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RESEARCH ARTICLE

Serosurveillance to H9 and H7 Avian Influenza Virus among Poultry Workers in Punjab Province, Pakistan

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ABSTRACT

Among the different subtypes of avian influenza virus (AIV) H9 and H7 are able to infect poultry and human. These viruses in some countries has been isolated from the occupational hazard group of people, like veterinarian, poultry attendant and poultry retailers. The aim of this study is to determine the seroprevalence of H9 and H7 AIV subtype in different occupational people who are directly or indirectly involved with the poultry industry. Antibodies to H9N2 and H7N7 avian influenza virus were measured by modified horse RBC hemagglutination inhibition (HI) test using receptor destroying enzyme (RDE) treated sera. Total 465 human sera samples were analyzed who were directly exposed to poultry industry and 25 samples were taken as control that was not exposed to poultry industry. The highest (85.7%) seroprevalence against H9 was recorded in vaccinator and the lowest (30.4%) was recorded in veterinarian. On district wise the highest (82.1%) seroprevalence against H9 was observed in Toba Tek Singh district and the lowest (9.7%) was observed in the Islamabad. In case of H7 AIV subtype the highest (44.4%) seroprevalence was recorded in lab technicians and the lowest seroprevalence (11.1%) was recorded in butcher. By district wise the highest (57.9%) seroprevalence against H7 was recorded in Haripur district and the lowest (4.6%) was recorded in Gujranwala district.

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INTRODUCTION

Influenza A virus is classified on surface glycoprotein which are designated as hemagglutinin (HA) and neuraminidase (NA). So far 16 HA and 9 NA have been identified in wild migratory birds and in poultry around the globe (Fouchier *et al*., 2005; Cheema *et al*., 2011; Hadipour *et al*., 2011; Khan *et al*. 2012). The H9 subtypes cause mild conjunctivitis and respiratory disease in human. It poses a similar threat likes H5N1 as the mild infection may become virulent through antigenic drift or shift (Li *et al*., 2003). The H9N2 were frequently isolated from China. The H9N2 subtype was isolated in Hong Kong from 2 children who are suffering from respiratory disease. In Hong Kong, it also showed that blood donors had neutralizing antibody against H9N2 suggestive that it had infection before blood donation (Peiris *et al*., 1999). In one study it shows that H9N2 avian influenza virus can replicates in murine respiratory tract without prior adaptation (Qi *et al*., 2011). Recently the virus has been isolated from an immunocompromised patient (Cheng *et al*., 2011). From northern area of Pakistan low pathogenic H9N2 was isolated from a poultry flock. Antibodies against serotype H9N2 was demonstrated in 7 wild bird species (Khwaja *et al*., 2005). The H9N2 infections in human were reported around Rawalpindi and Islamabad (Bashir *et al*., 2003). In Netherlands in 2003 H7N7 causes infection in 89 human with one case of fatality (Koopmans *et al*., 2004). In British Columbia in Canada human infections with H7N7 have been reported. Out of 65, 57 had suspected and two were laboratory confirmed avian influenza infection by H7N7 subtype AIV (Tweed *et al*., 2004). Natural infection of human being infected with H7N7 virus associated with conjunctivitis can readily

be transmitted either directly from birds to human or by seals (Banks *et al*., 1998). It has been reported antibodies against Influenza A (H3) and (H1) cross react with avian influenza virus A (H9N2) that infect human being (Peiris *et al*., 1999; Eick *et al*., 2000). However sensitivity of HI test for detecting antibodies against H5N1 in human being against H6N5, H11N6, H13N6, equine A (H7N7) influenza viruses could be improved by using horse RBC instead of turkey RBC (Stephenson, 2003). Recent increase in awareness and surveillance of AIV zoonosis we investigated the sero-surveillance in the poultry workers who are directly involved with poultry especially poultry attendant mainly who are at the most vulnerable section of the people.

MATERIALS AND METHODS

Sampling strategy

Selection of study population: From 23707 poultry farms of Punjab, study population was selected in two steps following simple random sampling method. In first stage sampling, all poultry farms of Punjab were taken as sampling frame and one poultry farm as sampling unit whereas; in second stage sampling, all poultry attendant (354) of selected farms constituted the sampling frame with sampling unit of "a poultry attendant". A total of 85 poultry farms were selected from Haripur, Sialkot, Toba Tek Singh, Multan, Gujranwala, Faisalabad, Bahawalnagar, Lahore, Rawalpindi, Chakwal, Islamabad and Sheikhpura districts 5, 6, 12, 6, 5, 7, 5, 9, 2, 17, 8 and 3, respectively in first stage sampling whereas; 354 poultry attendants of these farms were selected as study population in second stage sampling. Besides these 63, 18, 23, and 7 samples total 111 samples were taken from poultry butcher/ poultry retailer, lab technicians who work in diagnosis of poultry disease, poultry veterinarian who gives consultancy to different poultry farm and vaccinator who gives vaccine to different poultry respectively. As a control 25 samples were taken from individual who did not exposed to poultry industry. The sera samples were preserved at -80°C till processed. The samples were analyzed at University Diagnostic Laboratory, University of Veterinary and Animal Sciences, Lahore, Pakistan

Estimation of sample size: No previous report was available with respect to sero-prevalence of H9N2 in poultry workers of Pakistan. The value of expected prevalence (0.02) for estimation of desired sample size was therefore specified keeping in view the reported prevalence (0.017) from China (Jia *et al.,* 2009). The desired sample size was estimated following the model recommended by Thrusfield (2005).

$$n = \frac{1.96^2 P_{expected} (1 - P_{expected})}{d^2}$$

By specifying 0.05 as desired absolute precision (d), the desired sample size was (n=30).

During taking blood sample detailed information regarding age, type of farm, any signs and symptoms during work, location and how long attachment with this profession of the individual was also taken by filling the questionnaire. The samples were collected in the year 2011.

Horse RBC hemagglutination assay: Reference H9N2 antigen (A/turkey/Wisc/66), inactivated H7N7 antigen (A/tky/Eng/647/77), H7N7 anti serum (A/Tk/Eng/647/77) and reference H9N2 antiserum (A/Turk/Wisc/1/66) were procured from Veterinary Laboratory Agency, Surrey, UK.

Protocol: Horse blood was collected in 20 mL disposable syringe containing EDTA. Upon receiving the blood was washed three times in phosphate buffer saline (PBS). A solution of 1% RBC was prepared by adding pelleted horse RBC to PBS. The obtained sera were treated with receptor destroying enzyme (RDE) by diluting one part of serum with three parts of enzyme and incubated at 37°C for overnight. Sera were further diluted with PBS to a final dilution of 1:10 dilution. Two fold serial dilution of serum in 25 µl PBS were performed in 96 well U bottom micro titer plate. 25 µl of 4 HA unit virus were added upto 10^{th} well. 11^{th} and 12^{th} well were kept as positive control of virus and RBC, respectively (Kayali *et al*., 2008; Jia *et al*., 2009; Hadipour, 2010).

Statistical analysis: The data were analyzed by non-parametric Kruskal-Wallis one way ANOVA method using statistical software (SAS, 2004).

RESULTS

In this study HI titer having 1:160 or greater was considered to be positive which was also taken as a standard for human infection of high pathogenic H5N1 viruses (WHO, 2007). The highest percentage (82.1%) of sero-positivity against H9N2 AIV was found in district Toba Tek Singh followed by almost same 76 and 75% in Multan and Haripur district, respectively. However, the highest geometric mean titer (GMT) 160 was recorded in Haripur and Sialkot district followed by 149 in Toba Tek Singh district. The lowest sero-positivity and GMT were 9.7% and 26 respectively found in Islamabad. The GMT of Toba Tek Singh district people had significant ($P<0.01$) difference from the districts of Sialkot, Sheikhupura, Bahwalnagar, Chakwal, Lahore, Rawalpindi, Gujranwala and Islamabad. However, the non-significant difference among people of the districts from Haripur, Multan and Faisalabad was found (Table 1).

The highest percentage of sero-positivity (85.7%) of antibody titer to H9N2 was found in the vaccinator followed by poultry attendant 46.6%. The lowest sero-positivity (30.4%) was recorded in veterinarian. The highest GMT (139) was also recorded in the vaccinator but the lowest GMT (49) was found in butcher. It revealed that GMT of butcher, had significant ($P\leq0.01$) difference from GMT of laboratory technicians, poultry attendants and veterinarians but non-significant difference from vaccinators (Table 1).

Antibody titer against H7N7 AIV was also evaluated in the same population. The highest GMT (160) was observed in Haripur district. The lowest GMT (28) was observed in Islamabad. The highest sero-positivity (57.9%) was also observed in Haripur district and the lowest sero-positivity was observed in Gujranwala and Sialkot district and it was about 4.6% (Table 2). The titer of Toba Tek Sing district had significant difference with Sheikhupura, Bahawalnagar, Chakwal, Lahore, Islamabad,

Table 1: Antibody titer to H9N2 in population in different districts of Pakistan and occupational people (values in parenthesis indicate percentage)

Area/Occupation	No.	<1:20	1:20	1:40	1:80	1:160	1:320	1:640	GMT	Sum of titer ≥160
Districts										
Toba Tek Singh	28	-	-	1(3.6)	4(14.3)	18(64.3)	5(17.9)	-	149	23(82.1)
Multan	17	-	-	-	4(23.5)	12(70.6)	1(5.9)	-	139	13(76.5)
Haripur	57	-	-	2(3.5)	12(21.1)	27(47.4)	16(28.1)	-	160	43(75.4)
Sialkot	21	-	-	1(4.8)	7(33.3)	4(19.1)	9(42.9)	-	160	13(61.9)
Gujranwala	22	-	-	2(9.1)	7(31.8)	9(40.9)	4(18.2)	-	121	11(59.1)
Faisalabad	19	-	-	1(5.3)	7(36.9)	11(57.9)	-	-	113	11(57.9)
Lahore	46	-	9(9.6)	9(9.6)	11(23.9)	8(17.4)	9 (19.6)	-	75	17(36.9)
Chakwal	138	39(28.9)	24(17.4)	10(7.3)	14(0.1)	19(13.8)	27(19.6)	5(3.6)	49	51(36.9)
Bahawalnagar	37	-	-	3(8.1)	22(59.5)	9(24.3)	3(8.1)	-	98	12(32.0)
Rawalpindi	13	-	2(15.4)	1(7.7)	6(46.2)	4 (30.8)	-	-	75	4(30.8)
Sheikupura	36	-	8(22.2)	16(44.4)	6(16.7)	3(8.3)	3(8.3)	-	49	6(16.7)
Islamabad	31	12(38.7)	7 (28.6)	2(6.5)	7(22.6)	3(9.7)	-	-	26	3(9.7)
Control	25	22(88.0)	3(12.0)	-	-	-	-	-	11	0(0)
Occupations										
Vaccinator	7	-	-	-	1(14.3)	6(85.7)	-	-	139	6(85.7)
Poultry attendant	354	31 (8.8)	26 (7.3)	35(9.9)	97(27.4)	106 (29.3)	58(16.4)	1(0.3)	86	165(46.6)
Lab technician	18	2 (11.1)	3 (16.7)	3(16.7)	3(16.7)	4(22.2)	3(16.7)	-	65	7(38.9)
Butcher	63	17(26.9)	16(25.4)	6(9.5)	-	6(9.5)	14(22.2)	4(6.4)	49	24(38.1)
Veterinarian	23	1(4.4)	5(21.7)	4(17.4)	6(26.1)	5(21.7)	2(8.7)	-	61	7(30.4)
Control	25	22(88.0)	3(12.0)	-	-	-	-	-	11	0(0)

Table 2: Antibody titer to H7N7 in population in different districts of Pakistan and occupational people (values in parenthesis indicate percentage)

Area/Occupation	No.	<1:20	1:20	1:40	1:80	1:160	1:320	1:640	GMT	Sum of titer ≥160
Districts										
Haripur	57	-	-	6(10.5)	18(31.6)	9(15.8)	23(40.4)	1(1.75)	160	33(57.8)
Toba Tek Singh	28	-	-	1(3.6)	13(46.4)	12(42.9)	2(7.1)	-	113	14(50.0)
Faisalabad	19	-	-	1(5.3)	9(47.4)	9(47.4)	-	-	106	9(47.4)
Rawalpindi	13	-	-	-	7(53.9)	6(46.2)	-	-	106	6(46.2)
Multan	17	-	-	2(11.8)	7(41.2)	7(41.2)	1(5.9)	-	106	8(47.1)
Bahawalnagar	37	1(2.7)	1(2.7)	9(24.3)	13(35.1)	10(27.0)	3(8.1)	-	86	13(35.1)
Sheikhupura	36	2(5.6)	1(2.8)	7(19.4)	15(41.7)	7(19.4)	4(11.1)	-	80	11(30.6)
Lahore	46	8(17.4)	2(4.4)	10(21.8)	11(23.9)	10(21.7)	5(10.9)	-	61	15(32.6)
Chakwal	138	50(36.2)	10(7.3)	24(17.4)	7(26.8)	9(6.5)	8(5.8)	-	35	17(12.3)
Gujranwala	22	6(27.3)	2(9.1)	5(22.7)	8(36.4)	1(4.6)	-	-	35	1(4.6)
Sialkot	21	6(28.6)	1(4.8)	8(38.1)	5(23.8)	-	1(4.8)	-	35	1(4.8)
Islamabad	31	14(45.2)	7(22.6)	1(3.2)	2(6.5)	5(16.1)	2(6.5)	-	28	7(22.6)
Control	25	24(96.0)	-	1(4.0)	-	-	-	-	5	0 (0)
Occupations										
Vaccinator	7	-	-	1(14.3)	3(42.9)	3(42.9)	-	-	98	3 (42.9)
Lab technician	18	2(11.1)	1(5.6)	2(11.1)	5(27.8)	4(22.2)	4(22.2)	-	86	8(44.4)
Poultry attendant	354	57(16.1)	16(4.5)	58(16.4)	112(31.6)	68(19.2)	42(11.9)	1(0.3)	65	111(31.4)
Veterinarian	23	3(13.0)	2(8.7)	5(21.7)	7(30.4)	6(26.1)	-	-	57	6(26.1)
Butcher	63	25(39.7)	5(7.9)	8(12.7)	18(28.6)	4(6.4)	3(4.8)	-	32	7(11.1)
Control	25	24(96.0)	-	1(4.0)	-	-	-	-	5	0 (0)

Faisalabad, Gujranwala and Multan districts ($P \leq 0.01$). However, there was non-significant difference with Haripur, Sheikhupura and Rawalpindi districts (Table 2). The highest GMT (98) was observed in the vaccinator groups of people. The least GMT (32) was observed in the butcher groups who sell and slaughter the poultry. However, the highest seroprevalence (44.4%) were observed in laboratory technician followed by vaccinator 42.9%. The lowest seroprevalence (11.1%) was observed in the butchers. Among the butchers the highest percentage (39.7%) people had no detectable antibody titer. In the vaccinator all individuals had detectable antibody titer (Table 2). Antibody titer of butcher had differed significantly ($P<0.01$) as compared to group of vaccinator, poultry attendant, laboratory technician and veterinarian. In the control group, 22 individual (88%) had no detectable antibody titer (Table 1) against H9N2 and only 12% people had antibody titer of 1:20. In case of H7N7, 96% of people of control group had no detectable antibody titer while 4% had 1:40 antibody titer (Table 2).

DISCUSSION

The present study reveals that the highest sero-positivity (85.7%) against H9N2 AIV subtype was recorded in poultry vaccinators group who vaccinate poultry birds at different farms followed by poultry workers (46.6%). Vaccinators had high titer could be due to frequent handling and exposure to vaccine.

The least sero-positivity (30.4%) was found in poultry veterinarian, it may be due to adapting some precautionary measures like using mask while visiting poultry farms and using gloves during post mortem examination. In Iran in Fars Province it was found the antibody titers in poultry workers, poultry butcher, and veterinarians were in scale of 3 to log 8 $\log_2$ HI. The seroprevalence were 87, 76.2 and 72.5% in poultry farm workers, slaughter house workers and veterinarian, respectively. Samples were considered positive when the titer was >20 (Hadipour, 2010). In sero-survey in Xinjiang (XJ), Liaoning (LN) province of China 1.7% and 1.0% respectively positive serum sample was recorded in the farmers who lived in H5N1 poultry outbreak areas.

Whereas no positive sample was recorded in Shangdong (SD) and Shanxi (SX) provinces in poultry workers and farmers, respectively. Sero positive was considered when the HI titer was greater than 1:160. Age, gender and history of poultry contacts were not statistically significant with HI titer against H9 virus (Jia *et al*., 2009). In our study the highest sero-positivity (82.1%) were recorded of individuals belonging to Toba Tek Singh district followed by Haripur and Multan District. In Haripur and Multan District almost 75.0% sero-positivity were recorded. In Toba Tek Singh 28 samples were taken from poultry workers. All the poultry workers in Toba Tek Singh were male and their age ranged from 12 years to 58 years. However, most of the workers age was between 22-40 years. Out of total 14.3% samples were taken from laboratory technician who performs different routine serological tests. The tests include also HI test against various subtype of avian influenza and Newcastle disease. They also did the postmortem examination of dead birds. Haripur was the coolest place in compare to other district which may be allow to survival of the virus and circulate in the environment. Multan is a hot and humid place. This whether may be a stress factor which favors in effecting the disease. In our study we used WHO HI criteria for detecting H5N1 infection by a single serum sample collection (WHO, 2007). In USA human sera against antibodies were evaluated by comparing HI versus microneutralization assay. Human sera samples (n=75) were evaluated for antibodies against avian influenza virus by comparing hemagglutination inhibition versus microneutralization assay. Out of 75, 38 individuals were exposed to domestic and wild birds and 37 were not exposed. It was found that 93.3% population having less then <1:10. 5.3 and 1.3% population have the titer 1:10 and 1:20, respectively against avian H9 influenza virus (Kayali *et al*., 2008). As compare to high pathogenic H5N1 low pathogenic H9N2 subtype had not get priority in human infection. However, it has been isolated in many countries since mid 1990 (Alexander, 2007). In one study H9N2 virus which have been isolated from Hong Kong live bird markets showed receptor similarity to that of human H3N2 virus. Beside that mutation was observed in hemadsorbing site which is a feature to that of human H2N2 and H3N2 virus (Matrosovich *et al*., 2001). In one study antibodies against H9 were evaluated in poultry workers like poultry retailers in food market, poultry whole seller, workers in large-scale poultry breeding farm, farmers working in small scale poultry farm, workers in pig breeding enterprise and general population. Anti H9 was highest in poultry retailers (15.9%) in compare to other groups of workers. This was might be due to direct contact of poultry retailers with live birds specially during slaughtering of birds (Wang *et al*., 2009). Opportunistic viral infections can be occurred to human being. This was exemplified by two cases of human being who are immunocompromised and are infected with avian H9N2 influenza virus in Hong Kong in the year 2008 and 2009. One patient shed virus at 10 day after showing symptoms despite having acute lymphoblastic leukemia. In other patient it was graft versus host disease with respiratory failure (Cheng *et al*., 2011). Although infection of human being with H9N2 AIV is much lower than H5N1 but is found that when human macrophages and epithelial cells are infected with certain genotype of H9N2 viruses it express remarkable increase cytokines and chemokine levels as compared to influenza subtype H1N1 (Law *et al*., 2010). Pathogenicity and immunogenesis of an H9N2 avian influenza virus was investigated which have been isolated from a female patient in murine model (Qi *et al*., 2011). It showed that it can multiply in murine respiratory tract without prior adaptation. Highest antibody response titers were induced when the mice was challenged with a viral doses of 10^9 and 10^8 EID_{50}. The titer was measured 14^{th}, 28^{th}, 84^{th} and 196^{th} day post infection interval. The highest titer was observed 10.32 at 28 day interval of challenge by doze of 10^9 EID_{50} but after 84 and 196 days of post infection the titer level was not detectable. The lowest HI titer was observed in mice challenging with 10^4 EID_{50}. The HI titer was 4.32 and it persisted at 196^{th} day post infection. Splenic lymphocytes of infected mice exposed to H9 HA peptides and cytokine IFNγ showed that T cells were stimulated by H9 HA peptides and the cells were predominantly CD^{4+} T cells (Qi *et al*., 2011). In another study two commercial antigen capture enzyme assays were investigated to detect wild type (wt) and cold adapted (ca) H9N2 avian influenza virus in human. It showed both Directigen and X/pect assays give positive results in the nasopharyngeal washing when the samples have the concentration of 5 $X10^4$ $TCID_{50}$ (Fedorko *et al*., 2006).

Our study revealed that the highest GMT (98) against H7N7 AIV subtype in vaccinator groups while the lowest GMT (32) in butcher group which could be attributed to continual handling of killed vaccine of subtype of H7 and H9 AIV. However, there was a limitation of our study that numbers of the vaccinators were only seven in numbers. By district wise, the highest GMT (160) was observed in Haripur and the lowest GMT (28) was observed in Islamabad district. Haripur district (Punjab) is the most nearby district to Khyber Pakthun Khwa province having the same landscape of Punjab province. It is relatively colder than other districts which may favor the propagation and survivability of the virus. Due to this might be the virus perpetuates a considerable time and contaminate in vicinity of the poultry premises which in turn poultry workers got more infection with the virus. In Italy, a similar study was undertaken to evaluate the serum titer against H7 in workers who were exposed to AIV outbreak (Puzelli *et al*., 2005); they found 3.8% sero-positivity against H7 among the workers. Among the seropositive people only one person had the history of clinical symptoms (Puzelli *et al*., 2005). In February 2004, an outbreak of low pathogenic avian influenza occurred in the Fraser Valley, British Columbia. Out of 650, 77 people reported with symptoms. Out of 77, 55 had suspected and two were clinically confirmed avian influenza infection. Although people adapted protective equipment likes N95/North 7700 mask, gloves, goggles and biosafety suits (Tweed *et al*., 2004). In the present study on asking few people having high titer of antibody told they had history of respiratory symptoms although it was not confirmed clinically that it was case of influenza infections. In a serological survey in southern China in rural workers in a dense duck raising area had antibodies to subtype to H7 (Shortridge, 1992). A random 75 human sere sample were analyzed to H7 subtype in USA. Among

the 75 persons 38 were exposed and 37 were not exposed to domestic or wild birds. Sero-positivity to H7 was found in 1.3% and highest percentage 50.7% people had below the titer of 1:10. The sera were also analyzed by another method i.e., micro neutralization method. In that method no individual had infected titer and 77.3% of population below the titer 1:10 (Kayali *et al*., 2008). In northern China 583, 200, 277 and 407 sera samples were analyzed for antibodies to H7 from the province Xinjiang (XJ), Liaoning (LN), Shandong (SD) and Shanxi (SX) respectively. None of the samples were seropositive against H7. In all the provinces most of the people ranging from 90-99% have titer below 1:20. In the category of titer 1:20 highest percentage 6.2% was observed in XJ province and least percentage 0.2% was observed in SX province (Jia *et al*., 2009). In our study within the Punjab province the highest sero-positivity (50%) against H7 was found in Toba Tek Singh district. The lowest seroprevalence (4.6%) was found in Gujranwala district. The highest percentage (45.2%) of people that did not contain detectable antibody in Islamabad district followed by (36.2%) Chakwal district. Similar type of study was carried out in Netherlands. Thirty four, 508 and 63 sera sample from H7 infected person, poultry exposed and persons exposed to H7 infected persons respectively were analyzed for antibodies to H7N7. Using the cut off value of titer ≥10, H7 specific antibodies were detected in 29 (85%) in infected persons, in 251 (49%) in persons exposed to infected poultry and in 40 (64%) in the persons who are exposed to infected persons (Meijer *et al*., 2006). In our study the poultry attendant who helps in feeding, management in poultry farms only 57 persons (16.1%) had no detectable antibody titer. The highest number 112 (31.6%) of poultry attendant had the titer of 1:80. However, in the retailer and the people who slaughter the birds, 39.7% had no detectable antibody titer. Among the butchers, 4.8% had a titer of 1:320. In this study we found antibody titer in butcher was much lower than people who work in poultry farm. It might be poultry retailer sells off broiler chicken in which case influenza vaccine some time is not routinely used. But in the poultry attendant which includes the people who work in the broiler, layer, breeder farm where killed influenza is routinely used. The poultry shed the virus in the environment through their natural discharge and the poultry attendant got infected from the environment and develops high antibody titer to H7. H7N7 AIV was detected in three individuals who are not exposed to infected poultry and contact with family members who had conjunctivitis with H7N7 virus. It was speculated that the transmission of virus from human to human occurred in this case. Swab was taken from conjunctiva as well as from throat and nose. Virus load in the conjunctiva was higher than respiratory tract suggesting the virus replicates well in the cells of human being rather than spilled over in the environment (Fouchier *et al*., 2004).

This is the first time we studied the implication of H9 and H7 subtype in occupational groups of peoples who are directly or indirectly engaged with poultry industry in Pakistan. We found that occupational people like poultry attendant, poultry retailer, vaccinators, lab technician who are engaged with different serological test for diagnosis of this disease contains very high antibody titer to H9 and H7. Overall GMT of H9 was higher than H7 almost in all cases indicating H9 is more endemic than H7 in Punjab province. On filling the questionnaire some respondent told that they had occasional respiratory and conjunctival distress but which was not confirmed by laboratory diagnosis. The circulation of H9 and H7 AIV subtype indicates there is a risk for mutation or re-assortment of the virus in human being. Therefore public health surveillance needs to be established to monitor in occupational workers.

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