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# Isolation and Characterization of Infectious Bursal Disease Virus VP2 Gene Isolated from Tirupati Region of Andhra Pradesh, India.

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## ABSTRACT

Infectious bursal disease is a highly contagious viral disease of birds characterized by lesions in the bursa of Fabricius, immunosuppression, severe morbidity and mortality. Due to amino acids substitutions in this region results in change in the topography of hyper variable region of VP2, where most of the neutralizing epitopes are present. Cloning and characterization of hyper variable region of prevalent strains of IBDV in this part of the country will provide basis for development of better vaccine for future. In the present study, we cloned four full length sequences of IBDV-VP2 with hyper variable region and found that all the cloned sequences have similar epitopes. Phylogenetic analysis showed that they are closely related to each other and are also closely related to strains of Luxembourg, China, and USA. This study will enhance the knowledge on IBDV circulating strains in this part of the country.



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**Key Words:** Infectious bursal disease virus; VP2; Polymerase chain reaction; Cloning, Sequencing; Phylogenetic analysis.

**Abbreviations:** IBD, Infectious bursal disease; IBDV, Infectious bursal disease virus; ORFs, Open reading frames; VP, Viral Protein; vvIBDVs, Very virulent infectious bursal disease viruses

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## Introduction

Infectious bursal disease is also known as Gumboro disease, It is acute, highly contagious, infectious and immune-suppressive [1] viral disease of young susceptible chicks [2,3] and it affects the poultry industries worldwide [4] caused by an RNA virus, infectious bursal disease virus (IBDV) [5-6] with a primary target organ of the bursa of Fabricius [7]. IBD virus remains infectious for a very long period of time and has resistance to commonly used disinfectants [8]. The emergence of antigenic variant as well as very virulent strains in vaccinated flocks considerably stimulated research efforts on both, IBD and IBDV. The disease causes heavy economic losses in poultry industries due to immunosuppression in subclinical cases [9] and in acute cases; it is associated with mortalities, haemorrhages and also bursal damage [10]. The disease is mainly affects young chickens of between 3 and 6 weeks of age characterized by enlarged bursa of Fabricius, watery diarrhoea, accumulation of urate in the urinary structure, and severe depression [2] IBD is a clinical disease solely in chickens but also turkeys, ducks, guinea fowl and ostriches may be infected.

IBDV is a non-enveloped icosahedral bi-segmented double stranded RNA virus [11-12]. IBDV is a member of the family Birnaviridae [11] and is a type-III virus in the Baltimore classification. Birnaviridae family has three designated genera which infect different animal species such as birds (Avibirnaviridae) [13], Insects (Entomo-birnaviridae) [14], rotifiers and fishes (Aquabirnaviridae) [15]. Avibirnavirus which include Infectious bursal disease virus that infects birds especially chickens [16]. IDB virus is classified as serotype I and II but only serotype I is pathogenic in chickens [2]. IBDV infects and destroys B-lymphocytes in the bursa of fabiricious, which ultimately results in infiltration of T-cells and immunosuppression. IBDV genome consists of two segments (i.e. A and B segments). Segment-A has two open reading frames (ORFs), ORF1 encodes a non-structural protein VP5 protein, whereas ORF2 encodes 115KDa precursor polyprotein, which is later cleaved into VP2, VP4 and VP3 [17-18]. Segment-B encodes RNA Polymerase (VP1) of 90 KDa size. Serotype specific antigenic determinants inducing neutralizing antibodies are located on VP2, whereas group specific monoclonal antibodies recognize antigenic sites located on structural proteins, VP2 and VP3 [19].

Aim of the present study is to generate a rapid protocol for IBDV detection using Reverse Transcriptase-PCR, which also enables identification of circulating strains of IBDV in Andhra Pradesh. In the present study, we determined nucleotide and amino acid sequence of VP2 of IBDV, which is prevalent in Andhra

Pradesh, India to understand the molecular epidemiology of circulating strains and their phylogenetic relation with other IBDV-VP2 sequences deposited in GenBank, NCBI.

## Materials and Methods

**1.RNA isolation from IBDV infected birds**

IBD suspected birds showing clinical symptoms like diarrhoea, depression, anorexia, ruffled feathers especially in the head and neck region were collected from Sri Krishna poultry farms, Karakambadi, Tirupati, Andhra Pradesh, India and were transported to Department of Veterinary Pathology, Sri Venkateswara Veterinary University, Tirupati for clinical and pathological confirmation. The infected bursa showing hemorrhagic characteristics of IBDV infection were collected for isolation and characterization of virus. Total RNA was extracted from suspected bursal samples using TRIzol Reagent (Thermo Fisher Scientific, USA) as described by the manufacturer's instructions and kept at -80°C until further use.

**2. Reverse transcription and PCR**

The first strand cDNA synthesis was carried out by using M-MuLV-reverse transcriptase (Fermentas, USA) according to the manufacturer's protocol. 3 µl of the isolated total RNA was added and subjected to reverse transcription (RT). A set of primers were designed to amplify VP2 gene (1356 bp). Forward primer (5'-ATGACA AACCTGCAAGATCAAACCC-3') and Reverse primer (5''-TTACCTTAGGGCC CGGATTATGTC-3') respectively. The optimized 25µl reaction mixture for the PCR cloning consists of 1X Taq DNA Polymerase buffer (20mM Tris-HCl (pH8.8), 10mM KCl, 10mM (NH4)2SO4, 0.1% Tween 20.), 0.2mM dNTPs, 10 pMol of each of Primers Forward and Reverse, 1.5mM $MgCl_2$, 20ng of DNA template and 1U of Taq DNA Polymerase (Fermentas, USA). Standardized PCR conditions for amplification of VP2 was performed in a thermal cycler (Applied Biosystems, USA) with initial denature--ation of 94°C for 5 min followed by 30 cycles of 94°C for 30sec, 62°C for 30 sec, 72°C for 2 min, and final extension at 72°C for 10 min. Amplified products were visualized in 1% agarose with ethidium bromide.

**3. Cloning and transformation of PCR Products and Sequencing**

The amplified products (~1.3 Kb) were purified using Qiagen gel extraction kit (Qiagen, Germany) according to manu--facturer's protocol and the gel eluted PCR products was cloned into pTZ57R/T vector (Fermentas, USA) using manu--facturer's protocol. The Ligated vector with VP2 gene was transformed into Escherischia coli DH5α competent cells as described previously by Sambrook and

Russel, 2001[20]. Plasmid DNA was isolated from E.coli cells and confirmed for VP2 gene by PCR using VP2 gene specific primers.

Positive clones were sequenced at SciGenom Labs, Kerala, India commercially using M13 Forward and M13 Reverse primers present at the either ends of the TA cloning vector pTZ57R/T (Fermentas, USA) multiple cloning site to achieve the VP2 gene sequence of IBDV.

**4. Phylogenetic analysis**

The sequenced contigs were manually assembled. Analysis of nucleotide sequence was performed using BLAST N and BLAST X programs and phylogenetic analysis was performed using Mega 7 by neighbor joining method with bootstrap values.

## Results

The 9 suspected chickens for IBD were collected from Sri Krishna Poultries, Tirupati were confirmed based on clinical symptoms and pathological lesions. Birds with haemorrhagic lesions in thigh muscles alone or in bursa of Fabricius in gross pathological findings at the time of post-mortem examination were suspected as IBD positive (Fig. 1), bursae with lesions were collected and stored for RNA isolation

Reverse Transcriptase PCR was performed to RNA isolated from IBD suspected chickens using forward and reverse primers designed for IBDV-VP2 gene, which yielded an amplicon of a ~1.35 kbp (Fig. 2). The primers used in the present study are good enough to screen IBDV-VP2 gene from the cDNA synthesized from the total RNA of suspected bursal samples. Four samples were shown positive among nine samples tested for IBDV. Amplified fragments were sequenced for confirmation of IBDV-VP2.

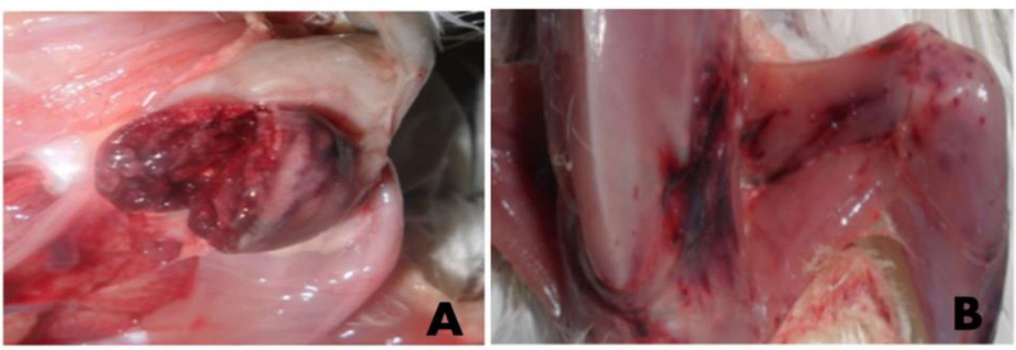


Fig 1: IBDV infected birds showing hemorrhages in Bursa of fabricius (A) and thigh muscles (B)

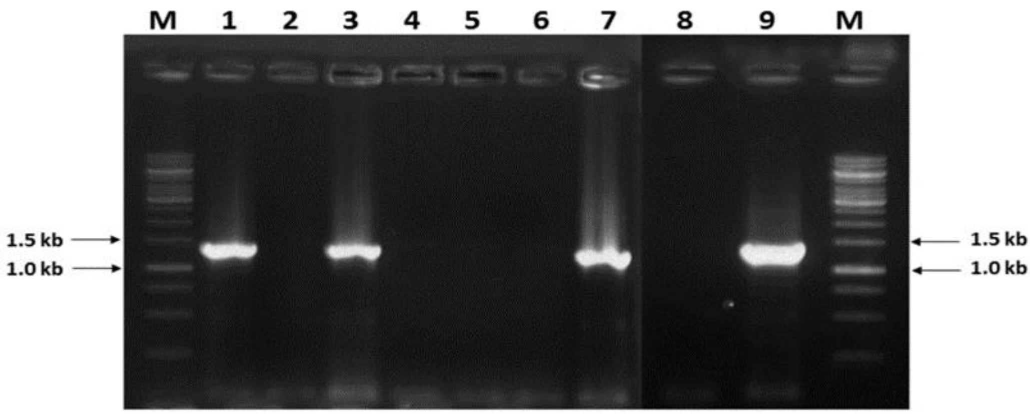


Fig 2: Identification of IBDV infected birds from suspected field isolates using PCR
Lane M: Marker; Lanes 1-9: IBDV suspected samples

PCR cloned sequences showed more than 96% identity with other IBDV-VP2 genes deposited in NCBI database. We determined nucleotide and deduced amino acid sequence of hypervariable region of four VP2 sequences cloned from chickens of four different sheds in poultry farm. All the sequences cloned in the present study were. submitted to GenBank, NCBI with accession nos. KU712259, KX078634, KX078635, KX078636. Multiple alignm--ent of nucleotide and deduced amino acid sequence with hypervariable region of cloned VP2 with other VP2 sequences was given in Fig. 3 respectively


Fig 3: Multiple alignment of deduced amino acid sequences of IBDV isolates
with special emphasis on amino acid substitutions in hyper variable region

Phylogenetic analysis of cloned VP2 sequence with other IBDV-VP2 sequences deposited in GenBank, NCBI showed cloned sequences shared same node with AF499929 (Luxembourg), AY321509 (China), JQ 411012 (USA). Sequences cloned in the present study are phylogenetically close to the sequence deposited from Tamil Nadu, India with accession no. KU891986 among Indian IBDV strains (Fig. 4).

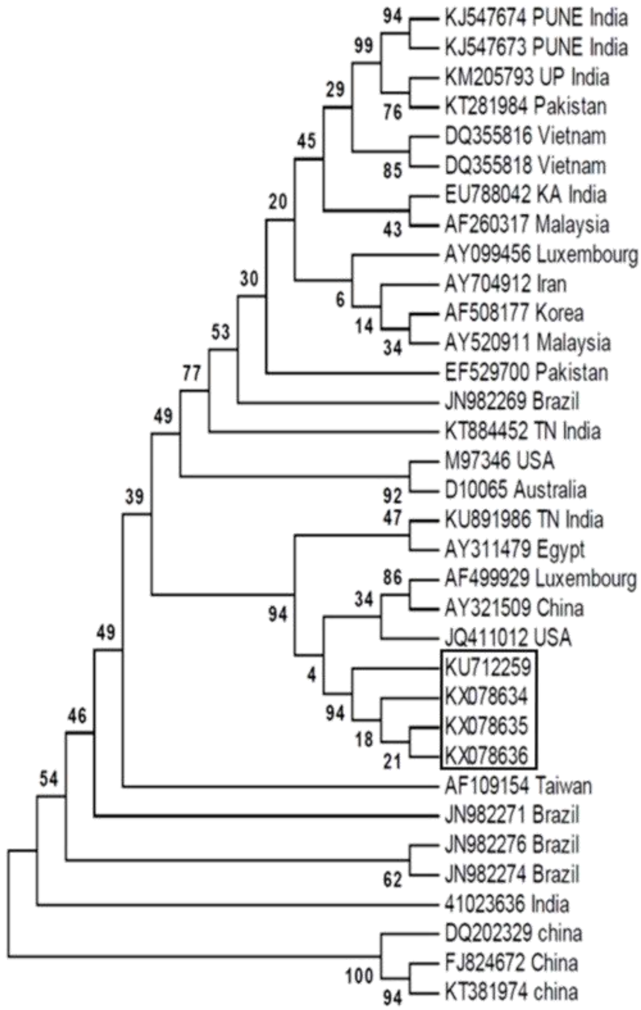


Fig 4: Phylogenetic analysis of cloned IBDV-VP2 with other IBDV-VP2 genes deposited in GenBank, NCBI

## Discussion

Poultry industry comprises one of the most rapidly growing food producing sectors in the world and keeps expanding with an increase in population. The production and consumption of eggs and poultry meat has been increasing worldwide over the last three decades as the consumption of eggs has doubled and that of chicken meat has tripled [21]. Indian poultry industry is booming and emerging as the world's 2nd largest market, growing at a phenomenal rate of 12 to 15% every year.

The flocks included in the present study were vaccinated with conventional vaccines. However, information on the extent of serological monitoring/evaluation of the flocks was not available. Outbreaks in vaccinated flocks are common due to their inability to effectively induce immune system against vaccinated diseases. The vvIBDVs have the ability to break through high levels of maternal antibodies and, in comparison to classical variant of IBDVs, can cause disease even in the presence of high titers of neutralizing antibodies produced by classical attenuated vaccine strains [22-23]. Therefore, highly attenuated vaccines that induce a low level of neutralizing antibodies may not provide adequate protection against vvIBDVs [24]. In the present study, we cloned four sequences of IBDV-VP2 from four different birds of different flocks in local poultry farm.

Furthermore, differences in amino acid sequence between the cloned sequences and other strains found in GenBank, NCBI was given in Fig. 3. Hyper variable region of VP2 is known for its ability to induce immune response in chickens and also for having hydrophilic region with key epitopes and amino acid substitutions in positions 222 (P/V/T/S/A), 249 (H/K/Q), 284 (T/A) and 299 (N/S) (Fig. 3). Four hydrophilic loops have been identified in VP2, and the substitution of amino acids in this region will induce selective pressure in the evolution of IBDV upon exposure to the host immune system [23].

In the present study, phylogenetic analysis showed that the cloned sequences were closely related to the IBDV strains of Luxembourg (AF499929), China (AY321509), and USA (JQ411012). Apart from this, cloned sequences were phylogenetically closer to the Indian counterpart isolated from Tamil Nadu region. Cloning and sequencing of hyper variable regions of IBDV-VP2 gene of circulating strains will enhance the knowledge regarding substitutions in hyper variable region to escape from the immune system of host. Understanding the molecular epidemiology of circulating strains of IBDV will provide basis for development of better vaccine to prevent the prevalence of IBDV in future.

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