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Full Length Article

Molecular Epidemiology and Pathology of Chicken Infectious Anemia in Day Old Broiler Chicks in Faisalabad, Pakistan

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Abstract

The present study was conducted to investigate the prevalence of chicken infectious anemia (CIA) in broiler chicks in district Faisalabad, Pakistan. For this purpose, 254 samples from chicks were collected from different broiler farms. These samples were analyzed for CIA through polymerase chain reaction (PCR). The hematological analysis of all collected samples was also performed. Results showed that 38 (14.96%) samples were positive for chicken anemia virus (CAV) through conventional PCR utilizing CAV1 and CAV2 primer pair amplifying highly conserved VP-2 gene. Hematological analysis of samples from CAV positive farms showed significantly decreased average values of red blood cell count (1.99 ± 0.37×10^6/µL), hemoglobin concentration (5.88 ± 0.77 g/dL) and hematocrit (18.74 ± 2.97%) as compared to samples from CAV negative farms. Results indicated that the disease is prevalent in commercial broilers and this might be the first report of presence of CIA in broiler chicks from Pakistan. The study also concluded that diagnosis through PCR assay in young broilers is highly suggestive in day old broiler chicks. © 2018 Friends Science Publishers

Keyword: Broilers; Chicken infectious anemia; Hematology; Liver; Spleen; Thymus

Introduction

Chicken infectious anemia (CIA) causes severe aplastic anemia, thrombocytopenia, lymphoid atrophy (Toro *et al.*, 2000; Ledesma *et al*., 2001; Mohamed, 2010), stunted growth and high mortality (Haridy *et al*., 2012; Rashid *et al.*, 2017). Mostly clinical symptoms are produced at the age of 10 to 14 days. The disease is very lethal at this age and produces anemia, intramuscular and bone marrow hemorrhages, atrophy of the bone marrow and thymus in young chicks (Adair, 2000). Older birds infected with chicken anemia virus (CAV) show no clinical signs but decreased immune response is observed in the form of poor response to vaccine with reduced resistance to other secondary infections and produce significant consequences on growth rate and profitability of flock (Adair, 2000). Vaccination in breeders (maternal antibodies) is an important source to protect from clinical infection with CAV in pullets up to 3 weeks of age (Yuasa *et al*., 1980; Otaki *et al*., 1992) because Maternally Derived Antibodies (MDA) in case of CAV are very strong. Regardless of vaccination clinical disease has been witnessed in chickens proposing the vertical transmission of CAV leading to clinical infection of CIA in the offspring. Susceptibility to the clinical disease; however, decreases after four weeks of age (Bhatt *et al*., 2011).

The virus causes severe immunosuppression and enhances the pathogenicity of other co-infecting organisms (McNulty *et al*., 1991). The co-infection of this virus with other viruses such as reticuloendotheliosis virus (REV), Marek's disease virus (MDV) and infectious bursal disease virus (IBDV) has the combined effect on induced immunosuppression and in the pathogenesis (De Boer *et al.*, 1992). The mortality and morbidity rates are very high in multiple infections with other pathogens (Schat *et al.*, 2008).

In the past, diagnosis of CIA was dependent on the necropsy and clinical signs produced by the infection. In recent times modern techniques are available for diagnosis of this important immunosuppressive disease by the isolation and identification of the CAV. Initially, it was known as a disease which was not reported before in young chickens caused by some viral agent. The virus was first isolated from commercial chickens during an outbreak investigation of a Marek's disease in Japan in 1974 (Yuasa *et al*., 1979).

In Pakistan, the disease has long been suspected on the basis of clinical manifestations and lesions. It was confirmed by virus detection through PCR in layers (Islam *et al*., 2013).

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Severe outbreaks of disease occurred in Pakistan during the year 2011–2012, with moderate to high mortality in 3–10 weeks old layer pullets in layer-growing areas of Punjab and up to 30% mortality was reported in young layer pullets (Islam *et al*., 2013). However, no report is available regarding CIA in broilers in Pakistan. Therefore, the present study was conducted to detect the CAV through PCR and to investigate the prevalence of CIA in young broiler chicks.

Materials and Methods

Study Area and Sample Collection

This cross sectional study was conducted in Faisalabad, Punjab which is the third largest city of Pakistan and is situated at latitude 31° – 26' N, longitude 73° – 06' E and altitude 184.4m. The blood/tissues samples of a day old broiler chicks from 254 farms were collected using simple random sampling @ 95% confidence interval. Broiler chicks (n=10) from each farm were selected randomly and brought to the Diagnostic Laboratory, Department of Pathology, University of Agriculture, Faisalabad. After killing, different organs including liver, spleen, and thymus from each bird were collected and tissue samples were stored at -20ºC for DNA extraction and CAV detection by PCR. A part of each tissue was fixed in 10% buffered formalin for histopathology (Bancroft and Gamble, 2008). Blood samples from these birds were also collected and immediately analyzed to check out the anemic status of birds by accessing the total erythrocyte count (TEC), hematocrit level and hemoglobin concentration (Benjamin, 1978).

DNA Extraction

Ten samples of liver and spleen collected from chicks of each farm were pooled and a representative sample for each farm was processed. Samples of liver/spleen were prepared for DNA extraction. The DNA was extracted using the genomic DNA purification kit (K0512, Fermentas EU).

CAV Detection by PCR

The quantified DNA was identified by PCR for CAV 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*., 1998). The PCR amplification was carried out in PCR buffer mixture containing $MgCl_2$ (1.5 mMol) 3 µL, buffer (10 X) 2 µL, dNTPs 200 µMol 4 µL, primers 10 pMol 2 µL each. Taq polymerase 1 U 2 µL, genomic DNA 0.2 µL and deionized water 6.8 µL and run on a fully automated thermal cycler (T100 BIO-RAD®), using following cycling profile; initial denaturation at 94ºC for 2 min, followed by 35 cycles of denaturation, annealing and extension at 94ºC for 30s, 60ºC for 30 s and 72ºC for 1 min and final extension was carried out at 72ºC for 7 min. PCR product was run with 1.5% agarose gel stained with ethidium bromide and then image was taken to check the presence of specific base pair by using gel documentation system. A DNA marker of 100 bp was loaded with samples for comparison.

Hematological Studies

Blood samples from all selected poultry farms were collected in anticoagulant (EDTA) coated tubes for the hematological variables (Benjamin, 1978) including hematocrit (Hct) (Microhaematocrit method), hemoglobin (Hb) concentration (Cyanmethemoglobin method) and total erythrocyte count (Heamocytometer method).

Histopathology

Samples of liver, thymus and spleen were fixed in 10% neutral buffered formalin for histopathology. After fixation 5 mm thick tissue pieces were further processed for routine paraffin embedding method and 4–5 µm sections were stained by hematoxylin and eosin stains to study tissues for microscopic changes (Bancroft and Gamble, 2008).

Statistical Analysis

The data of hematology and organ weights were subjected to analysis of variance (ANOVA) test and group means were compared with Duncan's Multiple Range (DMR) Test. Data of CAV positive and CAV negative samples was compared by "t"-test ($p \leq 0.05$) using MSTATC statistical software.

Results

Clinical Signs and Behavioral Alterations

The birds were normal in appearance because these chicks were collected at the one day age after hatching directly from hatcheries or soon after arrival at the farms. The birds were behaving normally in feed and water intake. At few farms, problems related to early chick mortality were present including ompahlitis.

PCR Results

In molecular analysis of liver samples, 30 out of 254 were positive for CAV, while eight spleen samples out of 254 were found positive for CAV using primers for VP-2 coding gene amplifying a 186-bp region (Fig. 1). Both the spleen and liver samples of 216 out of 254 were found negative through PCR for CAV infection.

Comparison of Bodyweights, Absolute Organ Weights and Relative Organ Weights of CAV Positive and Negative Chick Samples

The bodyweights and absolute organ weights of CAV

Table 1: Comparison of Bodyweight and Absolute organ weights average values in CAV positive and CAV negative Farms (Mean ± SD)

Parameters	CAV Positive Farms	CAV Negative farms
Body Weight	38.19 ± 2.49	38.35 ± 2.64
Liver	1.01 ± 0.17	1.06 ± 0.20*
Spleen	0.03 ± 0.01	0.03 ± 0.01
Thymus	0.97 ± 0.13	1.01 ± 0.19

*Significant difference in a row, CAV Positive Farms vs CAV Negative Farms (P≤0.05)

Table 2: Comparison of Bodyweight and relative organ weights average values in CAV positive and CAV negative Farms (Mean ± SD)

Parameters	CAV Positive Farms	CAV Negative farms
Body Weight	38.19 ± 2.49	38.35 ± 2.64
Liver	2.65 ± 0.44	2.79 ± 0.55*
Spleen	0.09 ± 0.03	0.09 ± 0.04
Thymus	2.56 ± 0.36	2.65 ± 0.54

*Significant difference in a row, CAV Positive Farms vs CAV Negative Farms (P≤0.05)

Table 3: Comparative Hematological Findings of CAV Positive and CAV Negative Farms (Mean ± SD)

Parameters	CAV Positive Farms	CAV Negative Farms
RBC ($\times 10^6$/µL)	1.99 ± 0.37	2.66 ± 0.35*
Hb (g/dL)	5.88 ± 0.77	9.12 ± 1.14*
PCV (%)	18.74 ± 2.97	29.62 ± 2.23*

*Significant difference in a row, CAV Positive Farms vs CAV Negative Farms (P≤0.05)

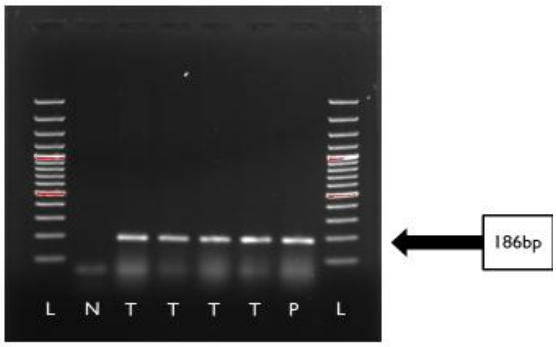

Fig. 1: Photograph of gel of PCR positive samples (Lane description: 1 and 8 ladder (100 bp), 2 control –ve, 3-6 positive test samples, 7 control +ve)

positive and negative chicks have been presented in Table 1. The difference in bodyweight of samples/birds from CAV positive farms and CAV negative farms was non-significant. The average values of absolute liver weight of samples/birds from CAV negative farms were significantly higher than CAV positive farms. However difference in the average values of absolute spleen and thymus weight of samples/birds from CAV negative farms and CAV positive were non-significant.

The bodyweights and relative organ weights of CAV positive and negative chicks have been presented in Table 2. The difference in bodyweight of samples/birds from CAV positive farms and CAV negative farms was nonsignificant. The average values of comparative liver weight of samples/birds from CAV negative farms were significantly higher ($P<0.05$) than CAV positive farms. Non-significant differences were observed in relative organ weights of Spleen and thymus of CAV positive and CAV negative samples/birds.

Hematological Findings in Birds from CAV Positive and Negative Farms

The hematological values of CAV positive samples and CAV negative samples have been presented in Table 3. The average values of total RBC of samples/birds positive for CAV was significantly lower as compared with average RBC values of the CAV negative farms. The same was true for average values of Hb concentration (g/dl) and Hct (%) in CAV positive and CAV negative birds.

Gross and Histopathological Findings

At necropsy, most of the birds were normal in appearance. The visceral organs including liver, spleen and thymus also appeared normal. However, in few birds liver was swollen, hemorrhagic, while spleen was quiet normal. In few birds liver was anemic. The thymus was normal in most of the birds. In some birds, yolk was present in abundant amount but this is a normal mechanism because yolk should be normally present in the young chicks.

The hepatic parenchyma of the birds collected from negative farms showed normal pattern of hepatocytes. The hepatocytes were arranged in fine hepatic cords, sinusoidal spaces were normal in appearance. The cytoplasm of the hepatocytes was fine pinkish, nuclei were normal having nucleolus and fine chromatin material.

In positive farms samples, all these features were similar in appearance except mild to moderate degree of cytoplasmic vacuoles. There were no significant changes in the spleen and thymus all were normal in appearance both in positive farms and negative farms.

Discussion

In Pakistan, during last 3–4 years, chicken infectious anemia cases in young layer pullets during the rearing period are drastically increasing. CIA was first time reported in Pakistan by Islam *et al.* (2013) from Central Punjab. They reported high mortality due to severe anemia at different scattered layer farms during early age of life. However, in the literature it was reported that birds during first 3–4 weeks of age are more susceptible to CAV clinical disease. But Islam *et al.* (2013) reported clinical cases of CIA up to 10–12 weeks of age. In case of CAV, maternally derived

antibodies (MDA) are very important. If parent flocks are properly vaccinated and immunized, the chances of clinical disease in progeny are almost zero as MDA antibodies protect the progeny chicks against horizontal field infections. In the present study, a total of 38 samples out of 254 (14.96%) were found positive for CAV infection through PCR. These positive samples were reconfirmed twice. Although this is not a high prevalence of CAV; however, it confirms the presence of CIA in day old broiler chicks, which is an indirect indication of vertical transmission of CAV from parent flocks to progeny chicks. Similar results of variable prevalence of CAV in different age groups have been reported as 73% in India (Wani *et al*., 2013), 78.38% in North India (Krishan *et al.*, 2015), 10.27% in China (Eltahir *et al*., 2011), 40–100% in Malaysia in chicken embryos from non vaccinated CAV breeder hens (Hailemariam *et al*., 2008), 58.3% in West Indies (Sharma *et al*., 2014). It is first ever report from Pakistan on the presence of CAV in broiler chicks. Previously, Rehman *et al*. (2011) and Islam *et al*. (2013) reported CAV from commercial layer pullets and breeders.

In the present study, blood samples were also collected from day-old birds for hematological evaluations. The results indicated that average hematocrit value in CAV negative farms was 29.62 ± 2.23% and in CAV positive farms was 18.74 ± 2.97%, which was significantly lower from CAV negative farms indicating severe anemia. The average hemoglobin concentration in CAV negative farms was 9.12 ±1.14 g/dl and in CAV positive farms it was 5.88 ± 0.77 g/dl which was significantly lower from negative farms. Similarly, RBCs value in CAV negative farms was $2.66 \pm 0.35 \times 10^6$/µL, while in positive farms it was $1.99 \pm 0.37 \times 10^6$/µL. All these values of Hb, Hct and RBC were significantly lower in positive farms as compared with negative farms. Similar results of severe anemia indicated by low hematocrit value, lower hemoglobin concentration and lower total erythrocytic count (TEC) count have been reported by Bhatt *et al*. (2011) from India, Islam *et al*. (2013) from Pakistan and Haridy *et al*. (2012) from Japan. These lower hematological values are an indicator for preliminary diagnosis of CAV even without molecular tools. However, it should be confirmed through PCR. In the present study, body weights, absolute and relative organ weights of spleen and thymus were non-significantly different between CAV positive and CAV negative farms. However, liver absolute and relative weight was significantly higher in negative farms as compared with positives farms. No one has previously reported such findings. In the present study, no significant histopathological findings were observed in different tissues of day old chicks. CAV is a potent immunosuppressive agent like IBD. It may lead to vaccination failure and aggravation of the pathogenicity of live vaccines strains secondary bacterial complications and ultimately production losses (Todd, 2000; Dhama *et al*., 2008; Bhatt *et al*., 2011; Gowthaman *et al*., 2013).

Conclusion

CIA is an emerging disease in national poultry industry affecting young broiler chicks. Keeping in view the emerging status of CAV and its increasing prevalence recent advances in the diagnosis, vaccination and therapy along with proper disease surveillance strategies should be started at national level to control the disease. There should be a legal binding to parent stock holder/breeding companies to ensure the proper immunization of their parent and grandparent flocks against CAV. The Government should also make it possible that throughout the year, CAV vaccine should be available in the country.

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