of H9N2 infections in poultry workers or the general populations indicating lack of exposure to the virus (Gray *et al*., 2008; Liebler *et al*., 2011). The highest percentage of seropositive samples was found in Pakistan, Iran, and China because H8N2 is endemic in poultry. Occupationally exposed people were consistently found more seropositive than the general people. Ahad *et al*. (2014) reported very seroprevalence of H9N2 antibodies (>50%) in farm workers from different areas of Pakistan. Moreover, workers were at the highest risk of H9N2 infection at floor reared breeder flocks in Pakistan (Ahad *et al*., 2014). Live poultry population is much greater in southern China, therefore greater numbers of samples were found positive from that area than those from Northern China. Poultry workers that had contact with live birds had a much higher prevalence of H9N2 infection than those that worked in slaughtering plants or in wild bird habitats (Li *et al*., 2017). In Iran, all studies showed occupationally exposed groups also had a higher seropositive rate than general population samples (Heidari *et al*., 2016; Anvar *et al*., 2013; Hadipour and Pazira, 2011). The seroprevalence were 87, 76.2 and 72.5% in poultry farm workers, slaughter house workers and veterinarian, respectively in Iran (Hadipour, 2010). Anvar *et al*. (2013) reported seroprevalence of 1.6% and 11.5 % by HI and ELISA assays, respectively. In Vietnam, relatively a low seroprevalence (<10%) was noted in human population at different time points (Hoa *et al*., 2017). Similarly, a low seroprevalence (6%) was reported from non-vaccinated poultry workers in India (Pawar *et al*., 2012). In Romania, a serological survey revealed a seropositivity of around 9% among poultry and swine exposed human population (Coman *et al*., 2013).

The present study reveals that the highest percentage

of seropositivity (13.7%) of antibody titer to H9N2 was found in the poultry butchers who slaughter poultry birds at different meat shops followed by poultry farm workers (11.4%). Poultry butchers had high titer could be due to frequent handling live poultry and exposure to poultry viscera. The lowest seropositivity (7.1%) was found in poultry veterinarian. The possible reason for low seropositivity in veterinarians may be due to adapting some biosecurity and precautionary measures (*e.g.* use of masks and gloves during farm visits and birds examination). Moreover, human contacts with live poultry is common in live poultry markets in Rawalpindi that may have led to transmission of H9N2 influenza virus into humans similarly as reported previously (Peiris *et al*., 1999; Guan *et al*., 2000; Butt *et al*., 2005). In accordance with previous studies, the present study reveals that exposure of workers to infected poultry could be a risk factor and could lead to anti influenza antibodies seropositivity or asymptomatic infections to humans. A significant higher (P>0.05) anti-H9 antibody titre (12.4%) was observed in males than females (8.5%). The best possible for this trend could be more frequent exposures of males with infected poultry or environment as females tend to stay at home most of the times in Pakistan. In Pakistan, poultry related or linked business is usually occupied by men *e.g.* feeding poultry, selling poultry, slaughtering poultry.

Interestingly, anti-influenza H9 antibodies titre in the general population among 16–39 was 25% and among 40–59 was 16.6%. It can be assumed that young people in general population may get H9N2 asymptomatic infection through their routine work (*e.g.* travelling, purchasing live poultry or fresh meat, touching dirty eggs, handling poultry meat for cooking *etc*.). Contaminated environment can pose a risk for AIV infections poultry as well as in humans. Researchers has elaborated the role of exposure to a contaminated environment in their previous reports and suggested contaminated water can contribute to enhance seropositivity against AIV infections (Vong *et al*., 2008).

Prevalence of H9N2 AİV has increased over the last few years in Pakistan in live poultry despite of vaccination against H9N2. The efficacy of AIV vaccine strongly depends on the similarity of virus causing diseases and vaccine viral strains being in use (Lee *et al*., 2004; Zhang *et al*., 2012). The differences between both of them might lead to vaccine failure or low immunity development. Therefore, it is necessary to update commercial vaccine H9 strains according to the prevailing vaccine strains to provide sufficient protection. It has been observed that AIV H9 has been the most common subtype of AIV in birds in Pakistan. Recent studies revealed the events of genetic reassortment among AIV H7N9 and H9 N2 leading to the emergence of a noval reassortant H9N2 AİV in Pakistan and China (Chaudhry *et al*., 2015; Feng *et al*., 2013; Liu *et al*., 2013). Keeping in mind the genetic reassortment events and great genetic diversity in H9N2 variants, live poultry markets can play a potential role in influenza pandemic and could be a major risk factor for AIV infection among poultry linked people and general non-working population in Pakistan in coming years. The best way to prevent human infections are to prevent exposure to the virus. Because H9N2 viruses are endemic in so many countries, it is unlikely that control measures such as test and slaughter approaches can be used because of the high economic cost. Therefore, practical tools should be considered to reduce human exposure in live bird markets. The use of vaccination of poultry likely provides the most practical control tool to reduce human exposure. Vaccination for humans is currently not a practical option for H9N2 avian influenza because the clinical disease currently observed doesnot justify the expense. In addition because of the wide antigenic variation observed between and within lineages, a single vaccine would likely not protect for all potential H9N2 exposures. Preventing a pandemic with H9N2 is possible with the right strategy for controlling H9N2 in poultry. This includes implementation ofmonitoring programs, vaccination, good biosecurity at farms and live bird markets.

There are certain limitations of our study. For example, samples number was small and only collected randomly which could leads to false estimation. Moreover, we did not check the cross reactivity with H1N1 and H3N2 which are known to cross react with antibodies of H9N2 AIV thus could cause false seropositivity. In addition, Hi assays are not 100% reliable and specific. It has been reported in past that HI assays shows relatively less sensitivity for antibody detections of AIV in humans sera (Profeta and Palladino, 1986; Rowe *et al*., 1999) while microneutralization (MN) assay shows more sensitivity in detecting AIV antibodies in mammalian sera. Therefore, it's necessary to further confirm samples with MN assay after HI assays. Furthermore, we used only one representative of G1 lineage as antigen for HI assays. The use of other antigen (*e.g.* antigen from Y280 lineage) could alter the sensitivity of the assays.

To recapitulate, our findings revealed potential transmission AIV H9 subtype from avian to humans and highlighted that occupationally exposed workers are at great risk of AIV infections than general population. Hence, further detailed and more systemic approaches to study seroprevalence of H9N2 AİV infections in human and live poultry population in all over Pakistan is recommended to determine the relations between clinical significance and seropisitivity in humnas and identify possible solutions to reduce the transmission of AIV in poultry workers from live poultry. İmmuninization of poultry with effective vaccines is required for the effective

control of the virus. Moreover, biosecurity measures should be put into practice to decrease the prevalence of H9N2 AIV in poultry population and reduce the risk of exposure in poultry operators.

*Statement of conflict of interest*

The authors declare that there is no conflict of interest.

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