Sequence analysis of a part of hypervariable region of VP2 gene of chicken embryo fibroblast adapted Infectious Bursal Disease virus isolates of Uttarakhand

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ABSTRACT

Infectious bursal disease (IBD) is an acute and highly contagious immunosuppressive viral disease of young chickens causing great economic loss to the poultry industry worldwide. The disease is caused by serotype 1 of IBD virus (IBDV) belonging to genus Avibirnavirus of family Birnaviridae. One of the most probable reasons for frequent vaccination failures is attributed to emergence of variant or very virulent strains. Hence the present study was carried out in an attempt to characterize the chicken embryo fibroblast culture (CEF) adapted IBD isolates which was originally characterized as very virulent in nature at molecular level. Amplification of 248 bp hyper variable region of VP2 gene was done by reverse transcription polymerase chain reaction (RT-PCR) using specific primers with RNA isolated from CEF culture infected with these field isolates. Though sequence and phylogenetic analysis of 248 bp RT-PCR product indicate that these field isolates are closely related to very virulent strains of virus there is change in nucleotide and amino acid sequences from virulent one which may be is due to cell adaption. However, further studies such as challenge studies, complete genome sequence analysis and site directed mutagenesis and reverse genetics approach will throw more light on correlation between genotypes and antigenic subtypes and pathotypes.

Keywords: IBD, VP2 gene, chicken embryo fibroblast, reverse transcription, polymerase chain reaction

INTRODUCTION

Scientific poultry production in India has made considerable progress during last three decades and has emerged as one of the fastest growing agro based industries in the country. Improvement in the productivity of the poultry needs to be supported by better health care and management practices, which can be achieved by control of infectious diseases with development of various diagnostic tests and methods of immunophylaxis. A shift to intensive poultry keeping has led to emergence of many devastating form of diseases. Infectious bursal disease (IBD) is one among them causing a huge economic loss to poultry farm every year. Infectious Bursal Disease, also known as Gumboro disease, is an acute and highly contagious viral disease of young chickens. The disease is characterized mainly by severe lesions in the bursa of Fabricius (BOF) and destruction of lymphoid cells in other lymphoid organs followed by immunosuppression [1].

The etiological agent, Infectious Bursal Disease virus (IBDV), is a member of the genus Avibirnavirus of family Birnaviridae [2]. After infection, the virus multiplies rapidly in the developing B lymphocytes of bursa of Fabricius leading to immunosuppression and increased susceptibility to other diseases [3]. IBD has shown different degrees of pathogenicity and mortality in chickens [4]. Although the disease causes severe losses, its effect in reducing the birds’ ability to develop immunity to other diseases seem to be of utmost importance [5]. The virus can be grouped into 2 distinct serotypes (1 and 2) based on...
cross neutralization test [6,7]. Serotype-1 is pathogenic to chickens and varies in its virulence. Thus the control of IBD is possible only by vaccination with attenuated or inactivated serotype 1 virus [8,5]. Ismail et al. [9] reported that serotype 2 isolated from turkey is apathogenic to both turkey and chicken.

According to antigenic variation and virulence, serotype 1 strains can be classified into several pathotypes: classical virulent strains, attenuated vaccine strains, antigenic variant strains and very virulent (vv) strains. This variation among viruses usually causes vaccination failure mainly when antigenic structures among field and vaccine strains no longer coincide [10,11]. The major host protective antigen responsible for induction of serotype neutralizing antibody is VP2. In this sense, VP2 gene region has been used to detect and identify IBDV subtypes [8,10]. Recent isolates of IBDV from India included very virulent phenotype [12,13]. The emergence of variants, as a result of mutation, is the major cause of vaccination failure [14]. Therefore, continued surveillance of emerging variants is essential to incorporate such strains in the vaccine so as to protect poultry from this dreadful disease. Control of the disease through sanitation and isolation is not practical for commercial poultry production, because of the stability of IBDV in the environment [15-17]. The principal method of control is, therefore, by vaccination. The level of passive immunity being variable and unpredictable, a common commercial practice is to vaccinate all chicks against IBD with a live attenuated vaccine during the first 3 weeks of life [18].

Nucleic acid based methods are useful tools for direct virus detection and subtyping without isolation and propagation in cell culture. On the basis of Reverse Transcription-Polymerase Chain Reaction profiles, field isolates could be compared and placed into different molecular groups and antigenic variations are detected in order to find a suitable vaccine for these isolates. Sequence analysis of genome of various strains of IBDV will not only provide information on molecular basis of antigenic and pathotypic variations, but also the evolutionary profile of these strains, which will explain genetic and environmental aspects of these variations. As the hyper variable region of VP2 encounters frequent mutations, which in turn results in changes in antigenicity and virulence, a thorough study on this particular region will give an overall understanding of antigenic and pathotypic variations. Moreover Indian field isolates have not been properly studied on this aspect. The present work was being attempted with the following objectives: i) to standardize RT-PCR for cell culture adapted field isolates of IBDV using specific primers for amplification of a part of hyper variable region of VP2 gene, and ii) sequencing and sequence analysis of the amplicons of these IBDV field isolates.

MATERIALS AND METHODS

Sodium chloride, potassium chloride, anhydrous disodium hydrogen orthophosphate, trypsin, streptomycin, penicillin and sodium bicarbonate (Himedia, India) were used for chicken embryo fibroblast culture. Isopropanol (SRL Mumbai), chloroform and isooamyl alcohol (SRL Mumbai) and ethanol (Redi-de Haen, Germany) were used for isolation of RNA. Methanol (S D Fine Chem. Mumbai) Giemsa (Himedia, India), May-Grunwald (Loba Chemie, Mumbai), glycerol (S D Fine Chem. Mumbai) bromophenol blue (BDH Lab chemical division), DPX mountant (spectrochem Pvt Ltd), xylene (Glaxo Laboratories), ethidium bromide (Sigma, USA) and agarose (Bangalore Genei, Bangalore) were used for preparation of buffer, dyes and gel preparation. MEM and foetal bovine serum (Himedia, India) were used to culture the chicken embryo fibroblast cells.

Total RNA extraction kit from Bangalore Genei was used for isolation of total RNA. One step RT-PCR kit (QIagen, Germany) was used for RT-PCR. RT enzyme mix, dNTPs from QIagen, Germany and trypsin from Himedia, India were used in the study. Primers (23mer) of Bangalore Genei for 248 bp amplification of VP2 gene fragment [12] used are as follows: Forward Primer 5’ GTAACAATCA CACTGTTCTC AGC 3’ 804-826; Reverse Primer 5’ GATGTGATTG GCTGGGTAT CTC 3’ 1029-
Nucleotide sequence was according to the numbering system of Bayliss et al. [2]. Field isolates of UA-BZ1, UA-BZ3 and UA-SP2 adapted in chicken embryo fibroblast were used as source of virus. Embryonated chicken eggs (9-11 days old) were procured from Instructional Poultry Farm, G.B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand and utilized for production of primary chicken embryo fibroblast cell culture.

Primary CEF culture was prepared as per the method described by Merchant et al. [19] with some modifications using 9-11 days old chicken embryos. The embryos were collected aseptically and placed in MEM. After removal of head, extremities and visceral organs, the remaining tissue was washed and minced using a sterile scissors. Then the tissue was digested with 0.25% trypsin solution for 20 minutes with constant stirring. The cells were filtered through sterilized muslin cloth and centrifuged at 2000 rpm for 5 minutes at 4°C. The cells were then washed in the Media. The washed cells were re-suspended in the Media and counted using Neubar’s chamber in a microscope. Finally the cell concentration was adjusted at the rate of 1x10^6 cells per ml of MEM containing 10% fetal calf serum (FCS) and distributed in 25 cm^2 tissue culture flask at the rate of 0.3 ml of cell suspension per cm^2. The seeded flasks were incubated in a CO2 incubator at 37°C and 5% CO2 till confluent monolayers were formed. Usually the monolayers were found suitable for virus inoculation within 24 hours. The culture flasks with complete monolayer of CEF cells were taken and the spent medium was decanted. The monolayers were washed once with 3-5 ml of fresh medium and then one ml of virus inoculum/25 cm^2 tissue culture flask was added and put in incubator at 37°C for about 1 hr for adsorption. The flasks were regularly tilted at every 6-8 minutes. Then the inoculum was decanted and fresh medium (with 2% FBS) added to the flasks and kept in incubator at 37°C for development of cytopathic effects. The inoculated tissue culture flasks were observed under inverted microscope and compared with the control for cytopathic effects. The cytopathic effect produced by IBDV in CEF culture were observed without staining daily at an interval of 12 hours post infection (PI) for appearance of CPE, or else cell cultures were prepared in coverslips and inoculated. The inoculated cultures were stained using May-Grunwald and Giemsa stains at 72 hours PI as per the technique described by Merchant et al. [19] with slight modifications. The presence of virus in the infected CEF was also confirmed in Agar gel precipitation test (AGPT) using hyperimmune serum available in the laboratory.

Total cellular RNA was extracted from the tissue culture as described by Chomczynski and Sacchi [20] and protocol supplied along with Total RNA isolation kit (Bangalore Genei). The media was discarded and culture washed with 5-8 ml of ice-cold phosphate buffer saline per 75 cm^2 tissue culture flask. Thereafter, the monolayer was detached by treating the culture with 1 ml denaturing solution. The cell suspension was then homogenized in sonicator. Then added 1ml of water saturated phenol followed by 200 µl of chloroform-isoamyl alcohol mix (freshly prepared in the ratio of 49:1), mixed thoroughly and incubated in ice for 15 m, then centrifuged at 10000 rpm for 20 minutes at 4°C. The upper aqueous phase was collected and added 1ml of 100% isopropanol to precipitate RNA and incubated at -20°C for 30 m. Then it was centrifuged at 10000 rpm for 20 m at 4°C. The supernatant was discarded and resuspended the pellet in 0.3 ml of denaturation solution and precipitated RNA by adding 0.3 ml of 100% isopropanol, incubated at -20°C for 30 m, centrifuged at 10000 rpm for 20 m at 4°C and discarded the supernatant. The RNA pellet was resuspended in 75 % ethanol and incubated at room temperature for 10-15 m to dissolve residual amount of guanidine and centrifuged at 10000 rpm for 20 m at 4°C. Then the supernatant was discarded and tubes were kept in the incubator at 37°C for complete evaporation of ethanol. The pellet was then dissolved in 100-200 µl of DEPC water and stored at -20°C for further use.

For Reverse Transcription-Polymerase Chain Reaction (RT-PCR) QIAGEN One-Step RT-PCR was used which provides a convenient format for highly sensitive and specific RT-PCR using any RNA. This allows both reverse transcription and PCR amplification to take place in ‘one-step’ reaction. Re-
Figure 1. CEF culture infected 72 hpi (MGG×400).

Figure 2. Agarose gel (1.2 %) showing total RNA isolated from monolayer inoculated with IBDV [Lane1: UA-BZ1, 2: UA-SP2 and 3: UA-BZ3].
verse transcription and PCR carried out sequentially in the same tube. All components required for both reactions were added during setup, and there is no need to add additional components once the reaction has been started. Thermal cycler program included steps for both reverse transcription and PCR and was programmed as one reverse transcription cycle of 30 min at 50°C. PCR conditions were as follows: initial denaturation at 95°C for 15 min, 40 cycles [denaturation at 94°C, 1 min; annealing at 60°C, 1 min; extension at 72°C, 1 min], final extension at 72°C for 10 min.

Obtained unpurified 248 bp amplified VP2 hyper variable fragment obtained from RT-PCR were sent to Chromous Biotech, Bangalore for nucleotide sequencing (Double pass analysis). 20 µl RT-PCR product and 100 picomoles each of forward and reverse primers were sent for each sample. Sequence analysis was done using Sanger’s sequencing method. Correct complete sequence was determined from the electropherogram and sequence data obtained using both forward and reverse primers. The nucleotide sequences and the deduced amino acid sequences were compared and phylogenetic analysis was done for sequence data available from the GenBank along with sequence data obtained from the study. Sequence data for IBDV isolates available in the department and cell culture adapted strains elsewhere were included for comparison. The sequence data were aligned by using the software Clustal W (1.82). Amino acid percentage homology was obtained by amino acid substitution score which indicates the number of amino acids that differed between strains in the variable region. Phylogenetic analysis was carried out using programs in the Phylogeny Inference Package (PHYLIP) version 3.6 (Joseph Felsenstein, Department of Genetics, University of Washington, Seattle, Washington, USA). For nucleic acids the phenograms were produced, based on nucleic acid distance, using DNASTAR. For amino acids the phenograms were produced, based on amino acid distance, using DNASTAR in conjugation with Clustal W (1.82).

RESULTS AND DISCUSSION

Virus used in the study (UA-BZ1, UA-SP2 and UA-BZ3) were propagated in CEF culture upto 10th passage. At this stage they started showing cytopathic effect 48-72 hpi. By 48 hpi, significant number of dead cells, which had become rounded, were seen scattered in the monolayer. There was marked granulation of cell cytoplasm particularly around nucleus. By 72 hpi, most of the degenerated cells had detached from the substratum, leaving micro-plaques in the cell sheet (Figure 1). The cells were clumped into patches, leaving behind large spaces and a few long cytoplasmic strands. The uninfected control did not show any changes up to 72 hpi.

Viral RNA was isolated from the CEF infected with UA-BZ1, UA-SP2 and UA-BZ3 field isolates, which were earlier confirmed by the observation of cytopathic effect and positive result in Agar gel precipitation test (Figure 2). RT-PCR was performed from viral RNA prepared from CEF infected with UA-BZ1, UA-SP2 and UA-BZ3 field isolates. RT-PCR using specific primers resulted in amplification of 248bp VP2 hyper variable region of IBDV. The authenticity of amplified fragment was confirmed by comparing its size with 100 bp molecular marker. In the present study, 1% agarose gel electrophoresis in which 100bp molecular marker, RT-PCR amplified 248bp VP2 hyper variable fragments of UA-BZ1, UA-SP2 and UA-BZ3 were run. RT-PCR amplified 248 bp fragment bands (Figure 3) of three field isolates were observed. These RT-PCR products were further used for sequencing.

The nucleic acid sequence of 248bp VP2 hypervariable region of the CEF culture adapted field isolates was directly determined using forward/reverse primers in double pass analysis. Based on the sequence data of both primers, a final complete sequence was determined (Figure 4). From the nucleic acid sequences, amino acid sequences (Figure 5) were deduced based on ORF determined by Bayliss et al. [2]. Sequence of 248bp VP2 hyper variable region determined in this study for three field isolates of IBDV were compared with corresponding sequence of different strains from the published data of cell
Figure 3. Agarose gel electrophoresis showing RT-PCR products [Lane 1 and 5: 100bp DNA ladder, Lane 2, 3 and 4: RT-PCR products for UA-BZ1, UA-BZ3 and UA-SP2 respectively].

Figure 4. Nucleic acid sequence of different field isolates 248bp hypervariable region obtained on double pass analysis using forward and reverse primers; numbering according to Bayliss et al. [2].

adapted strains, field isolated non adapted strains of Uttarakhand, along with cell adapted vaccine strain. Alignment and comparison of nucleic acid sequences was done. The field isolate UA-BZ1 has 10 nt exchanges with UA-BZ3, UA-SP2 has 7 nt exchanges with UA-BZ3 and UA-BZ1 has 5 nt exchanges with UA-SP2. UA-BZ considered as the reference strain and here UA-BZ1, UA-BZ3 and UA-SP2 has 62, 56 and 62 nt exchange with reference strain UA-BZ. Also the deduced amino acid sequence of studied field isolates of IBDV were aligned and compared with different field, cell adapted and vaccine strains of IBDV. On comparison UA-BZ1 differed from UA-BZ3 in amino acid sequences at position 234(S→I), 241 (A→E), 258 (L→V), 262 (R→C), 268 (D→G), 295(E→V). UA-BZ1 differed from UA-SP2 at position 234 (S→N), 256 (C→G), 258 (L→V), 262 (R→C) and UA-BZ3 differed from
UA-SP2 at position 234(I → N), 241(E → A), 268(G → D) and 295(V → E). UA-BZ1 shows 96.0 percent nucleic acid sequence similarity with UA-BZ3 and 97.6 percent similarity with UA-SP2. Similarly UA-BZ3 shows 96.0 and 97.2 percent nucleic acid sequence similarity with UA-BZ1 and UA-SP2 whereas reference strain UA-BZ has 76.2, 77.8 and 75.8 percent nucleic acid sequences similarity with UA-BZ1, UA-BZ3 and UA-SP2 respectively. As a general there is 74.2 to 78.6 percent nucleic acid sequences similarity between field isolated strains and the cell adapted strains in the study. It was found that there is 74.2 to 77.8 percent nucleic acid sequence similarity between various cell adapted strains from different places and UA-BZ1, UA-BZ3 and UA-SP2. On the basis of amino acid, UA-BZ1 has 92.7 percent sequences similarity with UA-BZ3 and 96.3 percent sequence similarity with UA-SP2, UA-BZ3 has 92.7 and 95.1 percent sequences similarity with UA-BZ1 and UA-SP2 respectively. There is 57.3 to 63.4 percent amino acid sequence similarity with field isolated strains and 57.3 to 65.9 percent similarity with various cell adapted strains from different places and our strains taken in the study. As a general, there is 74.2 to 78.6 percent nucleic acid sequences similarity between field isolated strains and the cell adapted strains in the study. It was found that there is 74.2 to 77.8 percent nucleic acid sequence similarity between various cell adapted strains from different places and UA-BZ1, UA-BZ3 and UA-SP2. On the basis of amino acid, UA-BZ1 has 92.7 percent sequences similarity with UA-BZ3 and 96.3 percent sequence similarity with UA-SP2, UA-BZ3 has 92.7 and 95.1 percent sequences similarity with UA-BZ1 and UA-SP2 respectively. There is 57.3 to 63.4 percent amino acid sequence similarity with field isolated strains and 57.3 to 65.9 percent similarity with various cell adapted strains from different places and UA-BZ1, UA-BZ3 and UA-SP2cur. On the basis of amino acid, UA-BZ1 has 92.7 percent sequences similarity with UA-BZ3 and 96.3 percent sequence similarity with UA-SP2, UA-BZ3 has 92.7 and 95.1 percent sequences similarity with UA-BZ1 and UA-SP2 respectively. There is 57.3 to 63.4 percent amino acid sequence similarity with field isolated strains and 57.3 to 65.9 percent similarity with various cell adapted strains from different places and our strains taken in the study. Phenograms for phylogenetic analysis for field isolates which has been studied earlier in the laboratory, cell adapted strains from elsewhere along with isolates in the study, produced using programs in DNASTAR in conjunction with clustal W (1.82) based on nucleic acid sequences and amino acids are presented in figure 6 and figure 7.

Sequencing of amplified RT-PCR fragment of VP2 hyper variable region of IBDV field isolates UA-BZ1, UA-BZ3 and UA-SP2 were carried out. Usually there is a loss of 30-40bp sequence data at one end of the fragment when sequencing of PCR product is being attempted. Here in this study also, the sequence data obtained was 20-30bp less than the expected size. However all the samples were submitted for sequencing by double pass analysis using the forward and reverse primers and finally the correct and complete sequence size was derived. In the present study, 248 bp VP2 hyper variable region was selected in which nucleotides positioned from 804-1051 and deduced amino acid sequences positioned from 225-307 [2]. This region lies between two major hydrophilic peaks A and B. It includes minor hydrophilic peaks 1 and 2. Nucleotide exchanges are observed in the studied field isolates with other very virulent and cell adapted strains also. However all the nucleotide exchanges may not be reflected in the amino acid sequence because of the possibility of silent mutations. The field isolate UA-BZ1 has 10 nt exchanges with UA-BZ3, UA-SP2 has 7 nt exchanges with UA-BZ3 and UA-BZ1 has 5 nt exchanges with UA-SP2. UA-BZ considered as the reference strain and here UA-BZ1, UA-BZ3 and UA-SP2 has 62, 56 and 62 nt exchange with reference strain UA-BZ. Similarly UA-BZ1 has 6 amino acid exchanges with UA-BZ3, UA-SP2 has 4 amino acid exchanges with UA-BZ3 and UA-BZ1 has 5 amino acid exchanges with UA-SP2. On comparison reference strain UA-BZ, UA-BZ1, UA-BZ3 and UA-SP2 has 31, 29 and 34 amino acid exchange with reference strain respectively.

On comparison UA-BZ1 differed from UA-BZ3 in amino acid sequences at position 234(S → I), 241(A → E), 258(L → V), 262(R → C), 268(D → G), 295(E → V). UA-BZ1 differed from UA-SP2 at position 234(S → N), 256(C → G), 258(L → V), 262(R → C) and UA-BZ3 differed from UA-SP2 at position 234(I → N), 241(E → A), 268(G → D) and 295(V → E). There is presence of H at 253, N at 279 and T at 284 position in all of the IBDV amino acid sequences in study. While the reference strain UA-BZ has Q at 253, D at 279 and A at position 284. Sequence comparisons have shown amino acid Q253 to be conserved in virulent strains and H253 in the chick embryo fibroblast (CEF) culture adapted avirulent strains [21]. Similar result obtained in our sequencing alignment for amino acids. They also showed that the amino acids at positions 279 and 284 were changed to amino acids N and T in in vitro passaged virus. Studies have shown that the tissue culture tropism of IBDVs depends on amino acids N279 and T284 [22]. Amino acids at positions 279 (D) and 284 (A) are conserved in all the very virulent IBDVs and in most of the virulent classical IBDV strains.

Aligning the hyper variable region of VP2, the three unique amino acids substitutions were attributed...
to vv IBDV at positions 222 (T, Q, P to A), 256 (V to I), 294 (L to I) [23]. Amino acid substitution at position 256 and 294 were found in UA-BZ1, UA-BZ3 and UA-SP2 indicating the very virulent nature. The position 222 does not fall within this 248 bp region. Yamaguchi et al. [24] showed that amino acid at position 256 (I) was substituted to T in the course of attenuation of the highly virulent OKYM strain indicating that amino acid residue at position 256(I) may be involved in virus cell interactions possibly improving the infectivity of vv IBDV and contributing to the highly virulent phenotype. Commonly found amino acids in attenuated strains at position 279 (N), 284 (T) are found in all cell adapted strain in study.

Considering all the three studied field isolates together, the field isolates shared 96.0 to 97.6% nucleotide and 92.7 to 96.3% amino acid similarity among themselves and 74.2 to 78.6% nucleic and 57.3% to 63.4% amino acid sequences similarity between very virulent field isolates and the viruses in the study. With other very virulent strains, if we consider amino acid characteristics at particular positions of previous studied strains in the present study, the phylogenetic analysis based on phenograms groups the field isolates UA-BZ1, UA-BZ3 and UA-SP2 nearer to vv IBDVs. The present study is in acceptance

![Figure 5](image1.png)  
**Figure 5.** Deduced amino acid sequences for different isolates in the study using nucleotide sequence obtained on sequencing for 248bp hypervariable region; numbering and ORF according to Bayliss et al. [2].

![Figure 6](image2.png)  
**Figure 6.** Phenogram displaying phylogenetic relationship of various IBDV field and vaccine strains, cell adapted strains from elsewhere and isolates in study based on nucleotide sequence of 248 bp hyper variable region of vp2 gene, generated using programs in DNASTAR in conjunction with clustal W.
with the study conducted by Viswas et al. [25], which indicated that two field isolates along with a reported very virulent Indian strains were closely related to European, Japanese and Chinese very virulent strains. Finally the nucleotide and amino acid sequences of these three isolates were phylogenetically compared with virulent isolates of the department and vaccine strains of elsewhere. The result indicated according to the expectation, i.e. the isolates in study clustered separately. Also the virulent and other vaccine strains which were derived from classical virulent clustered separately. There is yet no evidence that the mutations observed are responsible for their enhanced virulence [26]. The amino acid changes seen in vv strains perhaps indicate, why strong antigenic markers specific for vv viruses have not yet been identified. Most of the changes lie between two hydrophilic regions thought to form the discontinuous, conformational neutralizing epitope. Conclusively, in vitro passaging of the virus leads to molecular characteristics in accordance with attenuation of the virulence of the viruses. Sequence and phylogenetic analysis of the culture adapted isolates (UA-BZ1, UA-BZ3 and UA-SP2) indicated that they are closely related to each other.

REFERENCES


Figure 7. Phenogram displaying phylogenetic relationship of various IBDV field and vaccine strains, cell adapted strains from elsewhere and isolates in study based on amino acid sequence of 248 bp hyper variable region of vp2 gene, generated using programs in DNASTAR in conjunction with clustal W.