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Molecular and Cultures based Diagnosis of *Infectious Bursal Disease Virus* (*IBDV*) in Vaccinated and Non Vaccinated Poultry Flocks

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ABSTRACT

Background: In recent years, Infectious bursal disease is continuously occurring even after vaccination in India and requires an inclusive diagnosis. Therefore, the present study was undertaken to diagnose IBD through molecular and culture methods.

Methods: One pooled sample, from each of 54 flocks having birds with IBD like symptoms, was collected. History of bird type, age and vaccination was recorded. Samples were subjected to RT-PCR, egg embryo culture and chicken fibroblast cells culture.

Result: A total of 49669 out of 517900 (9.59 %) of birds, aging 3-6 weeks, were displaying the signs similar to IBD.In RT-PCR, 21 (38.88%) samples were found positive which belonged to11 (52.38%) vaccinated and 10 (47.62%) unvaccinated flocks.The RT-PCR positive samples were successfully cultivated for the virus through egg embryo and cell culture. The CEF culture was found least sensitive compared to egg embryo culture and RT-PCR.

Key words: Chicken embryo fibroblast (CEF), Egg embryo inoculation, Infectious bursal disease (IBD), RT-PCR, Vaccination.

INTRODUCTION

Infectious bursal disease (IBD) is probably most widespread and well recognized among three specific economically important non-oncogenic immunosuppressive viral diseases of chickens, *viz.* IBD, chicken infectious anaemia (CIA) and hydropericardium syndrome (HPS) (Balamurugan and Kataria, 2006). IBD is caused by *Infectious Bursal Disease Virus* (*IBDV*), a double stranded RNA virus of family *Birnaviridae.* The etiological agent has selective tropism for bursal B cells and induces massive destruction of the lymphocytes which results lymphopenia. Different techniques are used to diagnose the *IBDV* which are based on clinical signs and grossly visible lesions on the bursa of Fabricius. The virus can be isolated in embryonated eggs, cell cultures or by inoculation of susceptible birds. Current serological tests include agar gel precipitation test (AGPT), virus neutralization test and enzyme linked immunoassay (OIE, 2018). Other tests that have been used are fluorescent antibody techniques, immunohistochemistry and molecular techniques. Currently, reverse transcriptase-polymerase chain reaction (RT-PCR) is the molecular tool which is specific and sensitive method for diagnosis of *IBDV* infection (Kataria *et al.* 2001, Nandhakumar *et al.* 2020). RT-PCR can be further used in comparison of IBDV strains, especially for detection of vvIBDV strains, by supplementation with restricted fragment length polymorphism (RFLP)/ restriction enzyme digestion (RE digestion) and nucleic and amino acid sequencing methods (Jackwood *et al.*, 2018). Nonetheless, classical methods such as virus cultivation remain indispensable because of absence of false positive results and yield the virions for further experimentation. Thus, present communication is envisaged to employ cultivation method and RT-PCR of *IBDV* in an outbreak of IBD in the parts of Gujarat and Maharashtra state of India.

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MATERIALS AND METHODS

During the study period (Oct, 2018 - Mar, 2020), a total of 54 flocks, belonging to different commercial farms of north Gujarat area and Nagpur (Maharashtra), with history of infectious bursal disease like symptoms were visited. IBD was tentatively diagnosed on the basis of history, clinical signs displayed and post-mortem examinations. From these flocks, a total of 54 pooled samples of bursa of Fabricius (from two- three dead birds the pooled bursa was randomly collected in each affected flocks) were collected aseptically in 50% glycerol phosphate buffer solution. For the study

purpose, the samples were divided into two parts, one part was used for detection of IBDV by RT-PCR and another was kept for isolation of the virus.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Respectively, field samples and vaccine RNA (Hester Bioscience limited) was isolated using TRIZOL method (Invitrogen) and commercial RNA isolation kit, respectively, according to manufacturer's instructions. PCR was carried out in final reaction volume of 25 µl in thermal cycler using primer (5′- GGCCCAGAGTCTACA CCATAAC - 3′ and 5′- CCGGATTATGTCTTT GAAGCC - 3′) described as per Luka *et al.* (2014). Presence of 743 bp (Nt position 701-1444) in gel electrophoresis amplicon was considered positive.

Isolation of IBDV by chick embryo inoculation

The homogenized, clarified and filtered bursal tissue suspension was used for virus isolation.Ten days old live chick eggs embryo was inoculated at a rate of 200 µl per embryo through chorioallantoic membrane (CAM) route (Van den Berg *et al.,* 2000). Calcium Magnesium free-PBS (CMF-PBS) served as a negative control. Only embryos died after 48 hours were taken into recorded and chilled at 4°C. Three passages were given and embryo mortality pattern was studied in chick embryos for each passage material.

Isolation of IBDV by cell line

The primary chicken embryo fibroblast (CEF) cell culture was prepared from 10-day-old chick embryos (Rovozzo and Burke,1973). A suspension of 2×10^3 cells per ml in Dulbecco modified Eagle's growth medium (DMEM) containing 10 % fetal bovine serum and flasks were incubated at 37°C for 24 hours to form confluent monolayer.

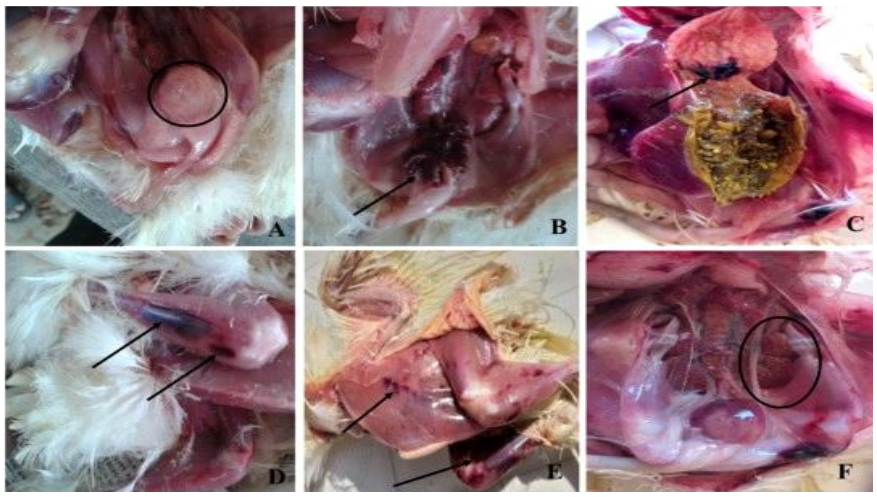
Propagation of virus in CEF cell culture

Both the reconstituted vaccine strain and local isolate previously confirmed by RT-PCR were used to infect the healthy, semi confluent monolayer of CEF cells. For virus isolation, 0.5 ml of IBDV inoculum was dispensed over each monolayer and flasks were incubated at 37°C for one hour. 10 ml of sterilized prewarmed maintenance medium was added in each flask and incubated them at 37°C in 5% CO_2. The flasks were examined at six hour intervals for cytopathic effects (CPEs). The virus infected cells and culture medium were frozen and thawed three times. The fluid used as IBDV inoculums inoculated in subsequent cell cultures for further passages.

RESULTS AND DISCUSSION

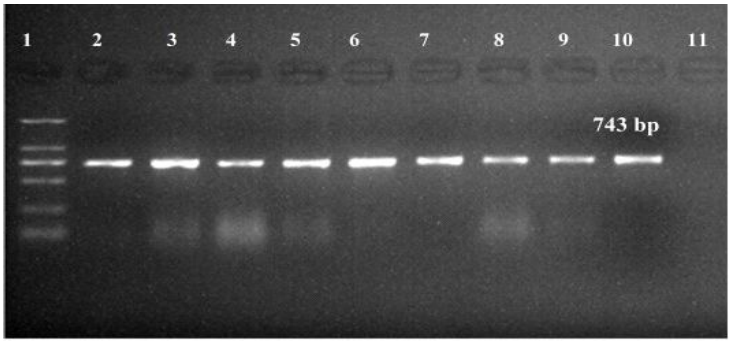
In the present work, birds were having the signs corresponding to IBD *i.e.* dullness-depression, stunted growth, dehydration, reluctance to move, ruffled feathers, decreased feed-water intake and closed eyes. The samples belonged to 21 layer and 33 broiler farms. Vaccination had been done in 32 (59.26%) farms. In the study area, mortality rate was reported between 0.67 to 30%. Gross lesions suggestive of IBD infection were found in bursa of Fabricius, proventriculus, kidneys and, thigh and breast muscles (Fig 1). Recently also, corroborating incidence level, based on signs and post mortem lesions, has been reported (Momin and Singh, 2017). Whereas lower than present level of incidence has been found as 8.89%, 4.5% and 4.55% by Jindal *et al.* (2004), Mor *et al.* (2010) and Preeti *et al.* (2018), respectively. The true prevalence of disease was further confirmed by RT-PCR and virus culture (egg embryo inoculation and cell culture) methods as few other diseases *viz*. chicken anaemia virus, fowl adenoviruses *etc*. and toxicities (Lukert and Saif, 2003) produce identical signs.

In RT-PCR, 21(38.89 %) and 33 samples (61.11%) were found positive and negative, respectively (Fig 2). In the 21 positive flocks, 11 (52.38%) were vaccinated, whereas 10 (47.62%) flocks were unvaccinated. Among the vaccinated



(A) Bursa was pale, enlarged and turgid (B) Hemorrhage at the bursal folds (C) Hemorrhage at the junction of proventriculus and gizzard (D-E) mild to severe ecchymotic hemorrhages on thigh and breast muscle (F) Enlarged and dehydrated kidney.

Fig 1: Gross pathological lesions observed during post-mortem examination of IBD suspected cases.



Lane 1: GelPilot mid-range ladder (Cat. No.239135);
Lane 2-10: Positive samples (743 bp amplicon); Lane 11: Negative control.

Fig 2: PCR amplification of *VP2* gene of *IBDV* isolates.

flocks, five flocks were of layer birds and six of broiler flocks. Similarly, among the unvaccinated positive flocks, one belonged to layer birds and nine were of broiler type.

RT-PCR is said to be most sensitive method of diagnosis (Jackwood *et al.,* 1997; Van den Berg, 2000). In the recent reports, approximate level of prevalence were recorded by Nandhakumar *et al.* (2020) from India and Mawgod *et al.* (2014) and Nwagbo *et al.*(2016) from Africa and Zahoor *et al.* (2011) from Pakistan, respectively, they recorded 53.65, 38.46 and 37.93 and 50.0 per cent of disease incidence using RT PCR. However, much higher rates as 73.33 and 85.0 per cent were revealed by Singh *et al.* (2014) and Mittal *et al.* (2005) from Madhya Pradesh and Haryana states of India. On the other side, Jackwood and Sommer-Wagner (2010) found that only 12 out 47 flocks (25. 5%) were affected by IBD, using RT-PCR.

The cause of such differences may be due to area specific spread of virulent strains of pathogen and husbandry practices, especially vaccination, bird's type (broiler or layer) and flock size. The two-tailed P value for comparison of layer and broiler farm positivity was found equal to 0.7759 and found statistically non-significant. Rather, it was noted that all positive flocks belonged to 3-6 weeks of age group. The age association was corroborating with Mor *et al.* (2010) and Kundu *et al.* (2018). Though, Preeti *et al.* (2011) reported occurrence of this disease more in young birds and Rashid *et al.* (2013) observed the disease in slightly older birds (8 weeks). The occurrence of disease in non-vaccinated flocks might be an anticipated phenomenon because of poor adherence of hygiene and biosecurity measures under Indian conditions of poultry farming (Kundu *et al.* 2018). But, occurrence of disease in vaccinated flocks was a matter of major concern and this phenomenon has already been reported by Patel *et al.* (2016) and Morla *et al.* (2016) from India and by Zahoor *et al.* (2011) from neighboring country Pakistan. Vaccination failure was most likely due to changes in the hypervariable region of VP2 of IBDV that may result from immunological pressure or presence of maternal antibodies.

In egg embryo inoculation the virus was successfully isolated from the 18 /21(85.71%) samples and the passage wise details of lesions are shown in Table 1. Haemmorhages below eyelids, behind the head and at toe, whereas 12 embryos showed generalized cutaneous body haemmorhages in 1st passage. The livers were swollen and mottled (with patchy congestion and pale yellow tending to green). Next common lesion was dwarfed embryos with oedema and congestion (Fig 3, A-C). Oedematous CAMs were observed

Table 1: Lesions observed on inoculated embryos.

Lesion observed	Passage 1	Passage 2
Early death of embryo	18/21(85.71%)	16/21(80.95%)
Dwarfed embryo	14/21(66.66%)	12/21(57.14%)
Haemorrhagic embryo	12/21(57.14%)	7/21(33.33%)
Oedematous embryo	8/21 (38.09%)	3/21(14.28%)
Enlarged and molten liver	17/21 (80.95%)	15/21(21.42%)
Haemorrhagic CAM	14/21(66.66%)	12/21(57.14%)

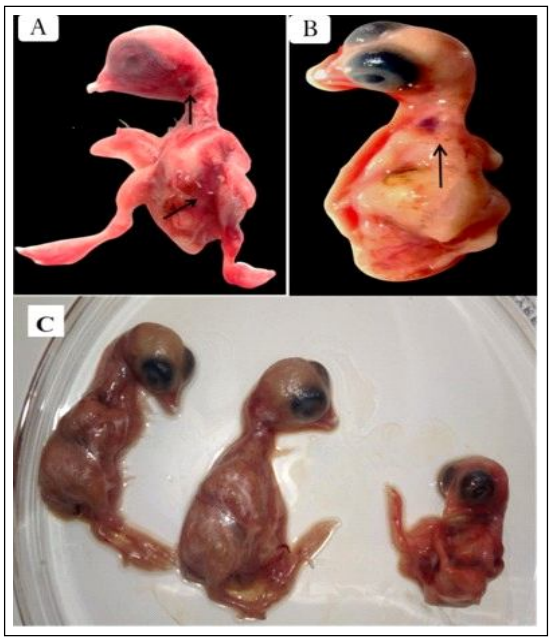
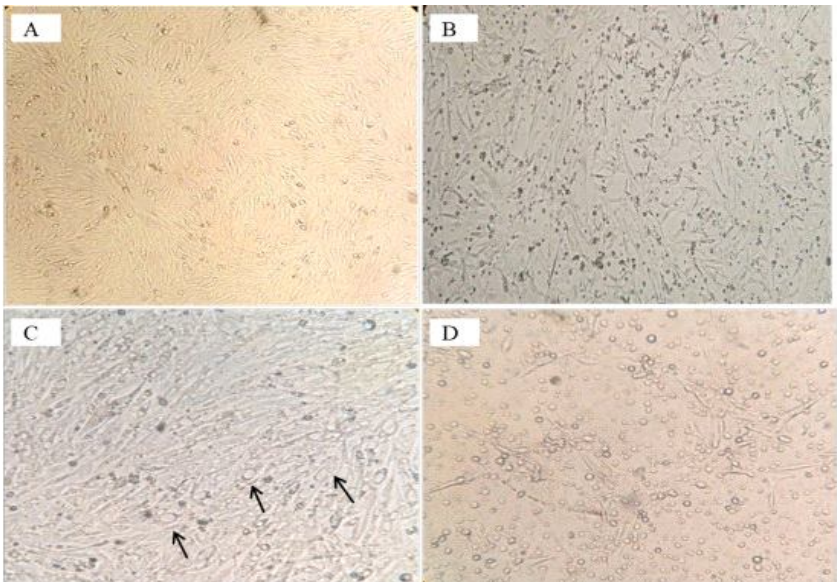


Fig 3: In (A-B) : 14th day old dwarf haemorrhagic embryo infected with IBD virus and (C) Dwarfed embryos with oedema and congestion and early embryonic death in second passage.



(A) Uninfected CEF. (B) 24 hours post inoculation of IBDV. (C) 48 hours post inoculation after 3rd passage, vacuolation, aggregations seen. (D) 72 hours post inoculation detachment could be seen.

Fig 4: Cytopathic effects on chicken embryo fibroblast cells.

with or without congestion or haemorrhages. Similar pathological lesions in embryos were recorded by Magwood *et al.* (2014) and Mutinda *et al.* (2015), with numerical differences only. The higher sensitivity of RT-PCR than that of egg embryo inoculation was ability of RT-PCR to amplify viral nucleic acid from both complete and disintegrated viruses. Whereas in the later test, viable virion are required to obtain positive results.

Upon CEF cells, out of 21 RT-PCR positive samples, IBDV was successfully isolated from the 15 (71.42%) samples. All the samples showed characteristics cytopathic effects on an average from the third passage and further the presence of the viral antigen was demonstrated by RT-PCR. During the first two passages, detachment of cells was observed at 24 hours post infection (h.p.i.), whereas typical CPEs like aggression and vacuolation were observed at 48 h.p.i. Further at 96 h.p.i. about 80 per cent cells were detached. Fourth passage onwards increased rounding and clumping of few cells at 72 hours PI and progressed to increased rounding and clumping of cells at 96 hours post infection, moreover a clear and distinct CPE was noted with increase in passage number (Fig 4, A-D). Comparison of RT-PCR with cell culture implicated that cell culture is even lesser sensitive method than egg embryo to detect IBDV. Similar results have been found by Singh *et al.* (2014) where 5/11 samples displayed CPE. This was due to better sensitivity of egg embryo inoculation than cell cultures because many hyper virulent strains failed to grow in cell culture until they are passaged in egg embryo (Van den Berg *et al.* 2000) or cell culture may be adversely affected by inhibiting substances (Fatima *et al.*, 2014). Other workers (Rekha *et al.,* 2014; Awandhkar *et al.,* 2018) reported similar CPE changes but in lesser time duration.

If three methods are compared on the basis of sensitivity, RT-PCR was found more sensitive for virus detection than egg inoculation and chicken embryo fibroblast culture because it can detect live as well as dead virus. Egg inoculation was found more sensitive than chicken fibroblast culture because most of the strains isolated in the field and especially the hypervirulent strains, cannot be multiplied in cell cultures without previous adaptation in egg embryos or several blind passages (Van den berg, 2000).

CONCLUSION

Gujarat and Maharashtra states have wide spread poultry establishment. Occurrence of IBDV in vaccinated flocks was also reported here, apart from other parts of India and was most likely due to changes in the hypervariable region of VP2. In this study, PCR was found more sensitive method of IBDV diagnosis than both cell culture and egg embryo inoculation. But sometimes PCR may also failed to detect newly emerged or uncharacterized genomes, therefore, both molecular diagnosis and classical cultivation methods should be employed to diagnose the etiological agent.

Conflict of interest

All authors declare that they have no conflict of interest.

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