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# Pathology of chicken infectious anemia (CIA) with concurrent infections

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**Abstract**
Poultry is constantly exposed to various immunosuppressive agents such as viruses, mycotoxins and environmental stress. Chicken Infectious Anemia (CIA) caused by a Circovirus a DNA virus is one of the very important viral diseases. Out of 100 clinically suspected outbreaks of immunosuppressive diseases in the current investigation, 11 outbreaks of CIA were diagnosed. The pathological parameters such as clinical signs, gross, microscopic and ultrastructural lesions were studied and characterized. All eleven cases were confirmed using PCR employing VP2 specific genes. Among the 11 confirmed outbreaks, based on necropsy findings, histopathology and bacterial culturing, 7 outbreaks showed concurrent infection with one or more secondary infections such as E.coli, Salmonellosis, Pasteurellosis, Gangrenous dermatitis, Cecal coccidiosis and aflatoxicosis.

**Keywords:** Chicken infectious anemia, circovirus, concurrent infection, immunosuppression, pathology

## Introduction
The poultry birds are constantly been exposed to various immunosuppressive agents such as viruses, Mycotoxins and environmental stress. This in turn is leading to secondary bacterial and viral infections, failure of vaccinations and severe economic losses. The important viral diseases involving immunosuppression include Marek's Disease (MD), Chicken Infectious Anemia virus (CIA), Infectious Bursal Disease (IBD), Reovirus, Avian Leukocis, Reticuloendotheliosis, Newcastle disease and Avian Influenza virus (Umar *et al.*, 2017; Gimeno and Schat 2018; Jordan *et al.*, 2018) [33, 10, 18]. The non-viral of immunosuppressive agents include Mycoplasma, Ammonia, Mycotoxin and heat stress (Umar *et al.* 2017) [33].
Chicken Infectious Anemia (CIA) caused by a Circovirus was first identified in the year 1979 (Yuasa *et al.*, 1979) [37]. as a new viral disease in young chickens. Circoviruses are small, non-enveloped icosahedral animal viruses characterized by circular single stranded DNA genomes. Their genomes were the smallest possessed by animal viruses (Crowther *et al.*, 2003; Todd *et al.*, 2003; Yao *et al*., 2019) [6, 32, 36].
The disease is known to have clinical picture of aplastic anaemia, generalized lymphoid atrophy, haemorrhages, increased mortality, and immunosuppression, drop in haematocrit values watery blood and morbidity and mortality of up to 100 and 60% respectively (Dhama *et al.*, 2008; Schat and Santen 2008; Wani *et al.*, 2013) [8, 30, 34]. With generalized lymphoid atrophy and immunosuppression, the disease is commonly complicated by secondary bacterial, viral, protozoan, mycotic infections (Bakshi *et al*., 2016) [2] or parasitic infestations.
Pathologically CIA presents varying lesions such as yellow fatty bone marrow (Pope 1991; Dhama *et al.*, 2008) [28, 8], generalized lymphoid atrophy particularly, hyperemic thymus, swollen and mottled liver and congestion and hemorrhages in visceral organs (Kuscu and Gurel 2008) [20] and hemorrhages on subcutaneous tissue, breast and thigh muscles, heart, and proventricular mucosa as noted by Islam *et al*., (2013) [16]. Polymerase chain reaction (PCR) has been considered as the most useful tool for confirmatory diagnosis of many viral diseases including CIA. The highly specific and conserved genes of putative scaffold viral protein VP2 are generally employed using thermal cycler for this purpose (Islam *et al.*, 2013) [16].
Therefore, the present investigation was carried out to investigate pathological features of CIA and generally accompanying concurrent infections in three southern states of India including Karnataka, Tamilnadu and Andhra Pradesh using various diagnostic tools such as gross,

microscopic and ultrastructural pathology, bacterial culturing and confirmation using PCR.

## Materials and Methods

The study was carried out in the Department of Veterinary Pathology, College of Veterinary Science, Sri Venkateshwara Veterinary University, Tirupati and Department of Veterinary Pathology, Veterinary College, Hassan of Karnataka Veterinary Animal and Fisheries Sciences University between January 2017 and February 2020. Poultry farms in southern districts of Karnataka and neighbouring states including Andhra Pradesh and Tamil Nadu were included in the study.

Detailed information on the farm outbreak/birds presented for post mortem with respect to type of birds (broiler, layer, breeder or country birds), flock strength in farm, breed/strain details, age, morbidity, mortality, clinical signs, previous history of outbreak and any other necessary details were collected.

Necropsy examination was carried out and vital organs such as liver, spleen, kidneys, heart and brain and lymphoid organs involving thymus, cecal tonsils, bursa, and femur with marrow and harderian glands were collected for histopathological examination. The representative tissue samples were collected for PCR in a sterilized tissue bottles and stored at -20 $^0$C immediately. Representative tissues were collected in 2.5% glutaraldehyde for electron microscopic examination.

The impression smears of various vital organs, lesions and bone marrow of the femur prepared during necropsy examination were air dried and fixed with absolute methanol and stained with Giemsa stain as per Luna (1968) [23]. Tissue samples collected were immediately fixed in 10% neutral buffered formalin and then processed by routine paraffin embedding technique and sectioned at five micron thickness. The sections were stained with routine Hematoxylene and Eosin (H & E) method. Special stains such as Masson's trichrome or Van Geison (Connective tissue), Shorr's staining (Inclusion bodies) and Perl's/Prussian blue stain (Hemosiderin pigments) were used on need basis to characterize the lesions (Luna, 1968) [23].

For electron microscopy, processed tissues were sectioned into ultra-thin (60 nm) sections with a glass knife on ultra-microtome (Leica Ultra cut UCT-GA-D/E-1/00), mounted on copper grids and stained with saturated aqueous Uranyl acetate (UA) and counter stained with Reynolds lead citrate

DNA was extracted from alkaline lysis method of extraction and subjected to conventional PCR. Amplification of VP2 targeting a 419 bp amplicons using specific primers as previously reported by (Ottiger 2010). The primers were synthesized and procured commercially from M/s Bio Serve Biotechnologies (India) Pvt. Ltd, Hyderabad. The primer sequence was 5'-CTAAGATCT GCAACTGCG GA -3' (CAV_P1, Forward) and 5'-CCTTGGAAGCGGATAGTCAT-3' (CAV_P2, Reverse). Thermal cycler conditions were 95$^0$C for 3 minutes (Denaturation), 94 $^0$C for 1 minute (Annealing), 72 $^0$C for 1 minute (Extension) and 72$^0$C for 10 minutes (Final Extension).

For culturing of co-infected bacteria, the samples collected aseptically during necropsy examination were used for culturing as per the standard protocols (Markey *et al.*, 2013) [24]. Various media/broth such as Eosin Methylene Blue and MacConkey Lactose Agar (for Escherichia and Salmonella species) and Mannitol Salt agar, Brain Heart Infusion broth and Baird parkers media (for Staphylococcus species). Further, bacteria were confirmed by gram staining and biochemical test.

## Results and Discussion

Out of 100 clinically suspected outbreaks of immunosuppressive diseases in the duration evaluated 11 cases of CIA were diagnosed and confirmed. This included 7 cases from broilers, 2 cases each from commercial layers and indigenous/upgraded breeds of chicken. The age group recorded varied from 3 to 30 weeks with highest recording of 4 cases accounting for 36% in the birds of 3-4 weeks of age. Morbidity and mortality varied from 3.3 to 15% and 6.3 to 25% respectively. Wide prevalence of CIA in India (Bhatt *et al.*, 2011; Wani *et al.*, 2013; Gowthaman *et al.*, 2014; Baksi *et al.*, 2016) [4, 34, 12, 2] and other parts of the world such as Switzerland (Hoop *et al.* 1992) [13], Mexico (Ledesma *et al.*, 2001) [21], Israel (Davidson *et al.*, 2004) [7] and China (Yao *et al.*, 2019) [36] has been well documented. Wani *et al*., 2013 [34] have recorded that the age wise prevalence of CIA in young chicks of up to three weeks was 80.3% of much higher value than the recorded value in the current study.

Birds affected with CIA showed anorexia, dullness, depression, stunted and reduced rate of growth, prostration, decreased production, and staggering gait with watery diarrhoea. Severe anemia indicated by pale comb, shanks and wattle, moderate to severe emaciation, dehydration, anorexia and ruffled feathers were also observed. These clinical signs matched with previous observations made by Dhama *et al.*, (2008) [8] and Wani *et al.*, (2013) [34].

Macroscopically, carcass of the birds appeared pale and anemic (Fig 1). Liver appeared swollen, mottled and enlarged in small percentage of birds with yellowish discoloration with distended gall bladder. Hemorrhages were observed both on proventricular mucosa and its junction with gizzard. Blue wing lesions and gangrenous type of dermatitis were recorded in few outbreaks. Thymus revealed moderate to severe lymphoid atrophy as a consistent lesion. These changes were as per previous observations made by Engstrom and Luthman (1984) [9], Pope (1991) [28] and Dhama *et al.* (2008) [8].

Bone marrows of femur appeared fatty, yellowish or pink coloured in most of the birds of affected flock (Fig 2) as per Kuscu and Gurel (2008) [20]. Bursal atrophy was observed in only small percentage of birds in few outbreaks only accounting to around 10 to 20%. The concurrent infections revealed corresponding gross lesions associated with the co-infected organisms such as fibrinous pericarditis and perihepatatitis in Ecoli (Fig 3), fatty liver in mycotoxicosis and hemorrhagic typhilitis in cecal coccidiosis.

Cytologically, bone marrow smears revealed severe decrease in cellularity indicating atrophy and aplasia of all cell types including granulocytic, agranulocytic and hematopoietic precursors. Adipose tissue in the form of vacuolar spaces and stromal cells were seen replacing hematopoietic cells (Fig 4). Large hemopoietic cells and hemocytoblasts with more cytoplasm and large nuclei were seen occasionally in some areas. These changes were in tune with Kuscu and Gurel (2008) [20] and anticipated ones as per the observations of Adair (2000) [1] who noted that the destruction of T lymphocyte, granulocytes, erythroblasts and progenitors of macrophage, non-availability of T helper cells for functioning of B lymphocytes was major pathogenicity pathway.

Histopathologically, bursa of Fabricius showed mild to severe atrophy of the lymphoid follicles, hydropic degenerative and

infolding changes in epithelial cells, multiple areas of necrosis characterized by pyknotic and karyorhexic changes of the nuclei, cystic bursal follicles and fibrous tissue proliferation (Fig 5). Thymus showed moderate to severe atrophy with lymphoid depletion in cortical lymphocytes. Atrophic changes were seen both in cortex and medulla (Fig 6). The cortical depletion led to thinning of cortex and appearance similar to medulla. These microscopic observations were in consensus with the findings of Kuscu and Gurel (2008) [20], Schat and santen (2008) [30] and Rimondi *et al.,* (2014) [29].

Bone marrow showed severe hypoplasia or atrophy of the hematopoietic tissues in the marrow and medullary sinuses with few mature erythrocytes or completely devoid of bone marrow cells, These findings have also been reported by previous researchers (Kuscu and Gurel 2008) [20]. Spleen showed various degrees of lymphocytic depletion and proliferation of macrophages and decrease in lymphocytes of the white pulp with proliferation of reticular and formation of bursa dependent follicles and hemosiderosis (Fig 7). Liver revealed mild to moderate degenerative and fatty changes in hepatocytes, necrotic areas and periportal leukocytic infiltration with hemosiderosis (Fig 8). Heart in few affected birds showed heterophils infiltration, sarcolysis, myocarditis along with congestion. The lungs were congested with occasional hemorrhagic areas. Kidneys showed pockets of hemorrhages along with nephritic changes. The affected skin with gangrenous dermatitis showed necrotic and inflammatory changes with infiltration of bacteria, heterophils and lymphocytes. The pathological changes were as per Todd (2000) [32], Schat and santen (2008) [30] and Narayani and Ghosh (2018) [26].

Electron microscopic investigation revealed thymic lymphoblasts in the outer cortex showed mild to moderate swelling with electron-opaque regions with microtubular aggregates in the cytoplasm (Fig 9). Spleen showed relatively decreased number of lymphocytes and increased number of small, dark, reticular cells (Fig 10). Occasional degenerating lymphocytes and microtubular accumulations were seen in the bursa of affected birds. CIA virions consisting of non-enveloped, icosahedral particles with approximate diameter of 25 nm were observed mixed organ suspension (Fig 11). These ultrastructural changes were in tune with the previous observations made (McNulty *et al.,* 1990; Jeurissen *et al.,* 1992; Krishan *et al.,* 2016) [25, 17, 19]. However, contained finely granular and homogenous intranuclear inclusion bodies in Thymus as noted by Goryo *et al.,* (1989) [11] were not noted in the current investigation.

The results of PCR confirmed CIA virus in all 11 suspected outbreaks. The 419 bp product amplicons (Fig 12) obtained was sequenced and phylogenetic tree was drawn and similarity in terms of percentage between various isolates was presented. This showed about 95% similarity with the complete genome of Taiwan, China, Japan and Vietnamese strains. Various researchers have employed specific genes of VP2 viral protein for confirmation of CIA (Basaraddi *et al.,*2013; Islam *et al.,*2013; Wani *et al.,* 2013; Wani *et al.,* 2014) [3, 16, 34, 35].

Among the 11 outbreaks of CIA, 7 outbreaks showed concurrent infection with one or more infections. This included E. coli (#4), Salmonellosis (#2), Fowl Cholera/Pasteurellosis (#1), Gangrenous dermatitis either due to Staphylococcus or Clostridium perfringens (#3), Cecal coccidiosis (#1) and aflatoxicosis (#1). The average morbidity and mortality in the outbreaks with concurrent infection were 11.7 and 15.5% respectively. These values in outbreaks without concurrent infection were 6 and 7.6%. Exacerbated clinical presentation with increased mortality has been reported in the past with concurrent infections (Yuasa *et al.* 1983; Hornok *et al.*1998; Hristova and Simeonov 2018) [38, 14, 15].

Similarly, many researchers in the past have documented concurrent infections with various other viral diseases such as reticuloendotheliosis (Yuasa *et al.,* 1979) [37], adenoviruses including inclusion body hepatitis/ hydropericardium syndrome (Yuasa *et al.,* 1983) [38], IBD (Engstrom & Luthman, 1984) [9] and Avian leukocis (LiTing *et al.,*2019) [22].

Other bacteria such as *Clostridium perfringens*, *Staphylococcus aureus*, salmonellosis and *Escherichia coli* leading to complications such as gangrenous dermatitis, colibacillosis and fowl typhoid have also been recorded as co-infectious agents in CIA (Bougiouklis *et al.,* 2007) [5]. Interaction of certain protozoans such as *Cryptosporidium baileyi* (Hornok *et al.,* 1998) [14] and *Plasmodium juxtanucleare* (Silveira *et al.* 2013) [31] has also been recorded. Chicken Infectious Anemia being an immunosuppressive disease causing dysfunction of both humoral and cellular immune response, increased susceptibility to secondary infections and vaccine failure was a common consequence (Adair 2000; Umar *et al.,* 2017) [1, 33].

**Fig 1:** Gross lesion: Note pale and Anemic carcass

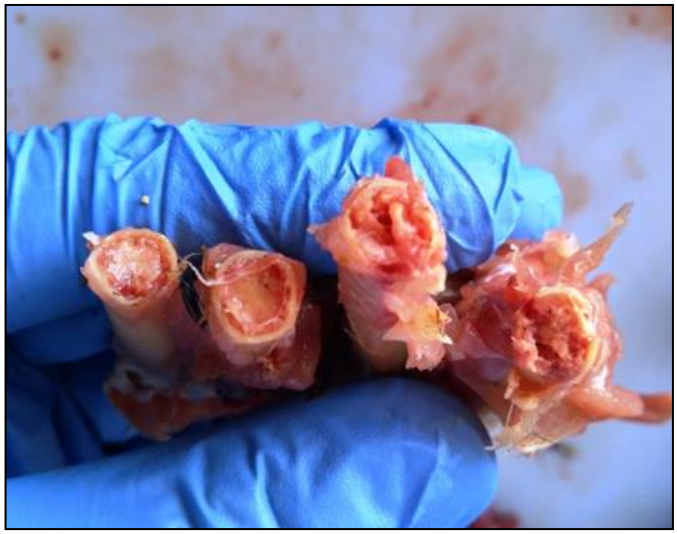

**Fig 2:** Gross lesion, Femur: Note pale and yellowish bone marrow

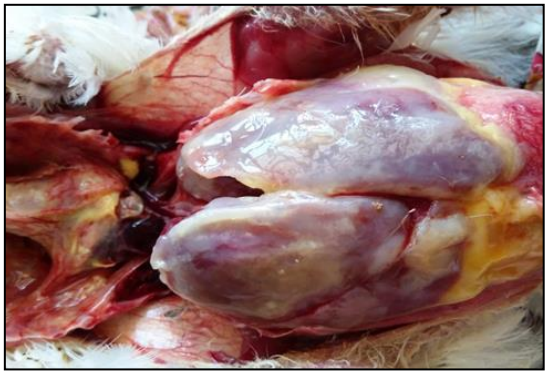

**Fig 3:** Gross lesion showing concurrent Colisepticaemia and fibrinous pericariditis and perihepatitis

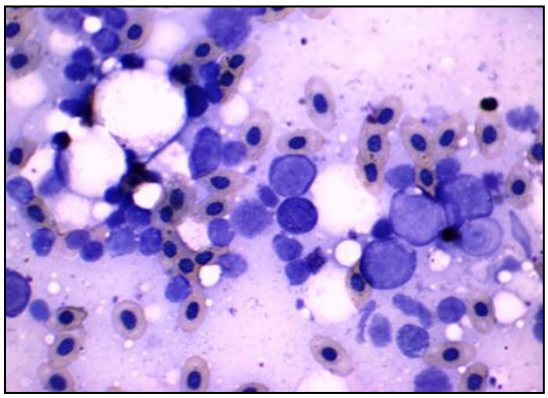

**Fig 4:** Cytology: Giemsa X 1000: Smear showing adipose tissue in the form of vacuolar spaces and stromal cells were seen replacing hematopoietic cells.

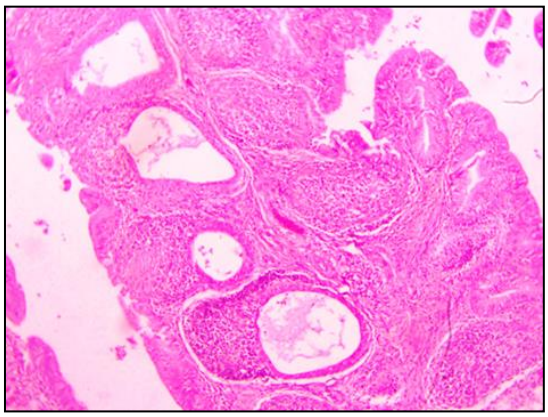

**Fig 5:** Histopathology: H& E X 100: Bursa showing severe atrophy of the lymphoid follicles

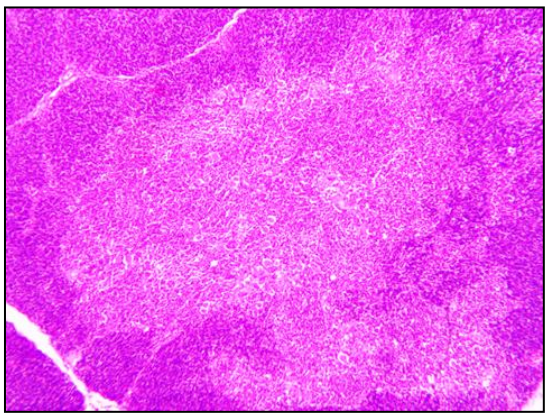

**Fig 6:** Histopathology: H& E X 100: Thymus showing atrophic changes in cortex and medulla

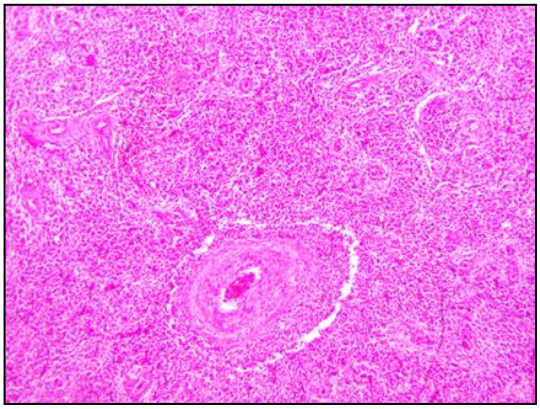

**Fig 7:** Histopathology: H& E X 100: Spleen showing lymphocytic depletion in white pulp and proliferation of reticular cells and macrophages

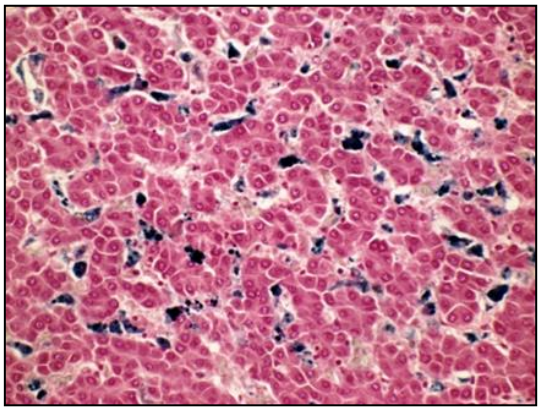

**Fig 8:** Histopathology: Perl's stain X 400: Liver showing hemosiderosis

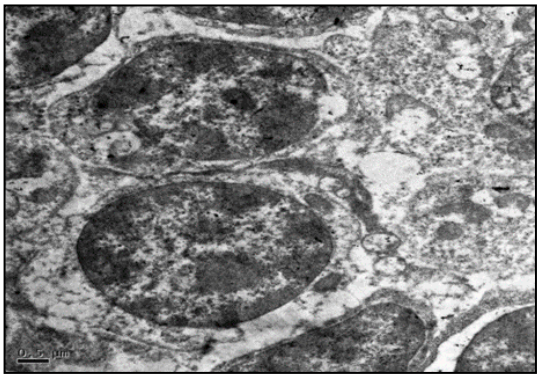

**Fig 9:** Electron Microscopy: Thymus: Lymphoblasts in the outer cortex showing mild to moderate swelling with electron-opaque regions

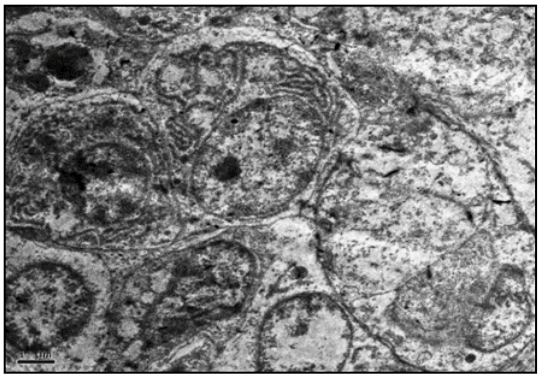

**Fig 10:** Electron Microscopy: Spleen: Hyperplastic epithelial reticular cells.

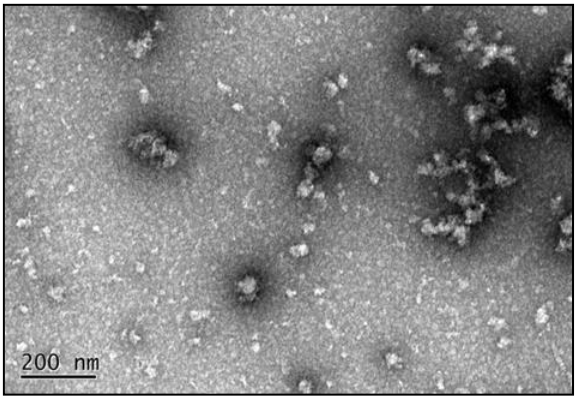


**Fig 11:** Electron Microscopy: Note CIA viral particles in negatively stained preparation.

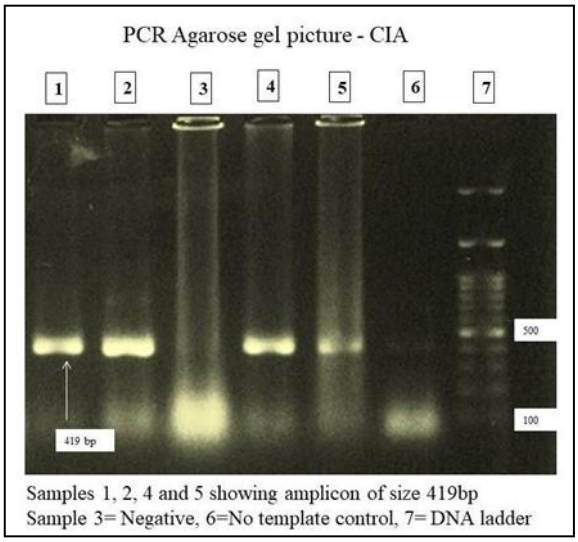


**Fig 12:** PCR gel doc picture: showing amplicons of 419 bp size

## Conclusion

In the current study carried out for 3 years duration between January 2017 and February 2020, a total of 100 outbreaks of immunosuppressive diseases of viral origin were studied and 11 outbreaks of Chicken Infectious Anemia were diagnosed and confirmed using history, gross, histopathology, ultrastructural pathology and PCR for identification of VP2 specific gene. Among the 11 confirmed outbreaks of CIA, based on necropsy findings, pathological lesionsand bacterial culturing, 7 outbreaks were also found to possess coinfections with one or more secondary bacteria, parasite or mycotoxicosis such as E.coli, Salmonellosis, Pasteurellosis, Gangrenous dermatitis, Cecal coccidiosis and aflatoxicosis. The secondary complications are due to immunosuppression brought about by CIA virus by the direct and indirect action on lymphoid organs such as bone marrow, thymus, spleen and bursa.

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