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Isolation, Identification and Characterization of a Unique Infectious Bursal Disease Variant Virus from a Layer Farm Having Recurrent Vaccine Failure

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Authors' contributions

This work was carried out in collaboration among all authors. Author JKP finalized the study conception and supervised comprehensive study and data analysis. Authors AK and SP performed Sample collection, sequencing, and data analysis. Author BT drafted the manuscript along with author SJ, and all authors contributed to the improvement of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The present investigation was conducted to evaluate and determine the genotypes of IBDV isolate associated with persistent outbreaks in fully vaccinated layer flocks from West Bengal, India.
Study Design: A recurring IBD outbreak was reported in three consecutive batches of approximately 32 to 36 days old chickens in a fully vaccinated layer farm in West Bengal, India. For etiological investigation, the bursal tissue samples were collected aseptically from the chicken assumed to have died due to IBDV infection.
Place and Duration of Study: The present study was carried out on a fully vaccinated layer (Babcobb) farm in West Bengal, India in April 2020.
Methodology: The bursal tissue samples were collected aseptically from the chicken assumed to have died due to IBDV infection. All the surviving birds were sacrificed and the bursa was collected for histopathological investigation and RNA isolation was done for the sequencing of the VP2 region.
Results: As a result, the population contains IBDV isolates with varying genotypic and phenotypic variability. The obtained isolate is denoted hereafter as WB/HBL/0320. The isolate was subjected to

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amplification and sequencing of the hypervariable region (HVR) of VP2. The previously reported IBDV strains were used as a reference for analyzing the inferred amino acid and nucleotide sequences of WB/HBL/0320. In the present study, the amino acid positions in the VP2 hypervariable region of WB/HBL/0320 isolate are indicative of the virulent strain, including 242Ile, 253Gln, 256Ile,272Ile, 279Asn, 284Ala, 294Ile, 299Ser, and 330Ser, which are very similar to the very virulent and intermediate plus virulent types with only novel substitution present as A222V. Unlike previously reported virulent genotypes, WB/HBL/0320 showed the unique substitutions V225E and Q304H.

Conclusion: The molecular analysis based on a nucleotide sequence of the HVR-VP2 showed close clustering of WB/HBL/0320 with previously reported very virulent strains. However, more investigations are needed to decide on the control of the WB/HBL/0320 variant and the effectiveness of available IBD vaccines for IBDV.

Keywords: Poultry; infectious bursal disease; virulence; vvIBDV; outbreaks genetic drift; VP2 hypervariable region.

1. INTRODUCTION

The young chickens are more prone to getting an infection with Infectious bursal disease virus (IBDV), which causes a severe invasive immunosuppressive disease commonly known as Infectious bursal disease (IBD). The IBD mainly affects chickens between the age of 3 to 6 weeks. At this point, maternal immunity would decrease, and immature B cells have occupied the bursa of Fabricius. After a successful infection and a 3-4 day incubation period, it develops clinical symptoms with high morbidity, mortality, and immunosuppression [1], while chickens infected before the age of 3 weeks frequently show few or no clinical symptoms. When the infection occurs in more than six weeks and even after 20 weeks, the birds become immunosuppressed, making them vulnerable to subsequent infections and a poor immunological response to immunization [2].

The first instance of classical IBDV (cvIBDV) arose in 1957 and quickly spread throughout the USA, Europe, Asia, and other parts of the world [3]. The infection is endemic in parts of Southern Asia, including India, Indonesia, South America, the Middle East, and Africa [4]. It has been clinically recognized in 80% of the countries that make up the World Organization for Animal Health [5].

Strict biosecurity measures are required when administering vaccination to a high-risk population due to the vaccine's highly contagious nature and resilience to inactivation. The majority of conventional live IBDV vaccines in the market now are based on classical virulent strains. The "Intermediate" and "Intermediate Plus" or "Hot" vaccinations have substantially superior efficacy and may overcome higher levels of maternally produced antibodies, in contrast to the vaccines made up of mild strains, which have poor efficacy [6]. However, regular outbreaks have been recorded frequently from various parts of the world despite routine immunization due to the antigenic changes in the IBDV genome [7]. IBDV is a non-enveloped virus that belongs to the *Birnaviridae* family and the genus *Avibirnavirus*. It has a bi-segmented and double-stranded RNA genome [8]. In poultry, the IBDV serotype one can cause disease, and antigenic variation across strains is possible.

The polyprotein encoded by the large ORF in segment A is cleaved by autoproteolysis to generate mature VP2, VP4, and VP3. The polyprotein encoded by the small ORF is cleaved by autoproteolysis to yield mature VP5. Another ORF of segment A encodes a nonstructural protein VP5 that may be involved in virus release. The virus polymerase, VP1, is encoded by segment B of the genome. The VP2 gene is the main host-protecting antigen of IBDV which comprises at least three distinct epitopes that elicit neutralizing antibodies; as a result, IBDV antigenic characterization has largely relied upon research involving the VP2 gene, where few of the amino acids alterations were proven to be the basis for antigenic disparity or anticipated as possible markers for very virulent IBDV (vvIBDV) [9]. The hypervariable region of VP2 (HVR-VP2) contains two hydrophilic domains, known as major peak A (amino acids 212 to 224) and peak B (amino acids 314 to 325) and minor peak 1 (amino acids 248 to 254) and peak 2 (amino acids 279 to 290) [10]. Immune evasion of the virus is caused by mutations in the amino acids between 206 and 350 because in this region, the attachment of neutralizing antibody occurs. The

virus has two serotypes. IBDV, serotype I strains are pathogenic and pose a huge challenge to the commercial poultry business, while serotype II can not cause any clinical disease or immunosuppression in chickens. Only one amino acid alteration, i.e., I272, can convert from vvIBDV to intermediate plus IBDV [11]. Previous studies on VP2 sequencing have reported the characteristic amino acids in hydrophilic peaks A and B to differentiate amongst cvIBDV, Variant, and vvIBDV. The cvIBDV has amino acids 222Pro, 249Gln, 286Thr, and 318Gly, while 222Ala, 2. At the same time,286Thr, and 318Gly amino acids characterize vvIBDV viruses [12], and antigenic variant strains (avIBDV) strains have been reported with specific amino acid residues 222Thr, 249lys, 286Ile, and 318Asp [13-15].

Several research studies on the evolution of IBDV throughout the world have been reported, which reveal the development of attenuated IBDV strain (atIBDV) [14] with recombinant [16], reassortant [17], and distinct [18,19] strains of the virus. These diverse antigenicities of the IBDV virus have made vaccination programs more challenging as vaccine failure occurs frequently. The studies done in India have unraveled the presence of the highly virulent strains, but it is continuously evolving to a more virulence type. Because of the significant possibility of IBDV strains evolving into new variants, the newly evolved isolates should be investigated to keep the inherent features of IBDVs up to date. In the present study, isolation and genetic characterization of IBDV was performed from a field outbreak in a fully vaccinated layer flock from West Bengal, India.

2. MATERIALS AND METHODS

2.1 Case History and Sample Collection

A recurring IBD outbreak was reported in three consecutive batches of approximately 32 to 36 days old chickens in a fully vaccinated layer (Babcobb) farm in West Bengal, India. The flock was vaccinated through oral route on 12th day and 20th day with Gumboro Intermediate plus live vaccine and outbreak started on 30th day of age. For etiological investigation, the bursal tissue samples were collected aseptically from the chicken assumed to have died due to IBDV infection in April 2020. The affected birds showed symptoms like depression, reluctance to eat, and within seven days mortality reached 30%. In the majority of the cases, an inflammatory bursa was full of exudate, a hemorrhagic bursa, and pinpoint hemorrhages in the breast muscle and thigh were noticed during the postmortem examination. The four to five infected bursa showing the clear lesion of Infectious bursal disease was collected for isolation of the virus. The samples were collected aseptically and brought to the laboratory under a cold chain, where they were maintained at -20^{o}C until virus isolation was performed.

2.2 Sample Preparations

The collected bursa samples were processed further by following the predefined method by OIE [4]. A 20% bursal tissue suspension was prepared in sterile phosphate buffer saline (pH 7.4). The prepared bursal suspension was centrifuged at 4,000 rpm for 5 min at 4 °C after 3 cycles of freeze-thaw. The upper aqueous phase was then filtered using a 0.45µm membrane filter and kept at -80 °C until further processing.

2.3 Isolation of Virus

The samples deposited at −80°C were defrosted and inoculated on the chorio-allantoic membrane (CAM) of 11-day-old embryonated specific-pathogen-free (SPF) (Venky's India Limited, Pune) eggs for virus isolation. The embryos were examined for IBD-specific lesions after inoculation. Each viral isolate's CAM was harvested individually in aseptic conditions using sterile scissors and forceps.

2.4 Reproduction of the Disease

Ten SPF chicks (Twenty-five-day-old) were inoculated with 30µl each of triturated bursal tissue samples by eye drop and observed for ten days. The autopsy investigation of the dead was performed. All the surviving birds were sacrificed on the tenth-day post-inoculation, and the bursa was collected and processed for histopathological investigation. For histopathology, tissues were fixed in 4% paraformaldehyde for 24h to dehydrate the tissue, then embedded into a paraffin wax block, and sections of 10-12µm thickness were taken, followed by staining with heamatoxylin and eosin [20]. The stained specimens were examined microscopically and photographed using a Carl Zeiss microscope (Axio Scope1, Jena, Germany).

2.5 RNA Isolation and RT-PCR

The bursal homogenate was prepared by using the 100 mg of bursal tissue in 1 mL of sterile

phosphate buffer saline using a tissue homogenizer. The bursal tissue homogenate was then centrifuged for 5 min at 1300 rpm before being processed for RNA extraction with a Purelink RNA extraction kit (Qiagen). After the RNA isolation, the isolated double-stranded RNA was denatured using dimethyl sulfoxide (DMSO). Then, reverse transcription was carried out according to the standard protocol in OIE [4].

2.6 RT-PCR and Sequencing

An RT-PCR technique targeting the HVR-VP2 gene was employed to examine the presence and identification of the IBDV from the isolated RNA. The complementary DNA was prepared using a cDNA synthesis kit (Qiagen) from the denatured RNA template. Further, the PCR amplification and sequencing of the IBDV were done by using the VP2 gene-specific primers VP2 forward 5'TCACCGTCCTCAGCTTAC3' and VP2 reverse 5'TCAGGATTTGGGATCAGC3' primers. The amplification was carried out following standard RT-PCR thermal cycling conditions employing annealing temperatures specific to the selected primers (Table S1). The final PCR product was checked on 1% agarose gel electrophoresis. The PCR product was purified through a DNA purification kit (D4033, Zymo Research, USA). The purified PCR amplified products were then sequenced by Sanger sequencing from the custom sequencing facility (Eurofins, Bangalore). The VP2 gene-specific PCR and sequencing of the IBDV Intermediate plus vaccine strain were as described earlier [21].

2.7 Data Analysis

The partial VP2 gene sequence of WB/HBL/0320 was aligned with known IBD variants or IBDV vaccine isolates using the clustalW algorithm. The BioEdit freeware v7.0.5.3 was used to evaluate the aligned sequences. The nucleotide sequences were translated to amino acids and compared to genomes of existing IBDV variants (Table 1) or vaccine strains to see if there were any alterations in the field isolates. The Tamura-Nei model and the maximum likelihood method were used to infer the evolutionary history of IBDV isolates [22]. A bootstrap consensus tree was built from 1000 bootstrap iterations to depict the alterations in the taxa investigated [23]. Branches that belonged to dividers with less than 50% bootstrap iterations were distorted. The unique phylogenetic tree for the empirical search was generated automatically using the Neighbor-Join and BioNJ program. The pairwise distances were calculated with the help of the Tamura-Nei model, then selecting the network topology with the log likelihood value. Codon positions 1st+2nd+3rd+Noncoding were included. After eliminating the positions with gaps and missing data, the total number of positions in the ultimate dataset was 349. The MEGA11.0.9 software package was used to conduct an evolutionary genetic analysis of IBDV isolates. Bioinformatics techniques were used to examine the epitopes found on a partial segment of the VP2 protein with the help of IEDB's Bepipred Linear Epitope Prediction 2.0 tool [24], which examines antigenic determinants based on the physicochemical and biological properties of proteins such as hydrophilicity, mobility, and surface potential.

2.8 NCBI Accession Number

The HVR-VP2 gene sequence of WB/HBL/320 isolate was deposited in Genebank (accession number OM674280).

3. RESULTS

3.1 Clinical Analysis

The clinically affected flocks with IBDV reported symptoms like depression, damaged feathers, drowsiness, inadequate dietary consumption, deprived development, and watery white excrement. The feathers around the cloaca were soiled because of watery fecal matter. During the postmortem examination, the layer flocks infected with vvIBDV were observed with various morphological abnormalities in the bursa of Fabricius. The bursae of Fabricius had significant edema, hemorrhages, and hypertrophy. In addition, several IBDV-infected birds had petechial hemorrhages on their pectoral and leg muscles.

3.2 Histo-pathology of the Bursa of Fabricius

Out of the 10 inoculated SPF chicks, three birds died, and the rest of the birds were sacrificed on the tenth-day post-inoculation. The infected embryos showed lesions of edema, hemorrhages and mottle necrosis of Liver (Fig. 1). Histopathological examination of bursa revealed more than 90% depletion of the bursal follicle indicating severe IBD (Fig. 2). Microscopic changes in the bursa of Fabricius were studied

and scored for each lesion. The bursa had lesion values ranging from 0 to +5 for normal to highly damaged bursa. The lumen had a significant increase in exudate, which could be detected in almost every 100X field. The exudate mainly consisted of degenerating heterophils, with a few erythrocytes, plasma cells, and macrophages incorporated in for good measure. Edema had developed in the serosa and submucosa. In all plicae and follicles, hyperemia and heterophil infiltration were visible. Many vacuoles could be seen throughout the plica at 45x, and the bursa appeared eosinophilic due to the lack of lymphocytes [Fig 2(B)]. The follicular architecture was significantly altered. In comparison to the control, the interfollicular connective tissue appeared to be quite loose [Fig 2(C)]. Along the mucosal epithelium, some follicles had ruptured into the lumen. The length and width of the plicae had become reduced, and the lumen was not as complete as it would be in a typical bursa [Fig. 2(D)].

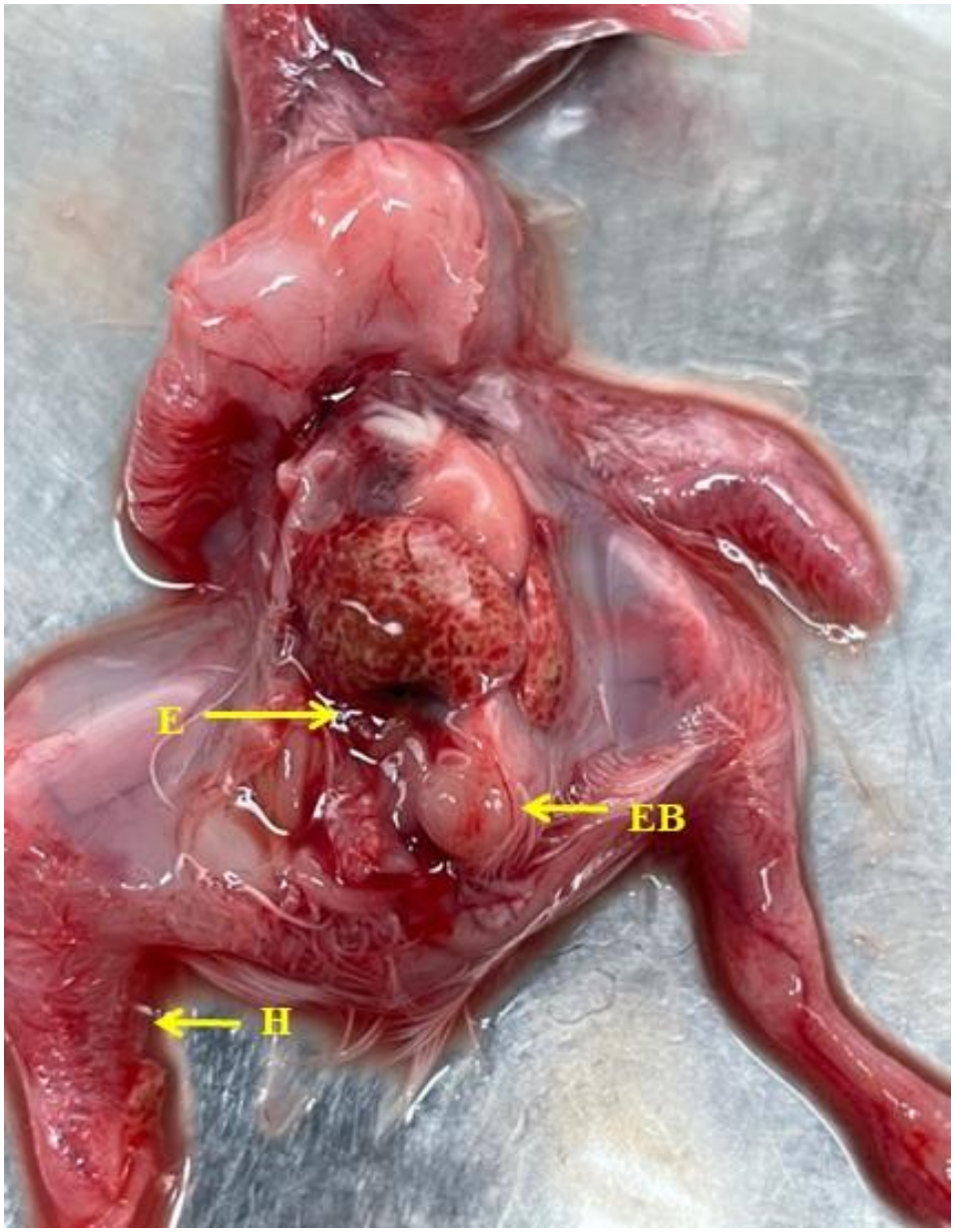

Fig. 1. SPF embryo showing: Edema, hemorrhages and mottle necrosis of liver, EB- enlargement of bursae of Fabricius, H- Hemorrhages, E- edema

Table 1. Unique amino acids substitutions in VP2 protein among IBDV virulent and attenuated strains used in data analysis. Amino acids highlighted in italics are known to enhance virulence Nucleotide and amino acid sequence analysis for HVR-VP2 gene of IBDV isolates

Strain	Virulence Phenotype	VP2														VP2 genotype
		222	225	242	249	253	254	256	272	279	284	294	299	304	330	
Guwahati/HBL-06-15	VV	*A*	*V*	*I*	*Q*	*Q*	*G*	*I*	*I*	*D*	*A*	V	*S*	*Q*	*S*	VV
Guwahati/HBL-07-15	VV	*A*	*V*	*I*	*Q*	*Q*	*G*	*I*	*I*	*D*	*A*	V	*S*	*Q*	*S*	VV
Hyderabad/HBL-07-15	VV	*A*	*V*	*I*	*Q*	*Q*	*G*	*I*	*I*	*D*	*A*	V	*S*	*Q*	*S*	VV
West Bengal/HBL-07-15	VV	*A*	*V*	*I*	*Q*	*Q*	*G*	*I*	*I*	*D*	*A*	*I*	*S*	*Q*	*S*	VV
Haryana/HBL-06-15	VV	*A*	*V*	*I*	*Q*	*Q*	*G*	*I*	*I*	*D*	*A*	*I*	*S*	*Q*	*S*	VV
Chikhodra/HBL-06-15	VV	*A*	*V*	*I*	*Q*	*Q*	*G*	*I*	T	N	*A*	*I*	*S*	*Q*	-	IPV
Napad/HBL-06-15	VV	*A*	*V*	*I*	*Q*	*Q*	*G*	*I*	T	N	*A*	*I*	*S*	*Q*	-	IPV
West Bengal/HBL-07-15b	VV	*A*	*V*	*I*	*Q*	*Q*	*G*	*I*	T	N	*A*	*I*	*S*	*Q*	*S*	IPV
BD3/99	VV	*A*	*V*	*I*	*Q*	*Q*	*G*	*I*	*I*	*D*	*A*	*I*	*S*	*Q*	*S*	VV
Uk661	VV	*A*	*V*	*I*	*Q*	*Q*	*G*	*I*	*I*	*D*	*A*	*I*	*S*	*Q*	*S*	VV
D6948	VV	*A*	*V*	*I*	*Q*	*Q*	*G*	*I*	*I*	*D*	*A*	*I*	*S*	*Q*	*S*	VV
OKYM	VV	*A*	*V*	*I*	*Q*	*Q*	*G*	*I*	*I*	*D*	*A*	*I*	*S*	*Q*	*S*	VV
HK46	VV	*A*	*V*	*I*	*Q*	*Q*	*G*	*I*	*I*	*D*	*A*	*I*	*S*	*Q*	*S*	VV
Cu-1WT	CV	P	*V*	*I*	*Q*	*Q*	*G*	V	*I*	*D*	*A*	*I*	N	*Q*	*S*	CV
002-73	CV	P	*V*	V	*Q*	*Q*	*G*	V	T	G	*A*	L	*S*	*Q*	*S*	CV
D78	AT	P	*V*	V	*Q*	H	*G*	V	*I*	N	T	L	N	*Q*	R	AT
CEF94	AT	P	*V*	V	*Q*	H	*G*	V	*I*	N	T	L	N	*Q*	R	AT
GLS	AV	T	*V*	V	K	H	S	V	*I*	N	T	L	N	*Q*	*S*	AV
Var-E	AV	T	*V*	V	K	*Q*	S	V	*I*	N	*A*	L	N	*Q*	*S*	AV
OH (Serotype II)	NP	P	*V*	V	*Q*	H	S	E	T	*D*	T	L	*S*	*Q*	*S*	NP
KT630856.1	VV	*A*	*V*	*I*	*Q*	*Q*	*G*	*I*	*I*	*D*	*A*	V	*S*	*Q*	*S*	VV
GI+IBD VP2	VV	*A*	*V*	*I*	*Q*	*Q*	*G*	*I*	T	N	*A*	*I*	*S*	*Q*	*S*	IPV
Recent most IBD isolate	VV	V	E	*I*	*Q*	*Q*	*G*	*I*	*I*	N	*A*	*I*	*S*	H	*S*	VV

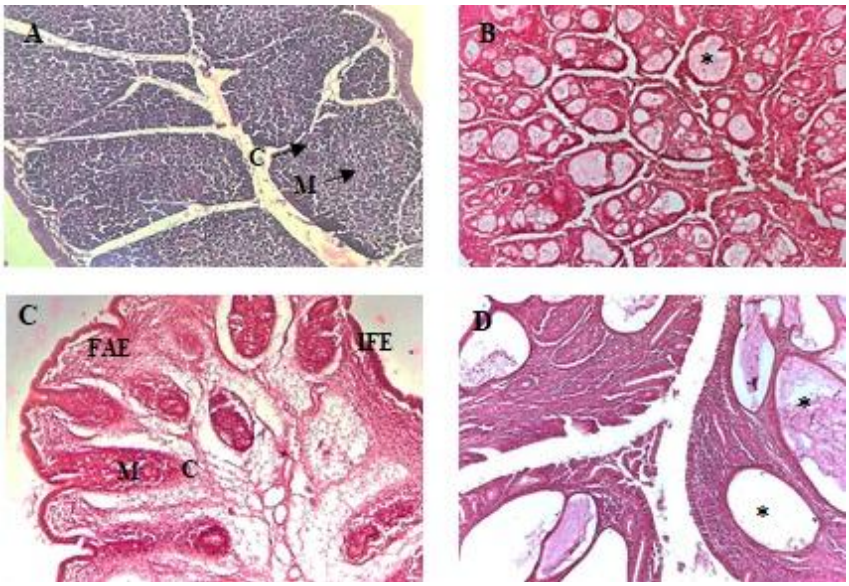

Fig. 2. Histological changes in the bursae of the Fabricius in SPF chicks, A) control, B) BLS+4 at 45X, C) BLS+4 at 100X D) BLS+5 at 100X; IFE: interfollicular epithelium; FAE: follicle-associated epithelium; M: lymphoid follicle medulla; C: lymphoid follicle cortex; Asterisks (*) specify large mucoid cysts in the lymphoid follicles

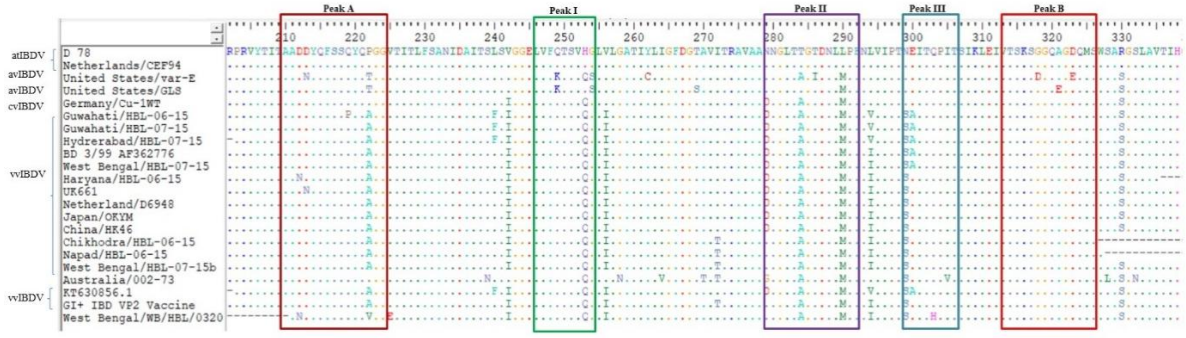

Fig. 3. Alignment of amino acid sequences of the HVR-VP2 between 202 to 334 of WB/HBL/0320, one intermediate plus vaccine and 21 known IBDV isolates. Major and minor hydrophilic peaks are boxed. cvIBDV: Classical virulent IBDV; avIBDV: antigenic variant IBDV; at IBDV: attenuated IBDV; vvIBDV: Very virulent IBDV

3.3 RT-PCR and Sequencing

The existence of IBDV in the inoculated embryos was established by RT-PCR, and the 743bp-long nucleotide sequence of WB/HBL/0320 IBDV isolates was sequenced. According to the Bayliss et al numbering system, the sequencing for the WB/HBL/0320 strain of IBDV covered nucleotide regions 625-1044 and amino acids locations 211-350 [25].

A fully immunized layer flock with IBD vaccine had its amino acid sequence of the HVR-VP2 genomic region aligned and compared to the amino acid sequences of the VP2 variable region of 22 globally reported sequences of IBDVs available on NCBI (Fig 3). Multiple sequence alignment revealed that the WB/HBL/0320 was similar to previously reported virulent strains. The WB/HBL/0320 isolate showed the presence of aa indicative of the virulent strain, including 242Ile, 253Gln, 256Ile, 272Ile, 279Asn, 284Ala, 294Ile, 299Ser, and 330Ser, which were similar to the intermediate plus virulent strain with only one exception of 222Val only present in isolate WB/HBL/0320 (Table 1). In the current analysis, WB/HBL/0320 was found to have two unique amino acid changes, i.e., V225E and Q304H, which have never been reported in any IBDV outbreak variant or attenuated vaccine strain.

Based on the surface chemistry and possibility of the amino acids' surface exposure in the HVR-VP2 gene, an essential hydrophilic peak A (amino acid 210 to 225) and peak B (amino acid 312 to 324) were identified, and small hydrophilic domains were recognized as peak I (amino acid 247 to 254), peak II (amino acid 281 to 292), and peak III (amino acid 299 to 305) [11]. In addition to the hydrophilic peaks, alterations were also detected in other regions. In north China, the D212N substitution was discovered to be quite prevalent [26] and has been noted in India [27]; it was also found in the WB/HBL/0320 isolate in the present study. The HVR-VP2 sequencing of WB/HBL/0320 clusters closely with previously described field strains from India, implying that they evolved from circulating field strains and such amino acid alterations in the HVR-VP2 region may have a role in not having protective immunity in these farms.

3.4 Phylogenetic Analysis of the WB/HBL/0320 with Reported IBDV Variants and Vaccine Isolates

The WB/HBL/0320 was found to be closely related to the previously identified very virulent strain of IBDV (Haryana/HBL-06-15) from India, according to genetic analysis based on the VP2 hypervariable region (Fig. 3). The proximity of WB/HBL/0320 with known vvIBDV field variants of India showed that they may have evolved from circulating field vvIBDV strains (Fig. 4, bootstrap value 96%).

3.5 The Antigenic Determinants of HVR-VP2 Protein

Epitope analysis of a deduced aa sequence of the partial segment of VP2 from the WB/HBL/0320 isolates, as well as the GI+ vaccine, revealed the existence of six immunogenic areas ranging from 6 to 40 amino acids, which could potentially contribute as linear epitopes (Table 2). The WB/HBL/0320 isolate was similar to the Indian vvIBDV, but in comparison to the GI+ vaccine IBDV strains; ^{225}ETI^{227} and ^{346}PL^{347} substitutions were located in antigenic sites 1 and 6, while 308S and 346A were located in the antigenic site 4 and 6 of the GI+ vaccine strain (Table 2). These alterations may affect the strain's antigenicity since they are altered in the antigenic areas, which produce neutralizing antibodies against the viruses.

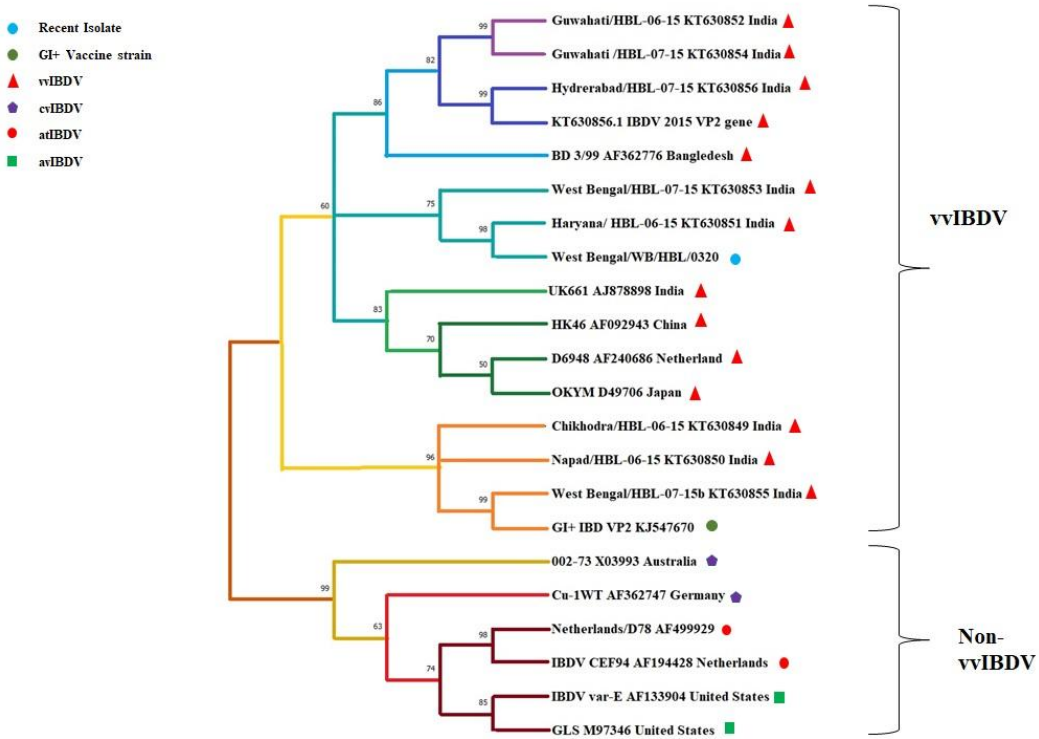

Fig. 4. Phylogenetic analysis of partial VP2 sequence of IBDV WB/HBL/0320 along with earlier reported IBDV strains. The percentage bootstraps likelihood as calculated by 1000 re-sampling is represented by the values at the nodes

Table 2. The amino acid sequence of the antigenic determinants with starting and end positions identified in 146 aa regions of VP2

No.	Start	End	Peptide	Length
Most recent Field isolate				
1	215	227	QFSSQYQVGGETI	13
2	235	242	DAITSLSI	8
3	252	259	SVQGLILG	8
4	269	307	GTAVITRAVAANNGLTAGTDNLMPFNIVIPTSEITHPIT	39
5	314	333	VTSKSGGQAGDQMSWSASGS	20
6	341	347	GNYPGPL	7
GI+ vaccine				
1	215	224	QFSSQYQAGG	10
2	235	242	DAITSLSI	8
3	252	259	SVQGLILG	8
4	269	308	GTAVTTRAVAANNGLTAGTDNLMPFNIVIPTSEITQPITS	40
5	314	333	VTSKSGGQAGDQMSWSASGS	20
6	341	346	GNYPGA	6

4. DISCUSSION

IBDV, like other RNA viruses, is genetically susceptible to mutation. The classical virulent IBDV, immunogenic variant, and vvIBDV strains have arisen in succession during the last 50 years, posing significant problems for the efficient prevention and control of IBD. Because of different conditions, there is a chance that IBDV will evolve into a more virulent and infectious strain [28,29]. Concurrently, pathogenic strains of the virus have been employed to develop attenuated IBDV vaccines (atIBDV). However, this is a time-consuming and labor-intensive method with dubious consequences for the advancement in vaccines. VP2 is a well-known antigenic determinant commonly used for IBDV molecular assessment [26,30]. The VP2 has recently been used to identify and analyze mutant IBDV [31,14]. The amino acids 222Ala, 256Ile, 279Asp, 284Ala, 294Ile, and 299Ser in VP2 were identified to be conserved in vvIBDV strains analyzed, whereas 222Pro, 256Val, 279Asn, 284Thr, 294Leu, and 299Asn were found to be preserved in atIBDV strains, and the amino acids 249Gln and 254Gly were found to be related with cvIBDV [14]. The nucleotides and aa sequences of the HVR-VP2 gene of the WB/HBL/0320 strain showed the highest similarity with vvIBDV strains.

The molecular analysis of the WB/HBL/0320 and the known vvIBDV strains revealed many substitutions, indicating that the virus in circulation has a significant genetic diversity. The most important measure for controlling IBD is vaccination; unfortunately, extensive use of active vaccines has led to the emergence of novel variants of IBDV [32]. Despite the normal immunization schedule, IBDV outbreaks are being reported worldwide. By carefully studying the evolution of IBDV, it is possible to pinpoint the cause of these outbreaks. According to the studies done by Fan and his team in 2019, both segment A and segment B genomic regions of IBDV appear to have a role in the virus replication. The majority of field isolates had a VP2 gene similar to that of a highly virulent strain, with some isolates having differential global positioning amino acid alterations. The discovery of a novel amino acid pattern in the highly contagious RNA polymerase HVR-VP2 in this study, including its significant genetic divergence from earlier described isolates, indicates that genome segment B has evolved and adapted and that it has reassorted to segment A of very virulent or vaccine strains, leading to the emergence of new strains. These new isolates may escape the immune system, resulting in enhanced pathogenicity or replicative potential [14].

None of the Indian isolates were 100% identical at the nucleotide level, having 6.16 and 8.22 percent divergence between them, which could be attributable to these isolates' significant chronological and geographical variation. Based on nucleic acid sequence analysis, the field isolates of India were found in the same cladogram cluster as particularly virulent isolates from Germany, the United States, the Netherlands, and Bangladesh, showing a closer evolutionary affinity [26]. Their discrepancy from other known very virulent strains was 0.5 to 3.5%. The VP2 hypervariable region analysis indicated the existence of virulence-determining

amino acids 222Ala, 242Ile, 253Gln, 256Ile, 284Ala, and 299Ser in all field isolates. In HVR, the eight amino acid substitutions (242Ile, 249Gln, 253Gln, 254Gly, 256Ile, 272Ile, 284Ala, and 299Ser and 330Ser) described for most Indian very virulent strains [29,33], is also identical to WB/HBL/0320 strain except for only one amino acid alteration, i.e., D279N, which was documented in some intermediate plus virulent strains. But the previous study done by Qi and his team had reported that both the replication efficiency *In-vitro* and the pathogenicity of IBDV to SPF chickens were unaffected by the mutation of residue 279 in VP2 [34].

The aa 222Ala, 256Ile, and 294Ile are highly conserved in vvIBDV strains, and the aa 242Ile, 256Ile, 294Ile, and 299Ser are regarded to be a primary defining trait attributable to the vvIBDV strain [35,36]. Among the analyzed vvIBDV strains, the WB/HBL/0320 possesses A222V, a novel mutation reported for the first time in Indian strains. As it is located at the extremity of a major peak A, the aa at position 222 is an important residue. An aa alteration at position 222 could cause vaccine failure [37] because these mutations originated in the antigenic domains of the viruses, which produce neutralizing antibodies.

The antigenic association between the vaccination and the circulating virus strains is important for IBDV protection [29]. Although all reported IBD-VP2 aa positions are more than 93% similar, variants between different isolates are often located inside virus-neutralizing epitopes, and modifications within a specific position of aa in the VP2 molecule give rise to differences in the immunogenicity of the virus. Data reported herein on the partial VP2 sequence of the WB/HBL/0320 IBDV immunogenic variants has given important information on the evolution of this isolate and molecular mechanisms for immunogenic and pathogenic variation. The presence of alterations in the predictive immunogenic determinants between the field isolate and the GI+ vaccine IBDV strain may explain the outbreak of the vaccine. The intensive vaccination of rearing flocks at various periods may have modified the field IBDVs.

In the modern scenario, the globalization of IBD virus vaccine strains may be the cause of the global emergence of new strains with different pathogenicity. Previous research on succeeding isolates of the hypervariable region was beneficial; the VP2 gene is an essential region that influences the genetic and immunological features of the IBDV [8]. The substantial genetic variation in IBDV HVR is not astonishing, as various mutations evolve over time due to constant vaccination, allowing viral adaptation to the biological environment.

5. CONCLUSION

Infectious Bursal Disease is the biggest problem of the poultry business globally. Although regular immunization programs are carried out, vvIBDV strain outbreaks occur often. The present study reports severe recurrent outbreaks with a high mortality rate in young poultry flocks. This study revealed that mutations in the VP2 main capsid protein of vvIBDV at A222V and V294I might be responsible for triggering resistance and vaccine failure, but not necessarily virus attenuation. The study also reports the novel substitutions V225E and Q304H in WB/HBL/0320, which have never been reported for IBDV strains. Such novel substitutions may also be responsible for vaccine failure. The vvIBDV strains persist in posing a serious menace to the poultry business, requiring continuous surveillance and genetic study to track the emergence and evolution of antigenically distinct IBDV strains or subtypes. These findings are essential for advancing our understanding of viral virulence mechanisms and the development of new IBDV-specific vaccines.

DATA AVAILABILITY

The datasets generated during and analyzed during the current study are available from the corresponding author upon reasonable request.

ETHICAL APPROVAL

The work described in this article was carried out under the approval of the animal ethics committee of Hester Biosciences Limited India, HBL-IAEC-09-2020.

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COMPETING INTEREST

Authors have declared that they have no known competing financial interests or non-financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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SUPPLEMENTARY MATERIAL

Table S1. Depicts the amplification conditions for the amplification of HVR-VP2 gene of IBDV

Sr. No.	Steps	Temperature	Duration (minutes)	Cycle
1	UDG activation	50°C	30:00	Hold
2	Dual-Lock DNA polymerase	95°C	15:00	Hold
3	Denature	95°C	00:30	40 Cycles
	Anneal	55°C	00:30	
	Extend	72°C	01:00	
4	Final extension	72°C	05:00	Hold

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