Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Cross Sectional Survey of Live Bird Markets and Zoo Birds for Circulating Influenza Subtypes in Pakistan

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ARTICLE HISTORY (16-297)

Received: November 12, 2016
Revised: December 05, 2016
Accepted: December 20, 2016
Published online: January 16, 2017

Key words:
Influenza
Live bird markets
Pakistan
PCR
Prevalence
Surveillance

ABSTRACT

In Pakistan, avian influenza surveillance has been both active and passive. Here, we present the results of a survey effort focusing solely on the live bird markets and wild bird species from different zoos and national parks to understand the impact of live bird markets on the spread of highly pathogenic avian influenza viruses. A cross sectional survey was conducted from Jan-Dec 2011 to identify and isolate the circulating avian influenza virus subtypes in live bird markets and wild birds from different localities in and around Islamabad Capital Territory. Swabs, tracheal tissues and sera samples were collected, screened and diagnosed by hemagglutination inhibition assay and RT-PCR. The highest seropositivity was recorded for H9 (100 %) followed by H5 (89.4%) and H7 (72.3%). All 27 isolates were of the low pathogenic H9N2 subtypes and no viruses could be successfully isolated of subtype H5N1 or H7N7. The higher prevalence of H5N1 (89.4%) observed in the present study was an alarming threat; therefore, we suggested immediate control strategies against this emerging risk of H5N1 for human in live bird markets in Pakistan. The factors unveiled in this study will help in understanding the lapses in controlling persistent outbreaks of avian influenza in country.

To Cite This Article: Fatima Z, Khan MA, Ahmad MUD, Muhammad K, Khwaja KN, Khan A, Anwar Z, Ahad A and Mahmood A, 2017. Cross sectional survey of live bird markets, and zoo birds for circulating influenza subtypes in Pakistan. Pak Vet J, 37(2): 185-189.

INTRODUCTION

The poultry sector is considered amongst the most vibrant sectors of agriculture in the country. Directly and indirectly, more than 1.5 million people are likely to have benefited in terms of income and employment from this sector (Naeem *et al*., 2007). Avian influenza (AI) outbreaks had devastating impacts on poultry sector in Pakistan and outbreaks of AIV subtype H9N2 (1998), H7N3 (1995, 1998, 2001-2002) and H5N1 (2006-2008) had been reported (Aamir *et al*., 2009; Abbas *et al*., 2010; Siddique *et al*., 2012). Food markets offering both live birds and poultry meat either for slaughter or for sale are collectively referred as live bird markets (LBMs). LBMs are the major part of supply food chain and are vital for maintaining nutritional and health status of the urban and rural populations, particularly in the developing countries (Yee *et al*., 2008).

During the last two decades, different AI subtypes including H5N1, H9N2, H7N7 and possibly H7N3 had been reported capable of zoonosis (Trampuz *et al*., 2004). Since 2003, more than 6500 H5N1 epidemics in 61 countries have been reported and human cases have also been recorded in 15 countries (Al-Natour and Abo-Shehada, 2012).

The poultry movement and transport through LBMs, which are common in almost all Asian countries due to cultural preference of consuming freshly slaughtered birds, has shown to be a vital factor in circulation of subtype A highly pathogenic H5N1 in Hong Kong and in Vietnam (Nguyen *et al*., 2005). HPAI surveillance programs conducted in several countries i.e. Vietnam, Cambodia,

Thailand, Hong Kong and China have demonstrated the circulation of HPAI/H5N1 in LBMs (Amonsin *et al.*, 2008). Information regarding epidemiology and dynamics of occurrence of different subtypes of AIV (especially H5N1, H7N7 and H9N2) in LBMs in Pakistan is poor and little is known about poultry market chains setup in HPAIV H5N1 and H9N2 endemic areas (Monne *et al.*, 2007). Keeping in view the economic significance of poultry industry in the country and epidemiology of AIV in LBMs as a major threat to public health, the present study was conducted to identify the circulation and prevalence of HPAI H5N1, H9N2 and H7N7 in LBMs located at territory of Islamabad, the Capital of Pakistan. The findings of this study provide insight into risks of spread of HPAVI H5N1 and H9N2 in the region.

MATERIALS AND METHODS

Study design and sample location: A cross sectional survey was conducted in Islamabad Capital territory (ICT) LBMs from January to December 2011. Field and laboratory examinations were performed in National Reference Laboratory for Poultry Diseases, NARC, Islamabad (33° 43' N, 73° 3' E). As AIVs have fecal oral route of transmission, therefore, cloacal swabs and serum samples from randomly selected markets located in Islamabad were collected (Fig. 2), twice a month. The primary sampling locations for random samples were LBMs and samples were also collected from wild avian species for isolation of influenza viral strains from national parks, lakes and zoos located in and around study area. We sampled chickens and some other avian species from zoo collectively termed as "minor poultry" which comprised of pigeons, silkie chickens, pheasants, guinea fowls, quails and ptarmigans and cloacal swabs and serum samples were collected.

Field work: Birds tagging was done in each selected market and newly entered birds were randomly sampled twice every month (pet, wild or indigenous poultry breeds). Sera and swabs samples were collected for detection of antibodies and antigens at different days i.e. entrance day as day 1, later at day 2, 3 and 4. Same samples were taken again from tagged birds to estimate any change in the antibody or antigen titers. The swab samples were then suspended in one ml of the storage medium (OIE, 2009) and transported in icebox to the National Reference Laboratory for Poultry Diseases, NARC per the OIE protocol, 2009.

Serology and screening of samples: Reference antigen and antiserum used for conducting of hemagglutination inhibition (HI) assay was performed as per procedure recommended by OIE (2009).

Virus isolation and identification: Initially all cloacal swab samples were inoculated into ten days old chicken embryonated eggs. After 72 hours of incubation at 35°C of incubation, the eggs were removed from incubator, chilled and harvested. Positive eggs were perceived by testing for HA of 0.5% (Chicken RBCs) following the procedures described (Thornton, 2011). The swabs samples were centrifuged (3000 rpm) at 4°C for 10 min. Supernatant was separated and stored at -70°C for virus isolation. The chicken embryonating eggs (9-10 days) were used for virus inoculation. Each sample (supernatant) was injected/inoculated into the chorioallantoic cavity of 3 eggs (0.2 ml/egg). Following that the eggs were incubated (at 35°C) and observed daily for death of embryos. After the death of embryo on 4th day, allantoic fluid was collected. The live embryos were killed by keeping at 4°C overnight and allantoic fluid was collected and tested for hemagglutination. The HA positive fluids were stored at -70°C for identification of viruses. OIE, (2009) protocol for influenza virus isolation was adopted during these procedures.

RESULTS

Influenza prevalence: A total of 2223 samples (swab, tracheal tissue and sera samples) from LBMs and wild birds (Table 4, zoo animals) in ICT and in its premises, were tested for H5N1, H9N2 and H7N7 antibodies and antigen through HI assay and RT-PCR. The sera samples were diagnosed with HI and swab samples with RT-PCR for the purpose of virus detection and isolation. Total of 77.4% samples were found positive on both HI and RT-PCR. Based on strains, 62.3% for H9N2, 0.0% for H5N1 and 0.0% for H7N7 but H9N2 was not isolated successfully. Hosts were considered AI positive if either tracheal swabs, cloacal swabs or sera samples tested were positive through HI assay or RT-PCR. In LBMs 100% sera samples were positive for H9N2, 89.4% for H5N1 and 72.33% for H5N7. Results showed equal prevalence in all three studied markets. The overall prevalence of H9N2 was 7.0% and 0.0% for both H5N1 and H7N7 in wild or pet birds studied outside the LBMs.

Geometric mean titers: Out of total 929 sera samples, the titers of 98 samples were zero against H5N1. However geometric mean titers (GMT) of antibodies against H5N1 were same throughout the year (Table 1). The range of GMT (log_2) was 2.65-4.32. In the tested samples, none of the sample had antibody titers above 64. LBMs same samples were evaluated against H7 (Table 2). Among the total 929 sera samples, 357 sera samples titers were zero. The range of GMT (log_2) was 1.59-0.87. In the tested samples, none of the samples had antibody titer above 32. All of these samples when evaluated against H9 (Table 3) were found positive through HI. The highest antibody titer (2048) was observed in the month of September. The range of GMT (log_2) was 6.0-8.17. GMT of all the three subtypes (H5, H7 and H9) of AIV were compared (Fig. 1) for each month for the year 2011. Data revealed highest antibody titer for H9 and the lowest for H7, among these three subtypes of AIVs subtypes. In case of H9 the highest GMT (8.17) was observed in March and the lowest GMT=log_2 (6.0) was observed in October.

Virus isolation: Attempts were made to isolate viruses from swab and water samples by inoculating into 9-day old chicken embryonating eggs. Randomly some chickens were tagged and they were sampled at consecutive days. 13 chickens were found infected at day 1 for H9 and 13 more infected with H9 on day 2. No H9 virus was detected in fecal and water samples from this stall until

Day 3. While H9 subtype was isolated from fecal and water samples of connected cages and nearby stand-alone cages on days 3 and 4. On the fourth day H9 was also isolated from water tanks used for washing the carcasses. Tracheal, blood and cloacal samples were collected from ten quails on day 4. Avian Influenza Virus sub type H9 was isolated from fecal swabs (two on day-2 and 3 on day-3). Tracheal swabs of pet roosters were found negative for NDV and AIV (Table 4). 12 NDVs were isolated from fecal samples (three on day 1, five on Day 2 and four on Day 3). Various samples were collected from wild bird species which are found in the study premises as shown in Table 4.

Mixed infection of different HA subtypes: Of the total 929 sera samples, positive for LPAI and/or HPAI different subtypes (in chickens and in minor poultry) showed 63.07% mixed infection. Among these concomitant (H5+H9) mixed infections were 89.46%, subtypes (H9+H7) were 63.07%, (H5+H7) were 53.2% and (H5+H9+H7) subtypes mixed infections were 63.07%. Mixed infections of H5 and H9 subtypes were the most prevalent of all mixed infections in the studied samples. However, samples collected from wild birds and backyard poultry from different zoos, aviaries and lakes (migratory birds) were having no mixed infections.

DISCUSSION

The growing poultry industry in Islamabad and its premises poses an immense public health and avian risk for highly pathogenic AIVs. Nonetheless, epidemio-logical disease surveillance of HPAIV and LPAIV in Pakistan is largely unknown. Therefore, prevalence of AIV specific subtypes in domestic and commercial poultry settings is significant for planning and implementation of cost effective veterinary and public health preventions. In Asian countries, LBMs remain the main source of HPAI H5N1 i.e. in Hong Kong (Thornton, 2011), Vietnam (Guan *et al*., 2000) and in China (Cameron *et al*., 2000). Mixing domestic poultry with water fowl and terrestrial poultry in LBMs is a common practice in Pakistan, where domestic and wild birds imported or caught are kept in very close proximity that imposes a higher cross contamination risk facilitating virus evolution and dissemination.

Table 1: Serological evaluation of live bird market samples using HI antigens of AIV subtype H5N1.

Months	No. of samples	Samples showing zero titer	No. of positive samples under each titer group											HI GMT (log_2)
			2	4	8	16	32	64	128	256	512	1024	2048	
January	84	12	-	8	18	20	26	-	-	-	-	-	-	3.33
February	81	10	12	4	15	17	-	23	-	-	-	-	-	3.34
March	73	6	2	5	18	23	7	12	-	-	-	-	-	3.63
April	88	15	7	12	-	21	9	14	-	-	-	-	-	3.72
May	83	11	-	7	23	20	10	12	-	-	-	-	-	3.43
June	70	6	2	12	16	14	-	20	-	-	-	-	-	3.57
July	88	11	-	10	15	16	17	19	-	-	-	-	-	3.72
August	83	12	-	13	17	14	25	12	-	-	-	-	-	3.97
September	74	7	12	12	9	13	9	12	-	-	-	-	-	3.13
October	61	8	6	13	16	8	10	-	-	-	-	-	-	2.65
November	70	-	10	10	13	9	11	17	-	-	-	-	-	3.74
December	74	-	4	16	10	22	13	19	-	-	-	-	-	4.32

Table 2: Serological evaluation of live bird market samples using HI antigens of AIV subtype H7.

Months	No. of samples	Samples showing zero titer	No. of positive samples under each titer group											HI GMT (log_2)
			2	4	8	16	32	64	128	256	512	1024	2048	
Jan	84	35	23	20	4	-	-	-	-	-	-	-	-	0.89
Feb	81	30	33	12	6	-	-	-	-	-	-	-	-	0.92
Mar	73	25	21	15	12	-	-	-	-	-	-	-	-	1.19
Apr	88	30	25	17	13	3	-	-	-	-	-	-	-	1.25
May	83	28	22	19	11	3	-	-	-	-	-	-	-	1.26
June	70	32	20	13	5	-	-	-	-	-	-	-	-	0.87
Jul	88	28	25	16	9	10	-	-	-	-	-	-	-	1.40
Aug	83	36	19	10	7	8	-	-	-	-	-	-	-	1.10
Sep	74	24	17	11	9	13	-	-	-	-	-	-	-	1.59
Oct	61	25	10	9	8	9	-	-	-	-	-	-	-	1.44
Nov	70	35	11	9	9	6	-	-	-	-	-	-	-	1.14
Dec	74	29	15	10	8	12	-	-	-	-	-	-	-	1.44

Table 3: Serological evaluation of live bird market samples using HI antigens of AIV subtype H9.

Months	No. of samples	samples showing zero titer	No. of positive samples under each titer group											HI GMT(log_2)
			2	4	8	16	32	64	128	256	512	1024	2048	
Jan	84	15	-	5	11	-	11	11	7	8	10	10	11	7.25
Feb	81	17	-	-	12	12	9	6	9	5	11	9	8	7.07
Mar	73	10	-	-	-	-	13	11	8	16	12	10	3	8.17
Apr	88	309	-	-	9	10	19	12	20	-	-	12	6	6.36
May	83	28	-	-	-	20	14	-	11	12	-	17	9	7.13
June	70	39	-		6	8	9	9	8	8	7	9	6	6.97
Jul	88	44	-	17	9	-	20	-	12	-	10	11	9	6.18
Aug	83	21	-	10	11	8	13	-	5	9	7	11	9	6.37
Sep	74	21	-	-	-	-	12	17	11	9	12	-	13	7.70
Oct	61	12	-	11	7	12		-	12	-	09	11	-	6.00
Nov	70	31	-	-	7	9	13	11	8	-	12	10	-	6.45
Dec	74	39	-	-	-	11	13	9	-	13	16	-	12	7.33

Table 4: Serology and virus isolation in sampled wild species from different locations in Pakistan, 2011.

Sample source/type of bird	Location	Type of sample	No. of samples	HI antibodies titers of AIV (GMT = log2)*				Virological evaluation	Other isolation**
				H3	H5	H7	H9	AIV isolation**	
Pheasantry Hazara University (Ring Necked)	Mansehra	swab	05	-	-	-	-	Nil	Nil
Pheasantry Hazara University (Silver Necked)	Mansehra	Swab	06	-	-	-	-	Nil	Nil
		serum	03	0.0	0.0	0.0	0.0		
Pheasantry Hazara University (White Turkey)	Mansehra	Swab	03	-	-	-	-	Nil	Nil
		serum	03	0.0	0.0	0.0	0.0		
Terbela Lake (Migratory Birds)	Haripur	Fecal and nasal	46	-	-	-	-	Nil	Nil
Abottabad (backyard poultry)	Abottabad	serum	15	0.0	0.0	0.0	4.66	Nil	Nil
Livestock Research Station (Duck)	Islamabad	Swab	20	-	-	-	-	Nil	Nil
		serum	40	0.0	0.0	0.0	0.0		
Livestock Research Station (Guinea Fowl)	Islamabad	Swab	02	-	-	-	-	Nil	Nil
		serum	04	0.0	0.0	0.0	0.0		
Ayub National Park (Mixed wild birds)	Rawalpindi	swab	21	-	-	-	-	Nil	ND virus
Livestock Research Station (Desi Chicks)	Islamabad	Swab	11	-	-	-	-	Nil	Nil
		serum	30	0.0	0.0	0.0	0.0		
Live Bird Market (Grey Partridge)	Islamabad	Fecal and nasal	04	-	-	-	-	Nil	Nil

-, HI not performed; *only ELISA positive samples are serologically evaluated; **virus isolation (for influenza and New Castle Disease) was attempted by egg inoculation.

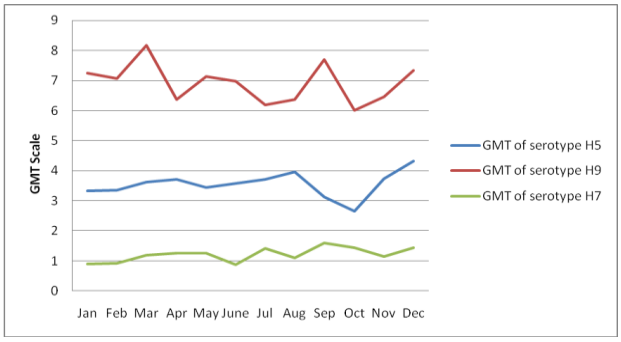

Fig. 1: Temporal distribution of GMT of antibodies against circulating serotypes of avian influenza viruses in LBMs of ICT for the year 2011.

Our results showed a high prevalence rate of LPAIV (H9) depicted in Table 3 in the LBMs in Islamabad Capital Territory. We identified H9 and H7 subtypes of LPAIV and H5 of HPAIV in chickens and minor poultry from this area. In addition, H9 viruses were isolated throughout the year, as the environmental condition of this area suits the persistence, transmission and survival of LPAIV. The presence of an elevated core is most likely to pose substantial challenge for the control of either HPAIV H5N1 or H9N2 due to flow of infected poultry through LBMs. As highlighted by the present results, the LBMs play an important role in Pakistan in facilitating the live poultry seasonal movements between different parts of the country. Similar observations were also reported by (Soares *et al*., 2010). LBMs once contaminated may even serve as a reservoir for viruses especially in mix type live birds market (Fournié *et al*., 2012). This depends on the incubation period if birds get infected from another bird during stay time in LBM.

No H5N1 virus was isolated although sero-conversion was found against H5. H9N2 was isolated both from fecal and water samples. Similarly, Fournié *et al*. (2012) isolated H9N2 from water sources of live poultry markets. H9N2 were mostly isolated in the present study from samples collected in colder months of the year i.e. December, January and February. These results were in line with that of Chen *et al*. (2006). However, low temperatures and humidity increase the virus survival rate in environment, which elevates the chances of viral transmission. We could not succeed to isolate any Avian Influenza Virus strain from quails and it might be due to the shorter stay of quails at LBMs. These results contrasted with the study of Ayaz *et al*. (2010). Nasopharyngeal samples were found positive more as compared to cloacal swabs that is a major concern regarding mode of transmission for viral infections. As reported, that several H9N2 virus subtypes can transmit via direct contact of susceptible healthy bird with infected ones (Shi *et al*., 2010). The higher prevalence of subtype H9N2 in LBMs may be of major concern to public health because many H9N2 subtype could cause infection in humans as reported by Wan *et al*. (2008). Current surveillance efforts revealed H9N2 as a primary subtype circulating in LBMs (Lee *et al*., 2010; Moon *et al*., 2010). Thus, though LBMs are dead end for the commercial poultry that are being slaughtered there, these are not the dead end for influenza viruses. Indeed, LBMs possibly help maintaining infections in poultry flocks especially in those that are situated in congested populated areas and it provides a potential location for intervening to control AIVs transmission (Webster *et al*., 2004). Studies to address the role of LBMs in maintaining AIVs circulation in countries like Pakistan where H5 and H9 are endemic are immediately needed.

In the context of baseline epidemiological data, the present study adds into the pool of avian influenza surveillance in Pakistan. Though it forms a baseline for in depth research in future to unveil the routes of transmission and role of LBMs in the spread and growth of HPAIV and LPAIV in Pakistan, it also has some shortfalls. Such as only HI positive samples were processed for isolation of viruses through RT-PCR. Different trading routes and networks of poultry source if observed and analyzed would have given a clear picture of LBMs role in the spread, growth and its persistence. Further studies may be conducted on these lines.

Conclusions: It was concluded that H9N2 and H5N1 are circulating in LBMs of Islamabad capital Territory that probably may be involved in the transmission and dissemination of low and highly pathogenic strains of influenza viruses into the commercial as well as backyard poultry through several means. There is a need for regular surveillance program and in depth research is required to unveil the routes of transmission for these viruses from markets into the commercial and back yard poultry.

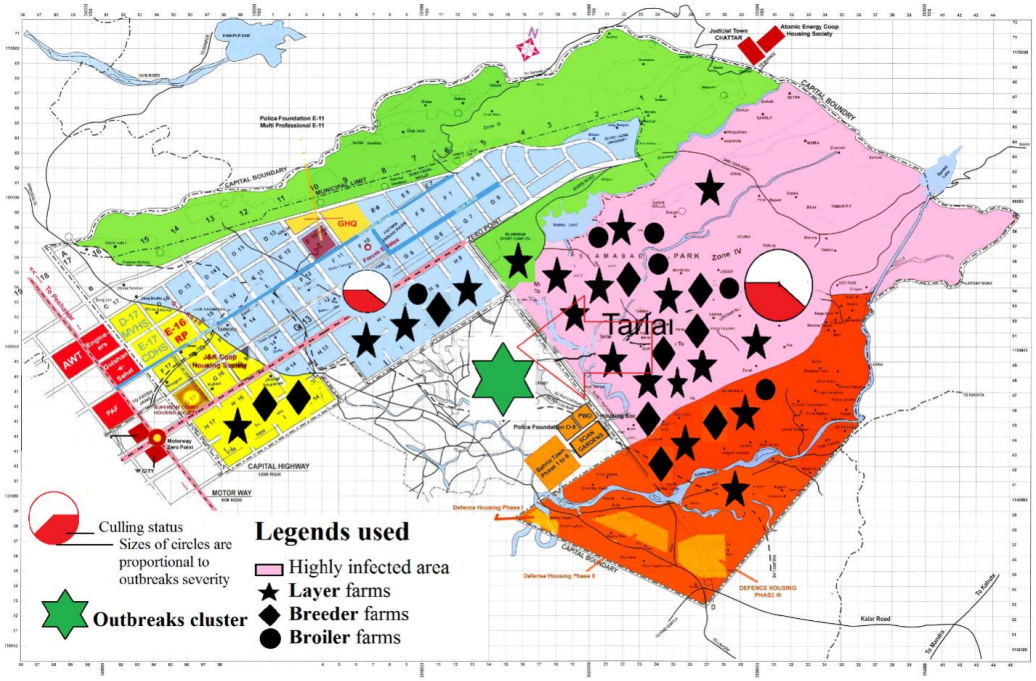

Fig. 2: Geographical location of sampled areas in the present study.

Authors contribution: ZF conducted the research as a major author and Contributed in the study designing, collection of data and write up. MAK and MUA supervised the research work and helped at each step of the work. KM contributed in the write up of the research article and diagnostics. KNK provided support in data collection and participated as a co supervisor. ZA, AK, AA and AM contributed in the laboratory work and data collection.

Acknowledgements: The authors highly acknowledge the Higher Education Commission, Islamabad (Pin AV4-220) for providing funds to undertake this research.

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