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Sero-conversion of Avian Influenza Virus Subtype H_9N_2 in Non-Vaccinated Commercial Poultry Flocks

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Abstract

Sero-conversion of pathogenic subtypes (H_5, H_7 and H_9) of Avian Influenza Virus types was studied in commercial poultry farms in the Province of Punjab, Pakistan during the years 2015-16. Out of 6731 morbid tissues, only 11 samples (4 from broiler, 4 from broiler breeder and 3 from commercial layers farms) were found positive for Avian Influenza Virus (AIV) H_9N_2 type. Only one isolate was found positive for H_9N_2 subtype from 17994 tracheal swab samples. All the 37163 cloacal swab samples were found negative. The sero-analysis of 133438 blood sera samples showed sero-conversion in 66219 samples (49.64%) against H_9 virus in the non-vaccinated poultry flocks, when tested with Heamagglutination Inhibition test (HI). Highest sero-conversion was recorded in broiler breeder (49.20%) followed by broilers (47.47%), layer (46.25%) and Native/Desi birds (45.59%). Highest HI titres were recorded in the flocks from Muzaffargarh, Rawalpindi, Toba Tek Singh, Sargodha, Gujrat, Faisalabad, Sheikhupura, Jhelum and Pakpattan while Bahawalpur, Gujranwala, Lahore, Jhang, Okara and Multan had relatively lower level of HI antibody titres. In conclusion only one type of AIV subtype H_9N_2 is prevailing in the province of the Punjab, Pakistan.

Keywords: Sero-conversion; Avian Influenza; Subtype H_9N_2; Commercial poultry flocks

Introduction

Avian Influenza (AI) is a contagious viral disease, having worldwide distribution affecting chicken of all age groups; it causes variable morbidity and mortality which has resulted into immense losses to the poultry industry during the last two decades in Pakistan. The AI infection has been diagnosed in all types of poultry including commercial broilers and layers; layer breeders, broiler breeders; fancy and migratory wild birds etc. [1]. Avian Influenza type A viruses can be classified into various subtypes on the basis of antigenic differences in the two surface Glycoproteins; Hemagglutinin (HA) and Neuraminidase (NA). Serologically, 16 subtypes of HA (H_1 to H_{16}) and 9 subtypes of NA (N_1 to N_9) have been identified [2]. In general, all HA and NA subtypes have been reported from the commercial and wild birds. However, in contrast, only three HA subtypes (H_1–H_3) and two NA subtypes ($N_1$and N_2) have frequently been reported in humans and are being isolated since 1918 [3]. Depending upon the pathogenicity, AIV is divided into two subtypes, one is called Highly Pathogenic AI (HPAI) and other is called low pathogenic AI (LPAI). Serotypes H_5 and H_7 have been classified in HPAI group of viruses for human infection. It has been observed that over the past few years, influenza A viruses such as H_5N_1, H_7N_7, H_7N_3 and H_9N_2 are being reported to infect the humans as well poultry [4]. The pathogenic Avian Influenza viruses have been involved in high mortality in poultry in over 63 countries of the world. In Pakistan AI outbreaks were initially recorded in October, 1994 [5]. The disease agent H_7N_3 virus affected the broiler breeders housed in the areas of Mansehra, Abbottabad, Rawalpindi districts and also in the adjoining areas and killed approximately half a million birds of various age groups. It affected all type of poultry farms of 7 to 66 weeks of age and overall mortality of 63% in the area of initial out break [5,6]. Many out breaks of AI have been recorded in Pakistan since 1994, which are attributed to virus types like H_7N_3, H_9N_2 and H_5N_1 and caused high economic losses to the poultry industry in Pakistan. The outbreaks of H_5N_1 in Pakistan were reported in 2006 [7].

The present project was designed to investigate Avian Influenza virus infections on commercial poultry farms using both serological and virus isolation techniques for understanding the circulation level of various high and low pathogenic subtypes of avian influenza virus.

Materials and Methods

Collection of samples

A total of 17994 tracheal swabs, 37163 cloacal swabs and 6731 tissue samples (lungs, liver and spleens) were collected, from the non-vaccinated commercial poultry birds having respiratory signs of disease suspected to be affected clinically with AIVs. The tissue samples were also collected from the sick/morbid birds submitted to Poultry Research Institute, Rawalpindi for diagnosis. The tissue samples were collected aseptically and processed for virus isolation/identification using the standard protocols [8]. Similarly a total of 133438 blood samples were collected by the staff of field diagnostic laboratories of North & South Punjab from the birds indicating signs of respiratory illness and those indicating very high mortality or drops

in egg production. The serum was separated, and stored in small aliquots at -20°C till used.

Virus isolation

The virus isolation was carried out in 9-11 day old Specific Antibody Negative (SAN) chicken embryos. The morbid tissue samples were triturated in sterile tissue grinder and a 10% tissue homogenate was prepared in PBS p^H 7.2. The homogenate suspension was filtered through 0.45 µm and 0.22 µm syringe filters to get rid of the bacterial and Mycoplasma contamination. A 0.1 ml quantity of the homogenate was inoculated via Chorio-allantioc (CAS) route. The swab samples were opened and their transport material was also filtered through 0.22 µm syringe filter. A 0.1 ml quantity of material was inoculated in 9-11 day embryonated chicken eggs via CAS route. After 18, 24 and 48 hours at 37°C post-inoculation, the dead and surviving embryos were chilled overnight and the Allantoic Fluid (AF) was harvested under aseptic conditions using biosafety cabinet. The AF samples were identified with known anti sera of avian influenza virus by Heamagglutination Assay (HA). The positive samples recovered during this study were also sent to the laboratory of National Program for the Control of Avian Influenza Islamabad for further identifications.

analysis

All the data was analysed by percentage prevalence and Geometric mean HI titres of positive Sera samples.

Antigen preparation

Antigens for various known subtypes (H_7N_3, H_9N_2 and H_5N_1) were prepared from the known cultured isolates during previous outbreaks and stored in Poultry Research Institute, Rawalpindi. A 0.1 ml virus suspension of each subtype was inoculated into batches of 100 embryonated eggs (9-11 days old non vaccinated flock) through allantoic route. After 72 hours of incubation all the embryos were killed with chilling and the allantoic fluid was collected aseptically and stored at -20°C until used for HA and HI test.

Inactivation of serum for heamagglutination assay

Prior to HA and HI test, all the serum samples were thawed and heat-inactivated for IgM at 56°C for 15 minutes in a water bath (it may be for inactivation of complement because the authors are dosing Antibody titre with HA/HI Assay and IgM is one of those). The inactivated serum samples were titrated through HI assay for the presence of antibodies against AIV subtypes H5, H7 and H9 as per procedure described by Hussain [9]. After the calculation of 4 HA, inactivated serum samples were titrated through HI as described [9].

Results and Discussion

A total of 31,698 morbid tissue samples were collected from various poultry farms, 11 samples were found positive for the presence of AIV H9N2 type. Infection of this virus was observed in 4 of the 2476 tissue samples from broilers, and in 4 of 2109 samples from the broiler breeder and 3 samples from commercial layers (Table 1).

Types of Samples	No. of Isolates/ No. of Samples				Total Samples	Total Isolates
	Broilers	Broiler Breeders	Layers	Native Birds		
Tissues	Apr-76	Apr-09	3/1123	0/1023	6731	11
Tracheal swabs	0/8002	Jan-60	0/2987	0/1445	17994	1
Cloacal Swabs	0/9500	0/9990	0/9500	0/8170	37163	0
Total	4/19978	5/17659	3/13610	0/10641	61888	12

Table 1: Isolation of avian influenza virus subtype H9N2 from broiler breeder, layer and native birds.

All the isolates were identified as H_9N_2 types. The results are in line with Naeem et al. [10], Khawaja et al. [11], Naeem et al. [12], Sidique et al. [13] and Subtain et al. [14]. They also reported the H_9N_2 type AIV in wild and poultry birds in Pakistan. All attempts to isolate any of the other two types of AIV subtypes (H_5 and H_7) were unsuccessful. This study indicated that in the province of Punjab Pakistan, only H_9 subtype of avian influenza virus was circulating in various types of poultry birds. During this study only one tracheal swab sample from the broiler breeders was found positive for the H_9 virus isolation. All other tracheal swab samples collected from commercial broilers, commercial layers and desi/native birds were found negative for the presence of any of the three subtypes of AI viruses. It was also observed that none of the 37162 cloacal swab samples was harbouring any of the three avian influenza virus subtypes suggesting that probably, the viruses were neither present in the gut of the sampled birds nor there was any shedding of this virus types in the environment through their faecal material.

The HI antibody titres in non-vaccinated commercial flocks and Desi birds against H_9 virus were at very high levels (Table 2).

Types of Birds	No. of Blood Samples	HI Positive Samples			Percentage
		H5	H7	H9	
Broilers	65770	-	-	31220	47.47%
Broiler Breeders	27880	-	-	13717	49.20%
Layers	21600	-	-	9990	46.25%
Native/ Desi	18188	-	-	11292	45.49%
Total	133438	-	-	62219	49.63%

Table 2: Sero-conversion of non-vaccinated poultry flocks of broiler, broiler breeders, Layers and native birds for different subtypes of AIV

The sero-analysis of 1,33,438 blood serum samples showed sero-conversion in 66219 samples (49.64%) against H_9 virus in the non-vaccinated poultry flocks when tested with Heamagglutination Inhibition test. The highest sero-conversion was recorded in broiler breeder (49.20%) followed by broilers (47.47%), layer (46.25%) and Native/Desi birds (45.59%). The highest GMT against H_9N_2 subtype was recorded in the Muzaffargarh, Rawalpindi, Toba Tek Singh, Sargodha, Gujrat, Faisalabad Sheikhupura, Jhelum and Pakpattan. The flocks housed in, Bahawalpur, Gujranwala, Lahore, Jhang, Okara and Multan had relatively lower level of HI antibody titre (Table 3).

District Name	GMT					
	H5		H7		H9	
	Maximum	Minimum	Maximum	Minimum	Maximum	Minimum
Rawalpindi	-	-	-	-	2.80	970.66
Jehlum	-	-	-	-	20	556.99
Gujrat	-	-	-	-	2.56	845.51
Gujranwala	-	-	-	-	0	318.04
SheikhuPura	-	-	-	-	0	749.8
Lahore	-	-	-	-	0	229.04
Okara	-	-	-	-	2.69	160
Pakpatan	-	-	-	-	2.32	511.13
Multan	-	-	-	-	0	147.66
Muzaffarh Garh	-	-	-	-	2.34	414.91
Faisalabad	-	-	-	-	0	837.33
Bahawalpur	-	-	-	-	0	441.67
T.T.Singh	-	-	-	-	2.11	899.98
Jhang	-	-	-	-	0	189.99
Sargodha	-	-	-	-	2.0	873.71

Table 3: District wise GMT of antibody titres against different subtypes of AI in Non-Vaccinated Flocks

Results are in accordance with Numan and Siddique [15], who also reported high incidence of H_9N_2 in Toba, Tek Singh and adjoining districts in non-vaccinated commercial layer flocks.

The presence of HI antibody titer in the sera samples from the non-vaccinated flocks is also an indication of infection of these flocks with H_9N_2 subtype of AIV, which might be circulating in the flocks and caused recurrent infections. High GMT against H_9N_2 in the non-vaccinated flocks throughout Punjab (Pakistan) is suggestive of wide spread of this infection. Rasool et al. [16] also studied commercial and desi layer breeds for low pathogenic avian influenza infection and found that the AIV, H_9N_2 isolate produced variable pathogenicity in Desi and commercial layer breeds. Although H_7 and H_5 viruses were isolated from poultry in Pakistan in past [5] but, these viruses could not be isolated during this study.

This study further indicated that developing chicken embryos were good source for isolation of the virus from the morbid tissues. Same has been reported by Muneer et al. [17,18] and Rasool et al. [16]. The circulation of H_9N_2 and other viruses in poultry flocks indicates poor or no practice of proper biosecurity measures adopted by the farmers at the flock levels. Presence of H_9 virus antibodies in the non-vaccinated flocks also provides the need for controlling the AI through strict biosecurity measures and awareness of the farmers on controlling this disease.

Conclusions

From this study it can be concluded that presently only one type of AI H_9N_2 type is present in poultry in Punjab (Pakistan). The authors feel that surveillance work be continued in future and good collaboration is required between Livestock Departments of all the provinces and special areas for generating national data and rapid responses to any AI outbreaks. The measures like vaccination and biosecurity must remain in practice in order to avoid any future outbreaks of low or high path avian influenza.

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