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Prevalence of Avian Influenza Virus (H_5) in Poultry Layer Flocks in and Around Faisalabad, Punjab, Pakistan

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Abstract.- Avian influenza virus (AIV: H_5) infections are persisting in wild birds in sub-clinical form and are source of dissemination to commercial birds. In an outbreak, rapid detection of the AIV is considered to be a valuable tool for its handling on poultry farms. In the present study, 975 samples of tissue homogenate, tracheal, and cloacal swabs (325 of each) were collected from 40 suspected flocks (10 layer breeder and 30 commercial layers) and were processed for AIV detection using reverse transcriptase polymerase chain reaction (RT-PCR), and virus culture and identification techniques. The AIV was detected from 92 (9.4%) samples (12 samples of tissue homogenate, 50 tracheal and 30 cloacal swabs) of 4 (10%) layer flocks (one layer breeder and 3 commercial layer) using RT-PCR technique while the virus was confirmed from 16 (1.6%) samples (10 samples of tissue homogenate, 20 tracheal swabs and 10 cloacal swabs) of 2 (5%) flocks (one layer flock of each category) using virus culture technique. Fifty tracheal swabs (15.4%), 30 cloacal swabs (9.2%) and 12 tissue homogenate (3.7%) showed presence of the AIV through RT-PCR and 20 tracheal swabs (6.2%), 10 cloacal swabs (3.1%) and 10 Tissue homogenate (3.1%) showed presence of the virus through the virus culture technique. In conclusion, AIV subtype H_5 was prevailing in poultry layer flocks in and around Faisalabad city, Pakistan. Tracheal swabs were the most reliable source of sample for detection of virus RNA using RT-PCR technique and isolation of the virus for subsequent confirmation through hemagglutination inhibition (HI) test.

Key words: Poultry layer flocks, avian influenza virus, RT-PCR, virus culture method.

INTRODUCTION

Influenza viruses continue to circulate among avian species and cause significant morbidity and mortality throughout the world, thus accurate identification and monitoring of circulating strains are essential. Therefore, early detection of newly emerging strains is important to control outbreaks (Zambon and Ellis, 2001). Influenza viruses of aquatic birds have been considered as ancestors of all influenza virus subtypes existing in human and other animals. Genetic variation of the haemagglutinin (HA) and neuraminidase (NA) genes resulting in the emergence of new influenza virus strains has frequently been recognized. The avian influenza virus (H_5N_1) has spread in Asian countries, where it is enzootic, causing multiple outbreaks in poultry. The AIV that posed a serious threat to public health has been directly transmitted from birds to humans. In Japan during 2003-2004, influenza outbreaks caused by an H_5N_1 virus in birds located at three distinct chicken farms and among a group of chickens raised as pet birds. However, route of infection and dissemination of virus remains unknown (Mase *et al.*, 2005). Outbreaks of avian influenza caused by H7 and H9 in Pakistan have been recorded since 1995. An outbreak of highly pathogenic avian influenza (HPAI) caused by a virus of H_7N_3 subtype affected Northern areas of Pakistan for the first time in 1995, causing death of 3.2 million birds, primarily broiler breeders and commercial broilers (Naeem and Hussain, 1995). Respiratory problems of unknown etiology have been seen in breeder and commercial layers in Faisalabad and surrounding poultry raising areas (Samundari, Tandlianwala, Jaranwala, Khurerianwala, Sayedwala and Chiniot). In some of these outbreaks, clinical signs and postmortem lesions have been like that of HPAI. Different methods like isolation of virus, reverse transcriptase polymerase chain reaction (RT-PCR) technology are going to revolutionize the diagnosis and as well as

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monitoring of influenza viruses (Noroozian *et al.*, 2007; Horimoto and Kawaoka, 1995). The present study is undertaken with objective to monitor the prevalence of AIV (H_5) in commercial breeder and layer flocks in and around Faisalabad city, Pakistan.

MATERIALS AND METHODS

A total of 975 different tissues, and tracheal and cloacal swabs were collected from 40 layer flocks (10 breeders and 30 commercial) located in suburb of Faisalabad and surrounding poultry raising areas having a layer breeder population of 50,000 and commercial layers 100,000 for the surveillance of AIV (H_5).

Collection of samples

Each of the tissue samples such as brain, trachea, lungs, air sacs and spleen was collected from the same slaughtered morbid bird from culled stock of each farm in the sterile sampling bottles having sterilized glycerol buffer (Nguyen *et al.*, 2005). Tracheal and cloacal swabs were placed in the tubes having sterilized peptone water and transported to the laboratory for further studies.

Processing of specimens

The tissue samples were removed from the glycerol buffer, triturated and centrifuged at 10,000 xg for 10 minutes. The tubes containing swab samples were shaken gently and the swabs were removed. The remaining peptone water was centrifuged as described above. The supernatant of each sample was harvested and passed through syringe filter of 0.22µm porosity. The filtrate was used for both the virus culture through chicken embryo and the virus RNA detection through RT-PCR.

Virus isolation and identification

Embryo inoculation

The semipurified virus suspension (0.2 ml) was inoculated into the allantoic cavity of 9 days old chicken embryos. These embryos were procured from a reputable hatchery. Each sample was inoculated into five embryos. After 72 hours of incubation at 37°C, the embryos were chilled; allantoic fluid of each embryo was harvested and tested for any hemagglutinating (HA) activity. The allantoic fluid exhibiting no HA activity was passed 3 times using the fresh embryos. Each of the harvested allantoic fluid was used for virus detection by HI test.

Haemagglutination inhibition test

The HI test was performed for identification of AIV (H_5) using hemagglutinin specific antisera (National Agriculture Research Center, Islamabad) as described by Allan and Gough (1974).

Reverse transcriptase polymerase chain reaction

The RNA was extracted from the samples (tissue homogenate, tracheal, and cloacal swabs) by using RNA extraction Kit (Trizol, Invitrogen, USA). Extractions were performed according to manufacturer's instructions. Extracted RNA was dissolved in RNase free diethyl pyro-carbonate (DEPC) treated water (1mg/ml). RNA solution 5µl (approximately 5µg RNA), 2µl of random hexa-nucleotide primers (50ng/µl) and 3µl of DEPC treated water was taken in microfuge tube (Eppendorf, Germany). After 5 minutes of incubation at 70°C, the solution was cooled at room temperature for 10 minutes to allow primer annealing and centrifuged briefly for 16-20 seconds before opening. An amount of 4µl of 5X RT buffer (50 mM tris HCl (pH 8.3), 3mM $MgCl_2$ and 75mM KCl), 2 µl of acetylated bovine serum albumin (BSA, 0.1 mg/ml), 2µl DTT (0.1 M), 1µl dNTPs (10 mM each) and 1µl reverse transcriptase (200 Units) was used for each RNA sample. It was mixed gently, centrifuged briefly and incubated at room temperature for 5 minutes and then at 40°C for 30-60 minutes to obtain cDNA.

The AIV was identified by H_5 specific primers, (forward) 5'- ACA CAT GCY CAR GAC ATA CT-3' and (reverse) 5'- CTY TGR TTY AGT GTT TAT GT-3', R = A/G, Y= C/T (Lee *et al.*, 2001). Reaction mixture of 50µl containing 5µl of 10X PCR buffer (Fermentas), 250µM concentrations of each deoxynucleotide triphosphate (Fermentas), 50 pmol of each forward and reverse primers, 5µl of 25 mM $MgCl_2$ (Fermentas) , 2 U of Taq DNA polymerase (Fermentas) and 10µl of cDNA template was prepared. DNA amplifications

were performed using thermal cycler (Thermo Electron, USA) that was programmed to heat the DNA at 94°C for 5 minutes, followed by 35 cycles at three different temperatures and times (94°C for 1 minute , 50°C for 1 minute and 72°C for 1 minute). The final extension step was 72°C for 10 minutes PCR product of each sample was loaded on 1.5% agarose gel (containing 0.5µg/ml ethidium bromide) and electrophoresis was performed at 100V for 1 hour, visualized under UV light and photographed using Gel Doc System (Bio-Rad, USA) (Lee *et al.*, 2001).

Data Analysis

The data thus obtained was analyzed by the Chi Square Distribution Test for more than two proportions (Steel *et al.*, 1997).

RESULTS AND DISCUSSION

Avian influenza infections are persisting in migratory birds particularly wild ducks. These birds harbor the virus and are incriminated to transfer the infection from one country to another. The birds shed the infectious agent in droppings on lakes and ponds. These water reservoirs are source of infection for susceptible birds in surrounding areas (Alfonso *et al*., 1995). The virus enters via respiratory or oral route of the birds and replicate in the mucus membrane of conjunctiva, air sacs, lungs, trachea, and digestive tract (Uzma *et al*., 2009), replicate in predilection sites of the infected birds and induce lesions. The lesions vary with subtype of the virus, route of infection, susceptibility of birds etc (Perkins and Swayne, 2001). The virus is recovered from samples collected from the tissues showing lesions or swabs from trachea, or cloaca of the infected birds. In the present study, the AIV (H_5) was detected from 50 tracheal swabs (15.4%), 30 cloacal swabs (9.2%) and 12 tissue homogenate (3.7%) through RNA specific RT-PCR while the AIV was recovered from 20 tracheal swabs (6.2%), 10 cloacal swabs (3.1%) and 10 tissue homogenate (3.1%) through the virus culture technique (Table I). The virus in trachea and digestive tract encounter minimum interference by anti-virus specific factors. This could be plausible reason that more than 15 percent tracheal swabs and 9 percent cloacal swabs showed presence of the virus specific nucleic acid. In contrast the virus in tissues encounters specific antibodies such as IgG and IgM (Abbas *et al.*, 1991). These antibodies augment the process of virus inactivation and phagocytosis and thus mitigate recovery of the virus from the tissue samples. This could be logical reason of less than 4 percent tissue samples showed presence of the virus. The AIV not coated by specific antibodies can be detected through culture methods. In infected birds, chances of virus recovery through culture method mitigate as the specific antibodies increase in the circulation. Overall forty samples showed recovery of the AIV by culture method while 92 samples showed the presence of virus specific nucleic acid using RT-PCR method (Table I). In the present study, the results were obtained within 24 hours as compared to the 3-5 days for virus isolation and identification. Lee *et al*. (2001) reported that RT-PCR technique could identify the virus directly from a variety of clinical samples without virus cultivation, and thus shorten the time for identification of the AIV. Dmitry *et al.* (2005) used RT-PCR and HI tests for the detection of six strains of AIV and found RT-PCR a quick and reliable assay for rapid diagnosis of various strains of Influenza virus. RT-PCR method has previously been used to detect influenza type A virus in throat and nasal specimens collected from humans, pigs and horses and to detect avian respiratory viruses in clinical specimens (Oxburgh and Hagstrom, 1999). The present study showed that the specimens from respiratory tract (tracheal swabs) might be more reliable source for virus detection as compared to cloacal swabs and tissue homogenate because we

Table I.- Pattern for presence of avian influenza (H_5) virus in different clinical samples from poultry flocks.

Types of Samples (n=325)	Number of positive samples (%)	
	RT- PCR	Virus Isolation
Tracheal swabs*	50(15.4)	20(6.2)
Cloacal swabs**	30(9.2)	10(3.1)
Tissue homogenates***	12(3.7)	10(3.1)
Total samples	92(28.3)	40(12.4)

The samples having similar number of superscript satiric are not significantly different ($p< 0.05$)

obtained maximum detection from respiratory specimens ($p < 0.05$). Moreover, cloacal swabs were found to be the second sample of choice for detection of the viral RNA and isolation of the virus from culled birds. The results are in accordance to Koch (2003) who reported RT-PCR inhibitory substances in some tissue or faecal samples that may reduce the chances of detection of virus RNA. In the field studies, swab samples are generally taken from living birds and organ samples are taken from dead birds. Extracted RNA may be degraded more rapidly in organ samples containing higher levels of RNAse, so that swab samples may generally yield better results (Spackman *et al.,* 2002).

Moreover, the viral RNA was detected from birds of one (10%) layer breeder and 3 (10%) commercial layer flocks using the virus subtype specific primers while the virus was isolated and confirmed by HI test from 2 (5%) flocks (one layer flock of each category). Overall out of 40 layer flocks, 4(10%) and 2(5%) showed the virus through RT-PCR and virus culture technique, respectively (Table II). There is no significant difference of AIV (H_5) prevalence in breeder and commercial layer flocks ($p > 0.05$).

Table II.- Distribution of avian influenza virus among layer flocks.

Type of layer flocks	No. of flocks (birds) tested	Number of Positive flocks (%)	
		RT- PCR	Virus Isolation
Breeder	10 (50,000)	1 (10)	1 (10)
Commercial	30 (100,000)	3 (10)	1 (3.3)
Total	40(150,0000)	4(10)	2(5)

There is no significant difference of prevalence of AIV (H_5) on breeder and commercial layer flocks ($p > 0.05$)

Our results are in agreement with Massi *et al.* (2005), Lee *et al.* (2001) and Munch *et al.* (2000) who identified the viral RNA of subtype H_5 and H_7 using a set of specific primers for each viral strain. In the present study, we used primer that is specific for HA of influenza virus H_5 and successfully amplified a product of 545bp (Fig. 1). Dwyer *et al.* (2006) reported that detection of AIV by using RT-PCR with primers specific for various human and AIV strains can be performed on virus isolates or directly on clinical specimens that is more advantageous, quick and reliable. Outbreaks of HPAI infection caused by a virus H_7N_3 subtype were recorded first time amongst commercial poultry flocks in Northern areas of Pakistan during 1994-1995. The disease caused death of more than 3.2 million broiler breeders and commercial broilers and induced heavy economic loss to the poultry breeders (Naeem and Hussain, 1995). Second outbreak of AIV caused by low pathogenic strain of the virus (H_9N_2) among commercial poultry flocks in Pakistan (Naeem *et al*., 2003). The disease caused by AIV of H_5N_1 subtype (a zoonotic problem) was recorded in live bird markets in Vietnam during 2001 and 2004, in Hong Kong during 1997 and 2002 (Nguyen *et al*., 2005), in commercial poultry in Pakistan and surrounding countries during 2004-05 (Viseshakul *et al.,* 2004).

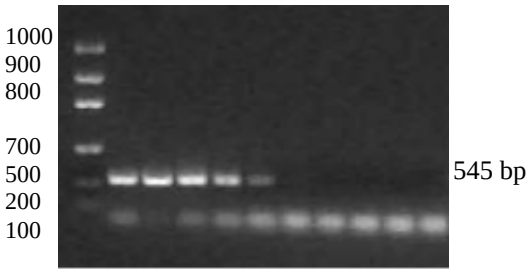

Fig. 1. Tile of RT-PCR based avian influenza virus amplicons.

Lane 1 is 100bp DNA Ladder. Lane 2 to 5 is H_5 positive flocks showing a product of 545bp. Lane 6 is control positive. The amplified products 545bp was observed with H_5 subtype specific primers.

In conclusion, AIV subtype H_5 was prevailing in layer flocks. Tracheal swabs were most reliable source of sample for detection of viral RNA using RT-PCR technique and isolation of the virus for subsequent confirmation through HI test.

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