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RESEARCH ARTICLE

Molecular Diagnosis and Pathology of Chicken Infectious Anemia in Commercial White Leghorn Layer Flocks in Pakistan

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ABSTRACT

The present field study was conducted for confirmation of chicken infectious anemia (CIA) in White Leghorn (WL) commercial layer birds. A total of 60 farms were investigated. Birds from each farm ware necropsied for the presence of lesions on different visceral organs. Samples of blood and different tissues were collected for hematology, histopathology, DNA extraction and PCR amplification using specific primers for CIA virus. There was severe anemia indicated by low hematocrit values (10.9 to 17.2%) and hemoglobin concentration (5.3 to6.7 g/dl). The petechial hemorrhages were present on subcutaneous tissue, epicardium, endocardium and gizzard mucosa. The liver and bone marrow were pale in appearance. The mortality ranged from 5 to14 % on different farms. Samples of liver and spleen from 15 farms were subjected to PCR analysis for CIAV infection by amplifying the186-bp region on highly conserved VP-2 coding gene using CAV1 and CAV2 primer pair. Presence of CIAV was confirmed in 67 and 33 percent samples of liver and spleen, respectively. A total of 13/15 farms (87%) were found positive for CIA. The results of present study confirmed the presence of CAV infection in WL commercial layer birds in current outbreak. It is concluded that extensive molecular epidemiological studies are required at national level to assess the prevalence of disease. Breeder flocks should be vaccinated to control CIA in commercial layer flocks.

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INTRODUCTION

The major economic losses in poultry industry are incurred by mortality, production losses, carcass condemnation because of infectious diseases caused by different viruses, bacteria, fungi and parasites (Hafez, 2011; Khan *et al*., 2011). In the recent years one of the most important viral infections of commercial poultry is Chicken Infectious Anemia (CIA). Chicken infectious anemia virus (CIAV) is an emerging poultry pathogen belonging to genus gyrovirus and family circoviriade. This virus was first reported as Chicken Anemia Agent in 1979 in Japan (Yuasa *et al*., 1979) and since then it has been reported worldwide in all poultry producing areas (Balamurugan and Kataria, 2006). Chicken Infectious Anemia virus induced immuno-suppression has gained considerable economic importance around the poultry globe in recent years (Toro *et al*., 1997; Dhama *et al*., 2008; Mohamed, 2010; Barrios *et al*., 2009; Bhatt *et al*., 2011; Rehman *et al*., 2011). Chicken infectious anemia is characterized by discernible immune suppression, subcutaneous hemorrhages, generalized lymphoid atrophy, thymic aplasia and aplastic anemia (McNulty, 1991;Todd, 2000).In young chicks it causes high mortality and stunted growth (Hadimli *et al*., 2008).

CIA induced immunosuppression predisposes the chicks to other diseases like Newcastle disease, Mareks disease, Avian coccidiosis, Gangrenous dermatitis and different respiratory infections. This disease is transmitted both horizontally and vertically and mostly it occurs in

young chicks at 2-4 week of age (Bhatt *et al*., 2011). Breeder hens vaccinated against CIA pass maternal antibodies (MDA) to their progeny which may give them protection from the field virus during first 3-4 weeks of life.

In field outbreaks, CIAVproduces few clinical signs of diseases but main concern isco-infections and secondary bacterial infections (McNulty, 1991). Chicks suffering from CIAV infection develop poor immunity against the causative viruses of Newcastle Disease, Mareks Disease, Laryngotracheitis and Fowl Pox resulting in vaccination failure, vaccinal reactions and development of new variants (Dhama *et al*., 2002). In Pakistan during year 2011-2012, severe outbreaks of a disease occurred in layer pullets of 3-10 weeks of age in layer growing areas of Punjab province including Faisalabad, Kamalia, Gojra, Samundri, Sargodha, Vehari, Hafizabad etc. This disease caused moderate to high mortality and the clinical picture of the sick birds resembled with that described for CIA in the literature. In field conditions the disease was tentatively diagnosed as CIA on the basis of clinical signs and gross lesions. However, the disease was not confirmed as CIA by any authenticated laboratory test. There has been a recent report describing the confirmation of CIA and avian adenovirus infections in breeder and commercial flocks by use of a duplex PCR assay (Rehman *et al*., 2011). In order to confirm the clinical picture based diagnosis of CIA in the above mentioned out breaks, attempts were made to conduct a molecular diagnosis based upon PCR assay for the detection of CIA virus in commercial White Leghorn (WL) layer pullets.

MATERIALS AND METHODS

Sample collection: During April-September 2011, sick/dead birds from 60 layer flocks were brought to the Diagnostic Laboratory of Department of Pathology, University of Agriculture, Faisalabad from different areas of Punjab. These flocks aged between 1-12 weeks and had a complaint of mortality of variable degree. The clinical picture and gross lesions observed in these birds were suggestive of CIA. To confirm this clinical diagnosis of CIA, a total of 15out of 60 farms showing the typical clinical disease and lesions and located in different cities of Punjab province were randomly selected for molecular diagnosis. Clinical signs and gross lesions on different visceral organs were recorded. From each flock, samples of liver and spleen were collected from 10 birds and stored at -20^{o}C. The blood samples of 10 sick birds from each farm were collected randomly for determination of hematocrit and hemoglobin levels to assess the anemic status of the birds.

DNA extraction: The10 samples of each organ were pooled up and a representative sample for each organ (liver/spleen) was prepared for DNA extraction. The DNA was extracted using the genomic DNA purification kit (#K0512, Fermentas EU).The DNA was extracted by the method of Eltahir *et al.* (2011). The DNA was quantified and stored at -20^{o}Cuntil PCR was performed.

CAV detection by PCR: The quantified DNA was identified by PCR for CAV DNA using specific primers , CAV1 : 5-GCA GTA GGT ATA CGC AAG GC-3 and CAV2: 5-CTG AAC ACC GTT GAT GGT C-3, covering and amplifying a 186-bp region on highly conserved VP-2 coding gene (Noteborn *et al*., 1992). The PCR amplification was carried out in PCR buffer mixture containing $MgCl_2$ 1.5mMol 3 µl, buffer 10X 2 µl, dNTPs 200 µMol 4 l µ, primers 10pMol 2 lµ each. Taq polymerase 1U 2 lµ, genomic DNA 0.2 µl and deionized water 6.8 µl and total PCR mixture was 20 µl and it was run on a fully automated thermocycler (PeqLsab Primus Germany 15383-01Q00430) using following cycling profile; initial denaturation at 94^{o}C for 2 min, followed by 35 cycles of denaturation, annealing and extension at 94^{o}C for 30 s, 60^{o}C for 30 s and 72^{o}C for 1 min, respectively, and final extension was carried out at 72^{o}C for 7 min. Then PCR product was analyzed with 1.5% agarose gel stained with ethedium bromide and image was taken. A DNA marker of 1 Kb was loaded with samples for comparison. This whole reaction was performed by modified method of (Eltahir *et al*., 2011).

RESULTS

Pathological and hematological findings: Birds were severely anemic, dehydrated, emaciated and had ruffled feathers. Feed intake of these birds was decreased. The comb and wattles were anemic in appearance. On necropsy examination hemorrhages were found on skin underlying the feathers of ventral side of wings, subcutaneous tissue, pectoral muscle and heart (Fig. 1). Hemorrhages on the proventriculus mucosa were also observed in birds from different farms. The liver and kidneys were swollen and anemic in appearance. The bone marrow was pale yellow in color as compared with healthy birds. These clinical signs and lesions have been presented in Table 1. The hematocrit values in the birds from farms under study ranged from 10.9 to 17.2 %. The average hemoglobin concentration on different farms ranged from 5.25 to 6.70 g/dl (Table 2).Blood was also thin in consistency. The average mortality on these farms was 14%.

PCR results: The liver samples from 10 and spleen samples from 5 farms were positive for CAV infection. Both the spleen and liver samples of 2 farms out of 15 were found negative through PCR for CAV infection (Table 2).When positive samples of both organs were run on gel they amplified required size band 186 bp (Fig. 2).

Table 1: Frequency of clinical signs and gross lesions

Age (weeks)	No. of flocks	Hemorrhages				Anemic appearance	Bone marrow (Pink/yellowish)	Mortality %
		Skin/Subcut.	Proventriculus	Internal organs	Heart			
1-2	8	+3	+1	+3	+2	+3	+3	12.5
3-4	9	+4	+2	+2	+3	+3	+3	14
5-6	8	+4	+2	+4	+4	+4	+4	10.8
7-8	30	+4	+3	+4	+4	+3	+3	12
9-10	9	+3	+1	+2	+1	+3	+2	9.5

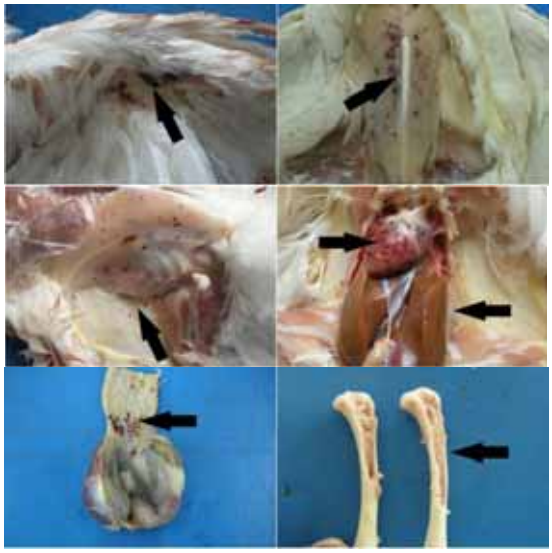

Fig. 1: Different gross lesions in visceral organs of birds infected with chicken Infectious anemia, skin underlying feathers of wings(a), hemorrhages on pectoral muscles (b), subcutaneous hemorrhages (c), hemorrhages on heart and pale liver(d), hemorrhages in proventriculus (e) and pale bone marrow (f) indicated by arrows.

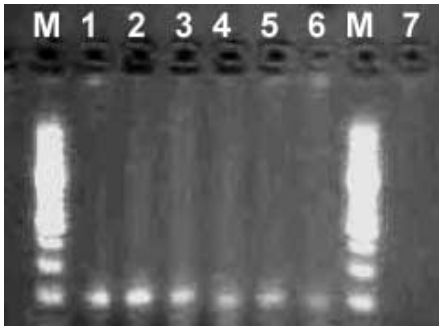

Fig.2: Detection of CAV by PCR and amplification of CAV, highly conserved VP-2 coding gene Specific PCR product (186 bp) detected in CAV-infected birds (M) 1 kb DNA ladder marker, Lane 1 positive control, 2-6 positive samples, lane 7 negative control.

DISCUSSION

The chicken infectious anemia is an immuno-suppressive disease which leads to heavy economic losses to the poultry industry. From April-September 2011, a total of 60 farms from different areas of Punjab had similar complaints of mortality, depression, anorexia, stunted growth, pale comb and wattles. Internally there was pale liver, pale bone marrow and hemorrhages on different organs. These flocks were suspected for chicken infectious anemia on the basis of clinical signs and gross lesions. These signs were similar to those previously reported by Hegazy *et al*. (2010) from Egypt and Hadimli *et al*. (2008) from Turkey. In present study gross lesions of pale liver, bone marrow and generalized atrophy of lymphoid organs were observed on all the farms. Similar lesions have been reported by other authors (Toro *et al*., 2000; Ledesma *et al*., 2001; Mohamed, 2010). Subcutaneous hemorrhages, hemorrhages on thigh muscles and pectoral muscles observed in present study have as also been previously reported (Kim *et al*., 2010).

Table 2: Mean hematocrit (%) and hemoglobin (g/dl) values and PCR results of birds from different farms suspected of CAV infection

Farm no	Hematocrit	Hemoglobin	PCR Results of tissues	
			Liver	Spleen
1	11.80	5.50	_	_
2	10.90	6.00	_	_
3	12.25	5.25	+	_
4	11.00	6.16	+	+
5	11.60	6.29	+	_
6	13.20	6.50	+	_
7	17.20	6.70	+	+
8	10.85	5.70	+	_
9	12.30	6.28	+	_
10	12.00	6.00	+	_
11	13.40	6.60	_	_
12	14.30	6.30	+	+
13	12.50	6.40	+	-
14	12.00	6.30	_	+
15	14.00	6.50	_	+

The mortality on different farms varied from 5-14% depending upon severity of disease and health status of the birds. These farms were divided into different categories on the basis of age of birds. The maximum mortality was in 3-4 week age group. The variation in mortality has also been reported by different workers. Hegazy *et al*. (2010) reported 4-21% mortality at different farms and correlated this variation with poor hygienic conditions, poor ventilation and lower quality feed. Similar to our results Jorgensen (1991) reported 19.2% average mortality with ranges of 8.4-33.9% and Rehman *et al*. (2011) from Pakistan reported up to 30% mortality in young layer pullets. In contrast to our results Chettle *et al*. (1989) reported higher mortality ranging from 20-60%.

The hematological findings in present study included very low hematocrit values (10.85 to 17.20%) and hemoglobin concentration (5.25 to 6.70 g/dl). These findings are in line with results of previous studies (Toro *et al*., 1997; Mohamed, 2010; Rehman *et al*., 2011).

For serological diagnosis of CIA different techniques have been employed by different authors including ELISA, florescent antibody technique etc. (Hadimli *et al*., 2008; Bhatt *et al*.,2011), However, it is difficult to confirm CIA infection by serological tests because maternal antibodies against CIAV are transferred to the chicks and both infected and non-infected birds may show presence of antibodies. Therefore, PCR is the most useful tool to detect CIAV in the tissues of diseased birds. The researchers in different regions of the World have used PCR to detect the nucleic acids of the CIAV for diagnosis of CIA (Mohamed, 2010; Kim *et al*., 2010; Eltahir*et al*., 2011; Snoeck *et al*., 2012; Hermann *et al*., 2012).

Among the farms selected for PCR assay, 87% samples were found positive for CAV infection through PCR using specific primers CAV1 and CAV2 for highly conserved VP-2 coding gene. The results are similar to the results of Eltahir *et al*. (2011) from China, Kim *et al*. (2010) from Korea and Hadimli *et al*. (2008) from Turkey, who used similar primers targeting the VP2 coding gene. Thirteen out of 15 farms were found positive for CIA.

Conclusion: The clinical picture and gross lesions observed in growing WLH pullets were highly suggestive of chicken infectious anemia. The clinical diagnosis was confirmed through PCR assay. The results of present

study indicated that the disease is highly prevalent in commercial poultry in Pakistan.

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