

Pathological Studies on Suspected Infectious Bronchitis – QX Strain Infection in Layer Birds

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Abstract

The pathological study of Infectious Bronchitis virus in a commercial layer flock comprised the information in relation to production, mortality, clinical signs, gross and histopathological examination, serological estimation, virus characterization by RT-PCR and subsequent gene sequencing. The mortality observed was 15.3% with typical penguin gait appearance with pendulous abdomen from 32 weeks onwards. Drop in egg production started from 20th week onwards with characteristically mis-shaped or soft shelled egg with watery albumin. Grossly cystic oviducts, accumulation of abnormally formed eggs or large masses of fibrinous yolk material in the oviduct observed, which microscopically confirm through severe decrease to complete absence of glandular tissue, atrophy of the villi with loss of mucosal epithelium and infiltration of mononuclear cells and plasma cells in the lamina propria oviduct; trachea and kidney parenchyma mononuclear inflammatory cells infiltration and mild to moderate interstitial nephritis. The typical dwarfing and curling of supernatant inoculated embryos confirmed the presence of the IB virus. Diagnostic RT-PCR with S1 gene targeted amplification performed and sequence analysis of isolates confirm 4/91 strain of IBV. In India this was the first study concluding the involvement of IBV 4/91 strain in inducing reproductive tract damages particularly cystic oviducts in commercial layer birds leading to production drop.

Keywords: IBV, Cystic oviduct, SPF, dwarfing, 4/91 strain.

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I. Introduction

Poultry production in India has taken a quantum leap in the last four decades, emerging from an unscientific farming practice to commercial production system with state of art technological interventions. India is the second largest producer of egg after China. Egg production at the end of eleventh five year plan (2011-2012) was 66.45 million tone which was 114.38 million tone in the 2019-20 (10.15%) and latest in the year 2022-23 it reaches 138.38 million tone (Annual report 2023-2024, Department of Animal Husbandry Dairying and Fisheries, GOI).

Farmers in the country faced challenges are high feed costs, inadequate cold chain and transportation infrastructure as well as high vulnerability to disease outbreaks. Among the important poultry diseases, Infectious bronchitis virus (IBV), a corona virus infection of chickens, is one of the major causes of economic losses in the poultry industry. The name of the disease refers to its most frequent clinical manifestation, although it can infect many other epithelial cells, including the kidney, genital organs and many parts of the alimentary tract (Cavanagh and Naqi, 2003). Pathogenicity for the reproductive tract depends on the age of the birds at the time of infection. Infection of laying birds produces a range of effects varying from eggshell pigment changes to production drops (Jones and Jordan, 1971).

Since IBV was first described, several serotypes in addition to the originally identified Massachusetts (Mass.) type of IBV were found (Fabricant, 1998). The epithelium of the oviduct of the female is highly susceptible to certain IBV strains. The infection is critical during production, where IB can cause a delay in onset of lay or a serious fall in egg production and in the days soon after hatching. If female chicks become infected by certain genotypes of IBV to which they have no maternal antibodies, the virus can replicate in the epithelium of the oviduct. This result in sufficient damage to the oviduct when the bird reaches sexual maturity, regions of the organ may have developed abnormally or not at all, so the bird is unable to lay eggs externally. Hens affected this way are called “false layers”.

In September 1996 an outbreak of disease reported in Qingdao, China and the associated virus was designated QX IBV (Yu Dong *et al.*, 1998). Egg production problems noticed in the period between 1995 and 2004 (Liu and Kong, 2004). Since early 2004, this infectious bronchitis (IB) strain has been detected in an increasing number in other European, Asian and African countries. One of the characteristic finding is that in the laying phase, the affected flocks showed a peak in egg production of only 36-50% due to many birds

showing cystic oviducts in the presence of active ovaries. These were diagnosed as false layers (Landman *et al.*, 2005). Around the year 2000, visceral gout and nephritis were observed in birds less than two weeks of age and several nephropathogenic strains of IBV were identified (Bayry *et al.*, 2005) in India. Since then, many nephropathogenic strains of IBV associated with gout in chicks have been isolated and genetically characterized. Yewale (2010) studied the visceral gout in broiler chicks around Anand region in Gujarat state, India and confirmed that the condition is associated with Infectious Bronchitis virus. Local isolates on sequencing of hyper-variable regions showed phylogenetic relatedness with 4-91 and mass strain. But the impact of IB virus on poultry production due to its tropism towards epithelial cells of oviduct has not been studied in a greater extent in India.

II. Materials and Methods

Pathological studies on suspected Infectious Bronchitis-QX strain infection in layer birds was undertaken in an affected farm near to Palanpur of Banaskantha district of Gujarat. The age of the affected layer flock was about 37 weeks where many of the affected birds showed clinical signs typical of 'penguin gait appearance' along with production drop from 90 to 70 percent. The affected farm was personally visited and information relating to clinical signs, mortality pattern and production drop were collected from the farm records. The blood samples were collected from ailing birds for serological testing. The postmortem examination of dead birds was conducted and tissue samples were collected for histopathology as well as isolation and subsequent conformation of virus isolate.

Pathological study

Clinical signs and pattern of production drop

During the farm visit a thorough history was taken regarding the clinical signs exhibited by the flock and the observed ones were recorded. From the farm records, relevant data like mortality rate and the production drop pattern was collected. Percentage Hen day egg production (%HDEP) was calculated on weekly basis from week 19 to week 54 and was compared with standard ones.

Gross Pathology

Necropsies were carried out on several carcasses of birds from the affected farm and also the birds showing the typical penguin gait appearance were sacrificed and grossly examined. Gross pathological lesions observed during necropsy were recorded giving special attention to ovary and oviduct. The severity of lesions was graded as mild, moderate and severe using an arbitrary scale.

Histopathology

During necropsy, organs including, liver, heart, spleen, lungs, trachea, kidney, parts of oviduct, ovary and proventriculus were collected in 10% formal saline and preserved for further processing. All the organs were processed in automatic tissue processor and then paraffin blocks of all the tissues were prepared. Sections of all the organs were cut at 5-6 microns thickness with automatic section cutting machine (Leica, Germany) and stained with Haematoxylin and Eosin (H & E) stain (Luna, 1968). The H & E stained slides were observed under light microscope and lesions were recorded.

At the time of necropsy examination, the samples of trachea, kidney, caecal tonsils and oviducts were collected aseptically from the birds showing typical lesion of cystic oviducts for subsequent virus isolation.

Preparation of inoculums for egg embryo inoculation

The collected tissue samples of kidneys, caecal tonsils and trachea were washed thoroughly in sterile Phosphate buffered saline (PBS) solution. The pooled samples were triturated with sterile sand particles along with chilled PBS in a sterile mortar and pestle. Then, the homogenates were subjected to three cycles of freezing and rapid thawing to release the virus from tissue cells. The supernatant of tissue suspensions were obtained through clarification by centrifugation at 7500 rpm for about 20 minutes at 4°C. Antibiotic was added and the supernatant was filtered through 0.45 µm syringe filter. Filtered suspension were stored at -20°C and used for inoculation in Specific Pathogen Free (SPF) embryonated eggs.

Virus propagation in embryonated SPF eggs

The tissue homogenates were inoculated into the allantoic cavity of embryonated SPF eggs. The eggs were candled on alternate days after fourth day to check the viability of the embryo and non-fertile eggs were discarded. After nine days of incubation, eggs were candled prior to inoculation to determine the position of the embryo and air sacs. The individual egg was laid on its side and a hole was punched where the embryo was located. Separate syringes were used for inoculation in different eggs. Needle was inserted vertically and slowly

200µl inoculum was delivered in the allantoic cavity. The hole was sealed with paraffin and all eggs were incubated in a horizontal position with the side of the hole facing up. Eggs were further incubated until death. Viability of the embryos checked daily by candling. Eggs containing dead or dying embryos and the remaining eggs at the end of the incubation period were first chilled at 4°C overnight and the allantoic fluid was collected. The air sac membrane was removed carefully using tissue forceps and a hole was made gently by microtip in allantoic membrane and slowly a clear, straw coloured allantoic fluid was aspirated by micropipette without disturbing the yolk sac. The collected fluid was used for second passage following the similar protocol. Likewise, three passages were given.

The embryos of the third passage were removed from egg shell, washed with Normal Saline Solution (NSS) and were kept in petri dishes. Egg embryos were observed for any defect in growth and development as well as gross lesions produced by the virus *i.e.* dwarfing and curling. RNA isolation was done from the allantoic fluid collected after third passage and stored at -20°C.

Serological testing

Collection of Serum Samples

A total of thirty five serum samples were collected randomly from the affected farm. About 4 ml of blood was collected from the jugular vein of the individual bird in clot activator vacuettes (Greiner Bio-one, Thailand). The vacuettes were kept in upright position at room temperature for about two hours, than after centrifuged at 5000 rpm for 15 minutes. The separated serum samples were collected in screw capped plastic vials preserved until i-ELISA test.

Indirect – Enzyme Linked Immunosorbant Assay for poultry serum samples

Infectious Bronchitis virus Antibody Test Kit, ELISA (Synbiotics Corporation, Canada) along with the user manual was procured and was used in the present study. The test was performed as per the protocol outlined in the user manual. Plate using the ELISA plate reader set at 410nm.

Detection of IBV BY RT-PCR

Extraction of Viral RNA

Viral genomic RNA was extracted from infected allantoic fluid using QIAamp Viral RNA mini kit according to manufacturer's instructions. RNA was quantified by spectrophotometric analysis through 40 cycle using NanoDrop1000 Spectrophotometer V3.7 as per the manufacturer's instructions.

Table 1 Details of Primers used for RT-PCR (Sigrist *et al.*, 2012)

Sr. No.	Primer code		Primer Sequences (5'-3')	Expected amplicon size
1.	IBV AV	Forward	TAGYGACYMTTBTGTVTGCACTATGTAG	636 bp
2.	IBV AV	Reverse	CTTGTGCDGTRCCATTAAYAAARTARGC	

To confirm the targeted PCR amplification, 5µl product from each PCR tube was mixed with 1µl of 6X gel loading buffer and placed in the well and electrophoresed along with DNA molecular weight marker (GeneRuler, MBI Fermentas) on 2% agarose gel containing ethidium bromide at the rate of 1µl/20 ml gel, at constant 80 V for 35 min in 0.5X TBE buffer. The amplified product was visualized as single compact band of expected size under UV light and documented by gel documentation system (Syngene, Gene Genius Bio Imaging System).

III. Results

The main objective of the present study was to know the strain of the infectious bronchitis virus involved and the pathological alterations induced by the virus in the affected layer birds. To accomplish the objectives autopsy as well as pathological and molecular methods were applied so that etiology and importance of the condition could be known. The results recorded during this study have been given under the following respective headings.

Clinical Signs

The most important clinical sign noticed was the birds sitting and walking upright like a penguin Fig. 1 (the typical “penguin gait appearance”) with a pendulous abdomen. It was due to the pressure exerted on the abdomen by the enormously fluid filled cystic oviducts which was later confirmed by necropsy. Those birds showing this characteristic sign were noticed to be non-laying and lethargic. The birds were at the age of 32 weeks. Birds laid either misshapened or soft shelled with watery albumin altogether affecting the production performance of the flock. There were no respiratory signs observed. The owner of the farm had purchased the flock (7170 birds) from another farm at the age of 16 weeks and hence he was unaware about the vaccination given to the birds before that age

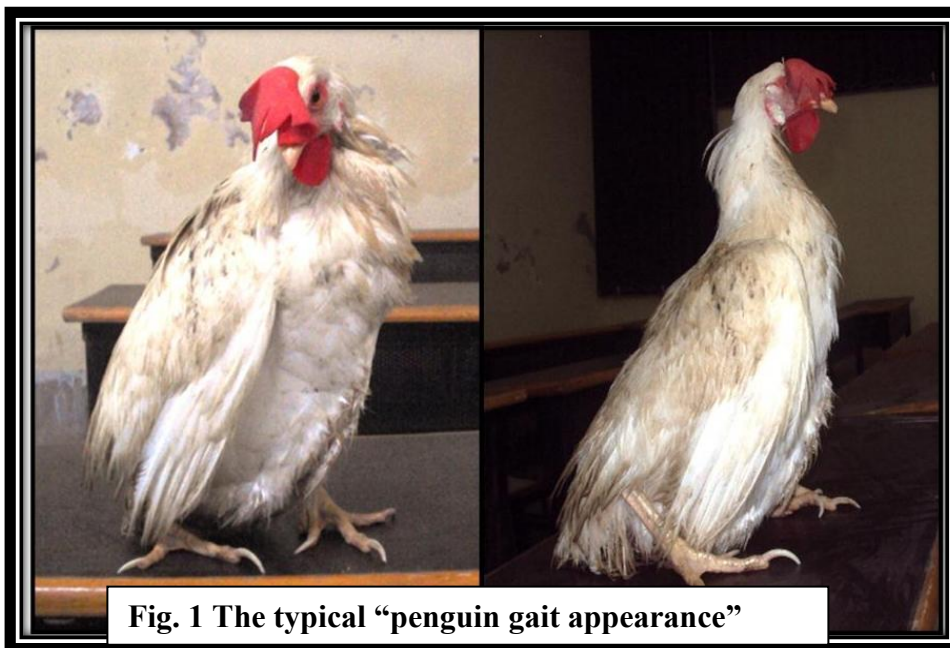


Fig. 1 The typical “penguin gait appearance”

The similar clinical signs were recorded by Landman *et al.* (2005) in 10 commercial chicken flocks in Netherlands. Sun *et al.* (2011) documented drop in egg production and deformed eggs as the prevalent signs in broiler breeders and layers affected with different strains of IB in China. Against our respiratory sign observation, Sigrist *et al.* (2012), Amin *et al.* (2012), Dolz *et al.* (2009) and Irvine *et al.* (2010) reported gasping, sneezing like respiratory signs in broiler breeder flocks affected with IB-QX strain.

Mortality

The pattern of mortality was almost regular during 26th to 54th week of age at the beginning of 26th week numbers of birds were 7072 and till 54th week 1084 birds died means 15.3% mortality. As owner culled the majority of the birds at end of 54th week of age, hence the mortality data were not available from then onwards.

The similar range of mortality was noticed by Picault *et al.* (1986) 2.6% at 40 weeks of age, Sun *et al.* (2011) 5% in layer and 30% and highest of 19% in a broiler birds Amin *et al.* (2012) due to QX-like IBV infection.

Drop in egg production

From the available farm records, the production drop based on Percentage Hen Day Egg Production (%HDEP) calculated on weekly basis and found peak production 89.5% at 25-37 weeks, and the lowest weekly production recorded was 75.13% on the 35th week of age. The production performance recorded upto 54 weeks showed that egg production drop ranged between 1.9 to 21.3 percent with an average production drop of 15.1 percent.

The findings regarding drop in egg production due to IB were in agreement with Picault *et al.* (1986) and Dergham *et al.* (2009). El Houdafi *et al.* (1986) reached only 70% of expected production. Jane and Huggins (1986) reported a production drop of 5-9% in 35 weeks old vaccinated layer flock. While Landman *et al.* (2005) reported maximum drop in egg production to the tune of 45 to 70 percent, Block (2009) reported maximum production achieved was between 41-50 percent.

Pathological Findings

Gross Pathology

Detailed postmortem examination of dead birds presented at the Dept. of Pathology from the affected flock as well as during personal visits of the farm was performed. The most prominent finding on external examination of the carcass was the highly enlarged abdomen. On internal examination gross lesions were mainly found in the organs like ovary and oviduct like, accumulation of large volume (more than 500 ml) of clear water like fluid, cystic dilatation (**Fig. 2**), some cases of impaction (**Fig. 3**). There were no appreciable gross lesions in other internal organs like liver, spleen, kidney, heart, intestine, proventriculus and gizzard. In few of the birds the tracheal mucosa revealed congestion and in majority of the cases ovary was found to be normal whereas in a few birds the changes varied from mild hyperemia to haemorrhages and distortion in shape.

Similar findings of cystic dilatation of oviduct was observed by Crinion (1972), Jones and Jordan(1972), Ballal *et al.* (2005), Landman *et al.* (2005), Benyeda *et al.* (2009).

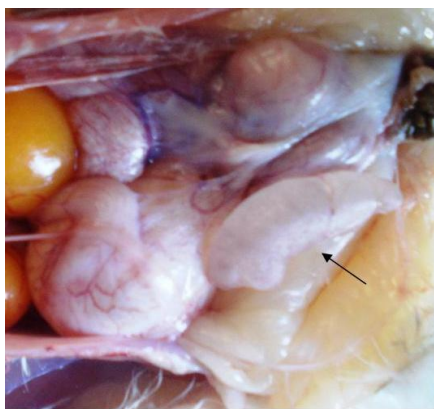


Fig. 2 Cystic dilatation of oviduct egg yolk



Fig. 3 Impacted abdomen with egg yolk

Histopathology

Histopathological lesions were mainly observed in tissue sections of trachea were deciliation and desquamation of mucosal lining epithelium with focal areas of congestion and mononuclear inflammatory cells infiltration in the lamina propria fewer necrotic cellular elements also there in tracheal lumen.

The oviduct cystic wall revealed a severe decrease to complete absence of glandular tissue reduced to absence of length of the villi (Fig. 4), the very thin and very often loss of mucosal epithelium muscular layer was noticed. Yolk material was observed in magnum.

Fig. 4 Oviduct showing atrophy of villi (H&E,120x)

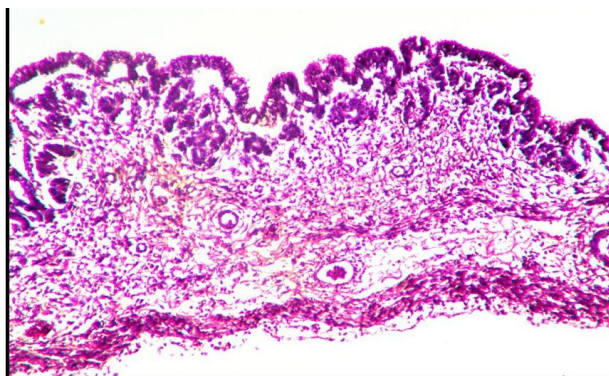


Fig. 4 Oviduct showing atrophy of villi (H&E,120x)

The kidney lesions were suggestive of interstitial nephritis characterized by mild to moderate infiltration of mononuclear cells in the interstitial space (Fig.5), fewer case of vacuolar degeneration in tubular parenchyma with albuminous casts in the tubular lumen.

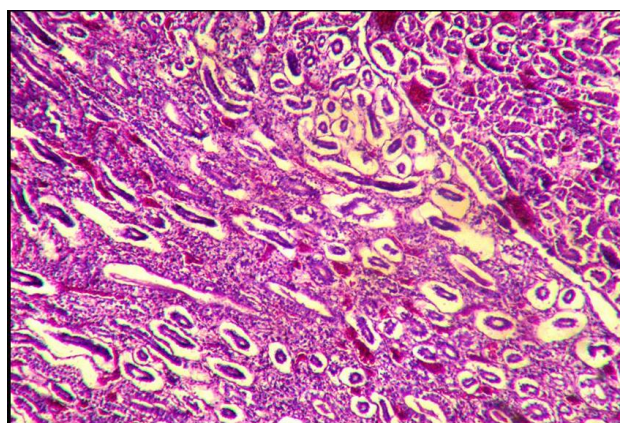


Fig. 5 Kidney showing infiltration of mononuclear cells with congestion (H&E,120x)

The other organs namely liver, spleen, heart, lungs and proventriculus did not show any microscopic lesions relevant to the Infectious Bronchitis infection.

The histopathological changes observed in the kidney during the present study were in agreement with the findings of Landman *et al.* (2005) Yewale (2010), Gaba *et al.* (2010), Sigrist *et al.* (2012), Sumi *et al.*, (2012) and Patel *et al.* (2015) but, Benyeda *et al.* (2010) observed thinning of the walls along with focal mononuclear cell infiltration beneath the epithelial layer or the serosal lining. In There appears to be no study with regards to the cystic dilatation of the oviduct and isolation of IB virus in our country.

Isolation and Identification of Infectious Bronchitis virus

Virus propagation in egg embryo

Seven days post incubation after the third passage dead embryos were noticed. Then the inoculated eggs were chilled at 4°C overnight and all the five inoculated eggs were opened with all aseptic precautions. All the dead embryos showed typical gross sign of dwarfing and curling which was confirmative for the presence of Infectious Bronchitis virus (**Fig. 6**). The allantoic fluid from SPF embryonated eggs was collected for further confirmation of IBV with RT-PCR.



Fig. 6 dwarfing and curling of IB positive embryo

Similar method of virus isolation from tissues by using SPF embryonated eggs through intra allantoic inoculation and the death of embryos with typical dwarfing and curling was reported by Sureshkumar *et al.* (2006), Terregino *et al.* (2008), Bhuvaneshwari *et al.* (2009), Gaba *et al.* (2010), Sun *et al.* (2011), Balasubramaniam *et al.* (2013) and Patel *et al.* (2015) during their study.

Serological detection of antibodies by i-ELISA

ELISA assay is a convenient method for monitoring of both the immune status and virus infection in chicken flocks. As per manufacturer's instruction, for the validation of result the corrected positive value must fall between 0.25 and 0.90 for positive control and below 0.25 for negative control. In the present study, the corrected positive control value obtained in the test was 0.40 which falls between 0.25 and 0.90 making the test a valid one as per the manufacturer's instruction and also the OD value of the normal control serum was 0.12 which falls below 0.25. The mean antibody titer value of the 35 serum samples was 11972 (Table 2) which was more than two fold of the targeted vaccine titer of 1000-2000 (Leerdam and Kuhne, 2009). Also the % Coefficient of variation was 46% which was less than the targeted % CV of 50-100 (Leerdam and Kuhne, 2009).

Table 2 Collected allantoic fluid, OD of same and antibody titer

Sample	1	2	3	4	5	6	7	8	9
Location	A4	A5	A6	A7	A8	A9	A10	A11	A12
OD	1.11	0.45	1.12	0.97	0.97	0.89	0.82	0.79	0.45
S/P Ratio	2.47	0.82	2.49	2.1	2.11	1.92	1.73	1.65	0.83
Titer	16303	2684	16573	12532	12556	10760	9074	8440	2724
Group	9	2	9	7	7	6	6	5	2
Sample	10	11	12	13	14	15	16	17	18
Location	B1	B2	B3	B4	B5	B6	B7	B8	B9
OD	0.7	0.42	1.38	1.48	0.51	0.73	1.18	0.76	0.77
S/P Ratio	1.43	0.73	3.13	3.37	0.96	1.52	2.63	1.6	1.61
Titer	6685	2222	24103	27159	3455	7364	18148	7966	8089
Group	4	2	13	14	3	5	10	5	5
Sample	19	20	21	22	23	24	25	26	27
Location	B10	B11	B12	C1	C2	C3	C4	C5	C6
OD	1.05	1.02	1.4	0.99	0.65	1.34	0.87	0.51	0.44
S/P Ratio	2.31	2.22	3.17	2.16	1.32	3.03	1.87	0.98	1.32
Titer	14613	13746	24576	13146	5851	22860	10373	3573	2565
Group	8	8	13	8	4	12	6	3	2

Sample	28	29	30	31	32	33	34	35	
Location	C7	C8	C9	C10	C11	C12	D1	D2	
OD	0.49	0.65	1.11	0.9	1.94	1.35	0.58	0.45	
S/P Ratio	2.47	1.32	2.47	1.93	4.53	3.06	1.15	0.83	
Titer	3223	5833	16330	10852	44126	23168	4625	2711	
Group	3	4	9	6	14	13	3	2	

Molecular detection of IBV by RT-PCR

The sample, one positive control and one negative control were tested for S1 gene using primers mentioned in Table 1, which specifically target the S1 gene of IBV. The expected amplified product of 636bp was detected in ethidium bromide stained agarose gel. The test sample and the positive control produced the expected amplicons of 636bp by RT-PCR, whereas no amplification was observed in negative control (Fig. 7).

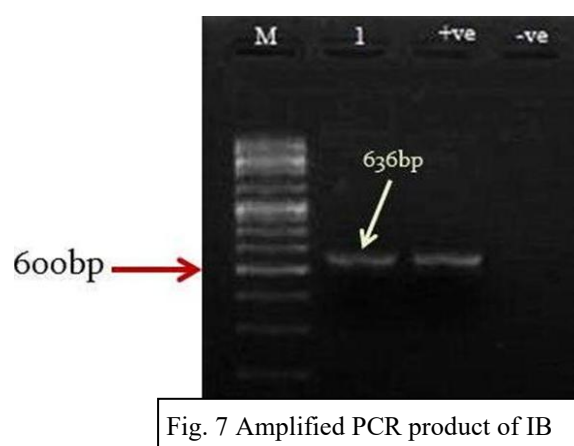


Fig. 7 Amplified PCR product of IB

IV. Discussion

The similar clinical signs penguin gait appearance, misshapened or soft shelled with watery albumin were recorded by Landman *et al.* (2005) in 10 commercial chicken flocks in Netherlands. Sun *et al.* (2011) documented drop in egg production and deformed eggs as the prevalent signs in broiler breeders and layers affected with different strains of IB in China. Against our respiratory sign observation, Sigrist *et al.* (2012), Amin *et al.* (2012), Dolz *et al.* (2009) and Irvine *et al.* (2010) reported gasping, sneezing like respiratory signs in broiler breeder flocks affected with IB-QX strain. The similar range of mortality was noticed by Picault *et al.* (1986) 2.6% at 40 weeks of age, Sunet *et al.* (2011) 5% in layer and 30% and highest of 19% in a broiler birds Amin *et al.* (2012) due to QX-like IBV infection. The findings regarding drop in egg production due to IB were in agreement with Picault *et al.* (1986) and Dergham *et al.* (2009). El Houdafi *et al.* (1986) reached only 70% of expected production. Jane and Huggins (1986) reported a production drop of 5-9% in 35 weeks old vaccinated layer flock. While Landman *et al.* (2005) reported maximum drop in egg production to the tune of 45 to 70 percent, Block (2009) reported maximum production achieved was between 41-50 percent. The gross observation revealed cystic dilatation of oviduct was observed by Crinion (1972), Jones and Jordan (1972), Ballal *et al.* (2005), Landman *et al.* (2005), Benyeda *et al.* (2009). The histopathological changes observed in the kidney during the present study were in agreement with the findings of Landman *et al.* (2005) Yewale (2010), Gaba *et al.* (2010), Sigrist *et al.* (2012), Sumi *et al.*, (2012) and Patel *et al.* (2015) but, Benyeda *et al.* (2010) observed thinning of the walls along with focal mononuclear cell infiltration beneath the epithelial layer or the serosal lining. In There appears to be no study with regards to the cystic dilatation of the oviduct and isolation of IB virus in our country. The nucleotide sequences of the isolate by BLAST analysis have similarity with Ballal *et al.* (2005) and Gaba *et al.* (2010).

V. Conclusions

The aim of our study was to differentiate whether the elevated titer levels were due to vaccine response or due to the exposure to infection since the flock had received a live H120 IBV vaccine after the clinical signs were noticed. On combining the serological findings with clinical signs and confirmation of viral isolation with egg inoculation, it could be undoubtedly concluded that the elevated levels of titer were due to the exposure to IBV infection only.

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