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Molecular Epidemiology of Infectious Bursal Disease Virus in Mizoram during 2021–2022

Naveen R. M.[1], Roychoudhury P.[1], Subudhi P. K.[1], Dutta T. K.[1] and Udaya S. S. T.[2]

[1]Dept. of Veterinary Microbiology, Central Agricultural University, Selesih, Aizawl, Mizoram (796 014), India
[2]Dept. of Veterinary Pathology, Assam Agricultural University, Khanapara, Assam (781 022), India

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Corresponding tellauday1592@gmail.com

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ABSTRACT

The study was conducted during the tenure period January, 2021–March, 2022 from different regions of Mizoram where outbreaks suspected to be IBDV were screened by agar gel precipitation test (AGPT) for molecular characterization of circulating Infectious Bursal Disease Virus (IBDV) carried out by targeting partial VP2 gene. Out of 32 samples, 12 tissue samples were found positive and further confirmed by Reverse transcription-Polymerase Chain Reaction (RT-PCR). A total of 4 field tissue samples were sequenced and phylogenetic analysis was carried out and found that one of the sequences clustered in Very Virulent IBDV (VVIBDV) group with sequence similarity of 95.3–98.6% and matching only 90.8% sequence similarity with Georgia vaccine strain and 95.6% with earlier reported VVIBDV strain (2015) of Mizoram. Other 3 sequences clustered in the Classical IBDV (CIBDV) group with sequence similarity of 98.9–99.8%. Deduced amino acid sequence of present study VVIBDV strain showed a unique very virulent amino acid marker at position A-222, I-242, S-299 and serine heptapeptide indicating very virulent IBDV. However, one unique virulent amino acid marker showed substitution at position 294 where isoleucine substituted by valine indicating the circulating VVIBDV in the North-eastern state differs from other parts of India. This study demonstrated that both classical IBDV and Very Virulent IBDV belonging to serotype-1 were associated in the outbreak of Mizoram.

KEYWORDS: Infectious bursal disease, polymerase chain reaction, sequence, phylogenetic

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1. INTRODUCTION

Infectious Bursal Disease Virus (IBDV) targets actively dividing and proliferating B lymphocytes, mostly in 3–6 weeks old chicks leading to 69.44% mortality by weakening the immune system and reducing vaccine response (Bhutia et al., 2017; Zhang et al., 2022). Infectious Bursal Disease (IBD) is a contagious infectious disease of young chickens caused by infectious bursal disease virus (IBDV), which belongs to the genus *Avibirnavirus* of the family *Birnaviridae* (Dobos et al., 1979). Two antigenically distinct IBDV serotypes were identified till date, serotype-1 and serotype-2. Based on clinical signs, serotype-1 again is classified into variant, classical and Very virulent IBDV (VVIBDV) (Jackwood et al., 1982). IBDV is a single-shelled, non-enveloped virus with icosahedral symmetry and a diameter varying from ~58–60 nm with two segments, A and B (Kibenge et al., 1988; Islam et al., 2021). The minor genome segment, "B" IBDV about 2.9 kb encodes one viral protein VP1 (91 k Da) which encodes RNA-dependent RNA polymerase (Bruenn et al., 1991). The larger segment of the dsRNA, "A", is approximately 3.3 kb and includes two partially overlapping open reading frames (ORFs). The first ORF encodes the non-structural VP5 protein (17 k Da) and this protein plays a role in anti-apoptotic function at an early stage of viral infection (Lombardo et al., 2000). The second ORF encodes a 110 k Da polyprotein which is cleaved auto catalytically to form precursor VP2 (pVP2) (48 k Da), VP4 (28 k Da) and VP3 (32 k Da) proteins (Kibenge et al., 1997).

VP2 and VP3 are major IBDV structural proteins (Dobos et al., 1979). The VP2 protein shows base, shell and projection, while base and shell region have conserved amino acids, whereas the projection region shows groups of sensitive amino acids which give rise to novel antigenic variants (Bayliss et al., 1990). The major capsid protein VP2 plays an important role in antigenic variants by which the virus can escape neutralizing antibodies, Thus the hyper variable region of VP2 gene is the target for IBDV detection, evolution and pathogenic variations (Liu et al., 1994; Enyetornye et al., 2024; Zhu et al., 2025; Cadiz et al., 2025). The hVP2 region contains two major hydrophilic domains, major hydrophilic peak A (aa 212–224) and peak B (aa 314–325), which form hairpin loops PBC (aa 219–224) and PHI (aa 316–324). The hVP2's minor hydrophilic peaks 1 (aa 248–254) and 2 (aa 279–290) form the loops PDE (aa 249-254) and PFG (aa 279–284) (Coulibaly et al., 2010). Based on these sensitive amino acids changes, recently IBDV has been classified into 7 genogroups (Michel et al., 2017). Molecular pathotyping of Indian VVIBDV strain shows constant presence of unique amino acids markers at position 222-A, 256-I, 294-I and 299-S (Kataria et al.,. 2001; Mittal et al., 2006; Dey et al., 2019; Pikula et al., 2017). Rajkhowa et al., 2018 reported a genetic reassortment that acquired segment A from very VVIBDV and segment B from non VVIBDV and even attenuated vaccine strains leads to outbreak of disease in North eastern India. Morla et al., 2016 and Rajkhowa et al., 2018 reported the circulating VVIBDV of North-eastern India that showed substitution in a unique amino acid marker at position 294I-V (Rajkhowa et al., 2018; Morla et al., 2016). Two neutralizing linear B cell epitopes were reported by Wang et al., 2005, at position 197–209 and 329–337 in VP2 gene (Wang et al., 2005). Islam et al., 2021. reported four epitopes in hypervariable regions of VP2 gene, epitope-1 (211–225), epitope-2 (245–256), epitope-3 (277–289), epitope-4 (313–331) (Islam, 2015). IBD outbreaks are frequently seen in the subcontinent's vaccinated chicken farms (Raja et al., 2016; Awandkar et al., 2018; Shinde et al., 2021). Either reassortment and recombination with field-circulating viruses or mutation in the major and minor hypervariable regions of the VP2 protein produced the novel IBDV strains (Jackwood et al., 2018; Raja et al., 2016). Virus is continuously evolving in the fields with changes in antigenicity and virulence, so closely monitoring the evolution of virus and finding out the cause of outbreak plays a key role. The aim of the present study was to characterize genetically the field IBDV involved in these outbreaks.

2. MATERIALS AND METHODS

2.1. Collection of samples

A total of 32 broiler chickens were suspected to suffer from IBD during the period January, 2021–March, 2022 in the state of Mizoram, and bursal tissue samples were collected, triturated and stored at-80°C in the Department of Veterinary Microbiology. Lyophilized freeze dried IBD virus-intermediate strain (Georgia) procured from Indovax was taken as positive control while bursal tissue from an apparently healthy bird was taken as negative control.

2.2. Agar gel precipitation test

AGPT was performed for the detection of infectious bursal disease virus (IBDV) antigen from the bursal homogenates. The test was performed as per the procedure described in the OIE manual with slight modification (Anonymous, 2018). Hyperimmune serum raised against Georgia vaccine strain virus was used for screening of field tissue samples. Briefly, 10% suspensions of the bursal tissue were prepared in PBS (pH 7.4) and centrifuged at 3000 rpm for 10 min. Supernatant was harvested and stored at-80°C for further use. Petridish were pre-coated with 0.5% agarose and 30 ml of molten 1% agarose gel and 2.4 g of sodium chloride was poured on it and allowed to cool down. Wells were punched

with one central well and 6 peripheral wells with 6 mm apart from the central well. Thirty µl of antiserum was placed in the central well and the tissue harvested antigen (30 µl) in the peripheral wells was loaded with a micropipette. One well was kept as positive antigen control, where vaccine antigen was loaded. Bursal suspensions from healthy birds in case of IBD were used as negative tissue control for standardization of the test. Slides were incubated at 37°C in a humid chamber for up to 48 hrs. Plates were observed against a dark background with an oblique light source for presence of positive precipitation reaction.

2.3. RNA extraction

RNA was extracted using commercially available RNA extraction kits (Nucleospin RNA mini kit-cat. No. 740956.50, Germany) as per manufacturer's instructions. The extracted purified RNA was stored at-80°C till further use.

2.4. Reverse transcriptase PCR

Reverse transcription of total RNA into complementary DNA (cDNA) was carried out using a cDNA synthesis kit (PrimeScript TM 1st strand cDNA synthesis kit, cat. No. #6110A, Takara, Japan) by following the manufacturer's instructions. The forward primers and reverse primers described by (Liu et al., 1994) were used for the amplification of 643 bp sequences of VP2 gene hypervariable regions. The incubation temperature and duration of each cycle of the PCR were 3 min at 94°C for Initial denaturation, 1 min at 94 °C for denaturation and 1 min at 56.7°C for annealing and 1 min at 72°C for extension. The amplification was carried out for 35 cycles. The PCR was terminated after the final extension step at 72°C for 10 min. The PCR products (5 ml aliquots) were separated on a 1.7% agarose gel stained with ethidium bromide and the size of the PCR products were compared with the 100-bp DNA Ladder (Takara).

2.5. Nucleotide sequencing

The PCR product of four field samples generated by using primer (19) were purified by using QIAquick PCR and Gel cleanup Kit (cat .NO. 28506, QIAGEN, Germany) as per manufacturer's instructions and cloned in pTZ57R/T cloning vector with the InsT/Aclonee PCR product cloning kit (Cat. No. K1213, Thermo scientific, US) as per manufacturer's instructions. White recombinant colonies were picked randomly and inoculated in 1.2–2 ml LB broth containing ampicillin (100 µg ml^{-1}) and incubated for 6–8 hrs at 37°C at 200 rpm in an orbital shaker, centrifuged and resuspended in 20 µl nuclease free water and confirmed by PCR. The recombinant plasmids containing gene fragments were sequenced at a DNA sequencing facility at Delhi University (South Campus, New Delhi, India).

A total of 4 IBDV isolates, representative of different outbreaks, were selected for genetic characterization. Sequences generated from all 4 samples were analyzed with the Mega-11 software. Nucleotide sequences from position 623 to 1264 for the VP2 Gene were aligned by clustal method using Mega 11 software (Michel et al., 2017; Rajkhowa et al., 2018; Morla et al., 2016). A phylogenetic tree was constructed using nucleotide sequences of the 643-bp region corresponding to the VP2 gene from 4 field samples sequences, with 36 reference sequences (9 sequences of genogroup-3 (vvIBDV), 2 of genogroup-2 (aIBDV), 6 of classic attenuated IBDV (caIBDV), 1 of genogroup-4 (latin america isolate), 1 of genogroup-5 (mexican recombinant classical and variant virus), 1 of genogroup-6 (Italian isolate), 2 of genogroup-7 (australia isolates) reference sequences retrieved from the NCBI GenBank nucleotide database (Table 1), along with a serotype II isolate. Phylogenetic and molecular evolutionary analyses were conducted with the neighbor-joining method (10, 12, 14, 15) in MEGA11. The robustness of the groupings in the neighbor-joining analysis was assessed with 1000 bootstrap replicates. Similarly, a sequence homology study of the present field samples was conducted using DNA Star software, and deduced amino acid sequences from positions 210 to 346 and neutralizing epitopes for the VP2 gene were aligned using the Clustal method with BioEdit sequence alignment editor software (Rajkhowa et al., 2018; Wang et al., 2005; Islam, 2015).

3. RESULTS AND DISCUSSION

3.1. AGPT

Virus detection by AGPT was performed for initial screening of field tissue samples from different outbreaks of Mizoram, as this test was most common, inexpensive and straightforward for detecting IBDV antigen using known positive hyperimmune serum (Bhutia et al., 2017). The AGPT was performed on bursal homogenates using IBDV hyperimmune serum. The white precipitation line reaction was observed during 36–48 hrs of incubation and was similar to that produced by the reference IBDV antigen (Figure 1). Such precipitation reaction was absent in the known negative bursal sample. Out of 32 samples tested, 12 samples were found positive for IBDV antigen.

3.2. PCR amplification and cloning

VP2 gene of four tissue samples of 4 different regions were amplified by PCR which gives the target amplification of 643bp without any amplification in apparently healthy unvaccinated bursal tissue (Figure 2). The PCR amplified product was cloned to pTZ57RT vector (TA cloning vector), sufficient white colonies were observed after 13–14 hrs of incubation, and few blue colonies were also seen on the plate (Figure 3). Colony PCR was performed by selecting 8–10 white colonies and detected positive by PCR amplification

Table 1: Reference sequences used for phylogenetic analysis

Accession number	Country	Year	Strain/isolate	Genogroups
KY828162.1	India	2016	IBD/MZ/IND/9/16	Genogroup 3
KU530208.1	India	2015	Kamrup/51/2015	Genogroup 3
AF362776.1	Bangladesh	2001	Bd3/99	Genogroup 3
KM20579.1	India	2007	Bareilly	Genogroup 3
KX342913.1	India	2015	IBD/MZ/IND/2/15	Genogroup 3
KX342912.1	India	2015	IBD/MZ/IND/1/15	Genogroup 3
KJ547670.1	India	2014	Ventri IBDV-plus	Genogroup3
DQ927040.1	Israel	2008	MB	Genogroup 3
EF517528.1	China	2007	Harbin	Genogroup 3
AY444873.3	China	2008	GXx	Genogroup 3
AY462027.1	USA	2007	9109	Genogroup 2
AF133904.1	USA	2000	Variant A	Genogroup 2
AF321056.1	China	2002	ZJ2000	Genogroup 1
AF321054.1	China	2002	HZZ	Genogroup 1
AF499929.1	Luxembourg	2004	D78	Genogroup 1
AF194428.1	Netherland	2000	CEF94	Genogroup 1
DQ906921.1	China	2006	B87	Genogroup 1
OK491857.1	India	2008	MH-HL-IVST1	Genogroup 1
JN982252.1	Brazil	2001	MG4	Genogroup-4
DQ916210.1	Mexico	2008	Mexico04M101	Genogroup-5
JN852986.1	Italy	2010	ITA.02	Genogroup-6
HM071991.1	Australia	2008	V877W	Genogroup-7
AF148080.2	Australia	2009	06/95	Genogroup-7
AF362773.1	Germany	2001	23/82	Serotype-2

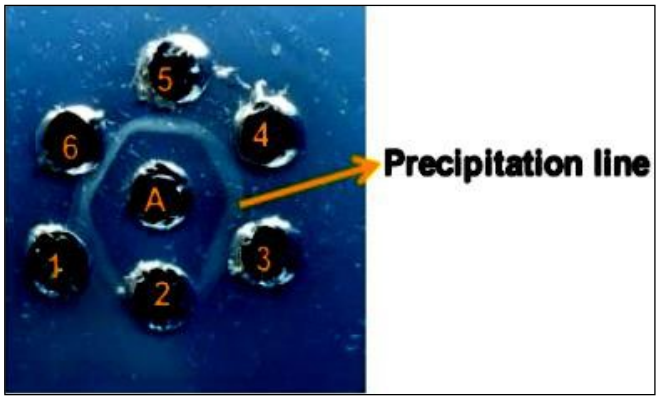


Figure 1: Agar gel showing white precipitation line indicating positive and no line means negative. Well 1: positive control Well 2: negative control, Well 3,4,5,6: sample

(Figure 4). The clone was sequenced by outsourcing (Department of biochemistry, university of Delhi) and also annotated and submitted to the Gene Bank-OP868849, OP868850, OP868851 and OP868852 are the accession number of the present field samples of Mizoram.

3.3. Phylogenetic analysis

The hypervariable region of VP2 was a key protein for molecular epidemiology and phylogenetic studies of IBDV (Michel et al., 2017; Mittal et al., 2006; Rajkhowa et al., 2018; Morla et al., 2016). Among the 4 samples OP868849 were observed to be clustered in VVIBDV genogroup showing 98.6% sequence similarity with Meghalaya VVIBDV isolate (KY828162.1), 96.8% similarity with Kamrup VVIBDV strain (KU530208.1) and 95.6% similarity with Mizoram previous VVIBDV isolate (KX342912.1) (Figure 5, Figure 6), whereas other 3 samples OP868852, OP868851 and OP868850 showing 99.8%, 99.3% and 98.9% sequence similarity with India classical strain MH-HL-IVST1 (OK491857.1) and clustering in classical IBDV genogroup (Figure 5, Figure 6). Present VVIBDV strain (OP868849) showing sequence similarity of 90.5%, 90.2% and 90.9% with others present classical IBDV strain (OP868851, OP868850 and OP868852, respectively)

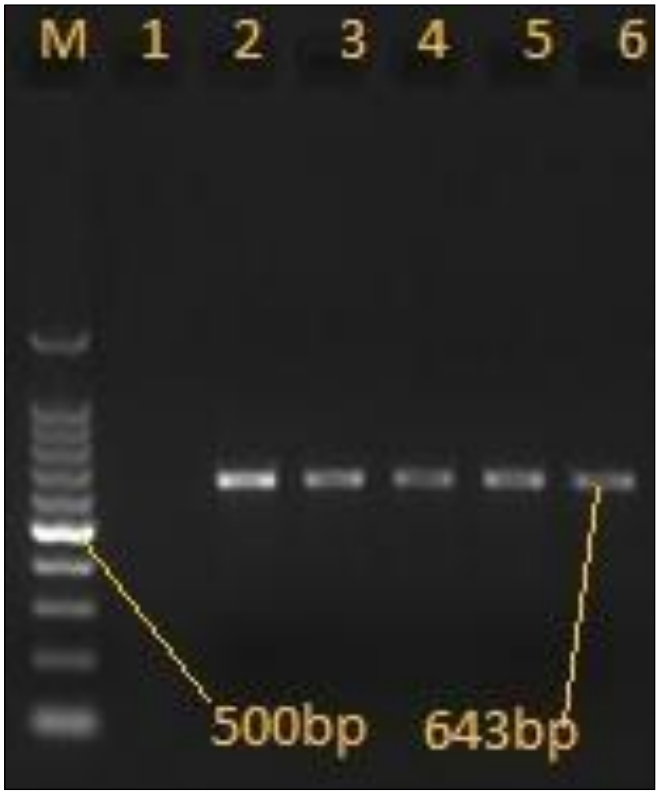


Figure 2: Agarose gel electrophoresis showing the PCR amplicons of VP2 gene (643 bp) of IBDV; Lane M: 100 bp DNA ladder (Takara); Lane 1: Negative control; Lane 2: Positive control; Lane 3-6: Field samples

(Figure 6). Georgia intermediate live vaccine virus strain (AJ249517.1) showing sequence similarity of 90.8% with present VVIBDV strain (OP868849) and 98.4%, 98.9% and 98.9% with present classical IBDV strain (OP868850, OP868851 and OP868852 respectively) (Figure 6). Present

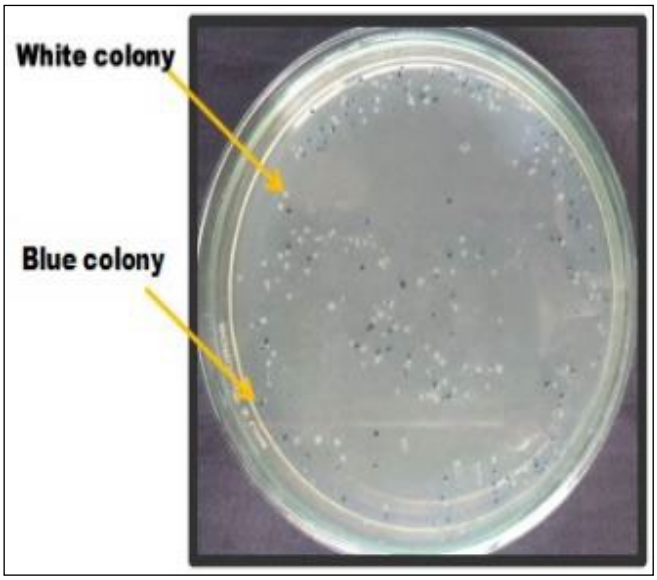


Figure 3: Blue white colony on Luria bertani (LB) agar plate

field strain OP868849 was seen clustering in genogroup-3, very virulent IBDV (VVIBDV) with sequence similarity of 95.3–98.6% (Figure 5, Figure 6), where as other present strain OP868851, OP868850 and OP868852 were seen clustering in genogroup-1, classical strain IBDV (CIBDV)

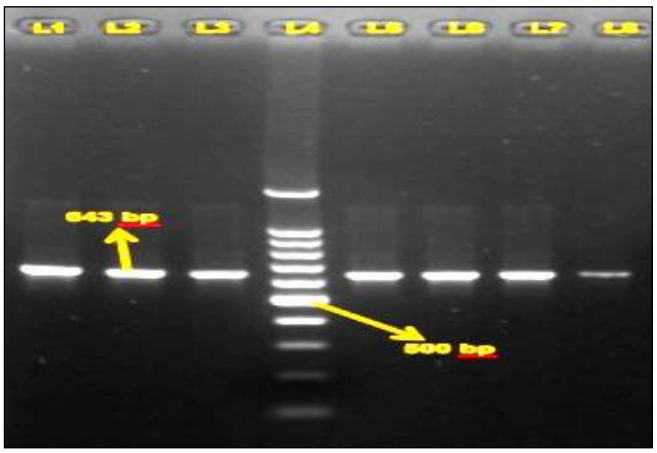


Figure 4: Agarose gel electrophoresis showing the PCR amplicons of VP2 gene (643 bp) of IBDV; Lane 4: 100 bp DNA ladder (Takara); Lane 1-8: Colony PCR of white colonies and positive showing amplification of ~643 bp

with sequence similarity of 98.9–99.8% (Figure 5, Figure 6). It clearly indicated that the present very virulent circulating IBDV was different from previously reported virulent virus of Mizoram (95.6%) and showing only 90.8% sequence similarity with Georgia live vaccine virus strain. This

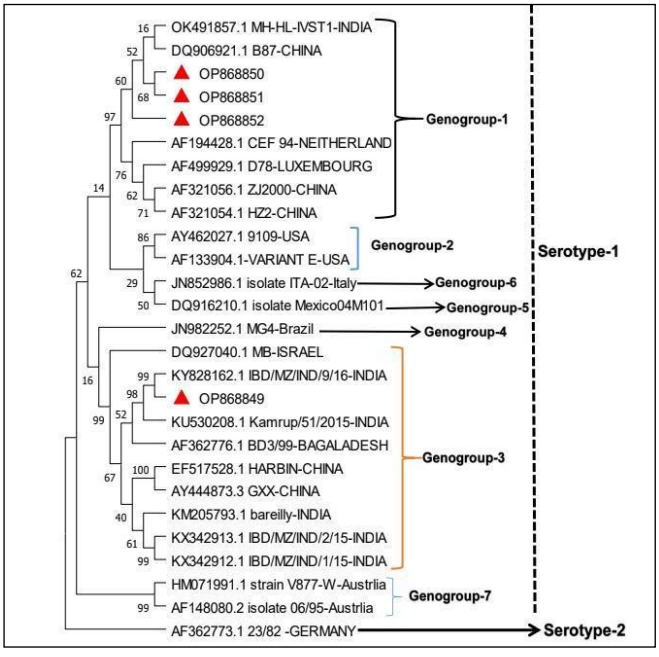


Figure 5: Phylogenetic tree generated by Neighbour joining method on the basis of partial VP2 gene (643 bp) of present isolate when compared with sequences of previous outbreak in India and others countries using MEGA 11 software

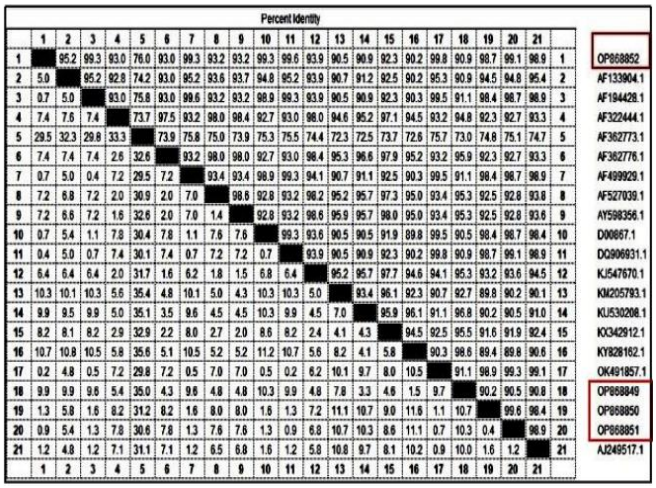


Figure 6: Divergence table on the basis of partial VP2 gene nucleotide sequence analysis by using DNAstar software

indicated that over a period of time there are differences among the isolates from North-Eastern India in terms of their sequence homology. As among RNA viruses the rate of mutation was higher, such types of differences were quite common among IBDV field isolates.

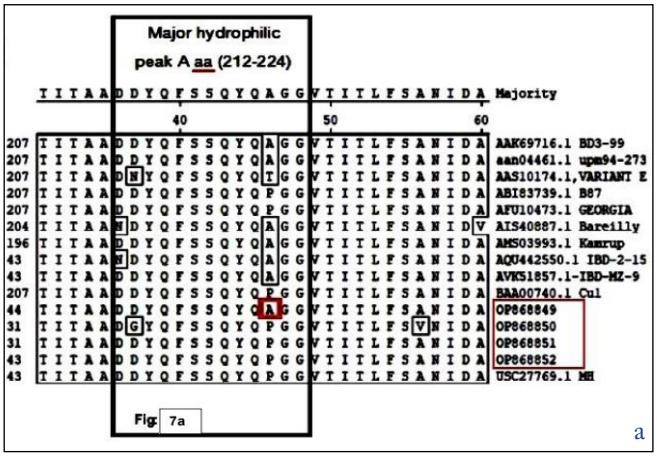
3.4. Deduced amino acid analysis

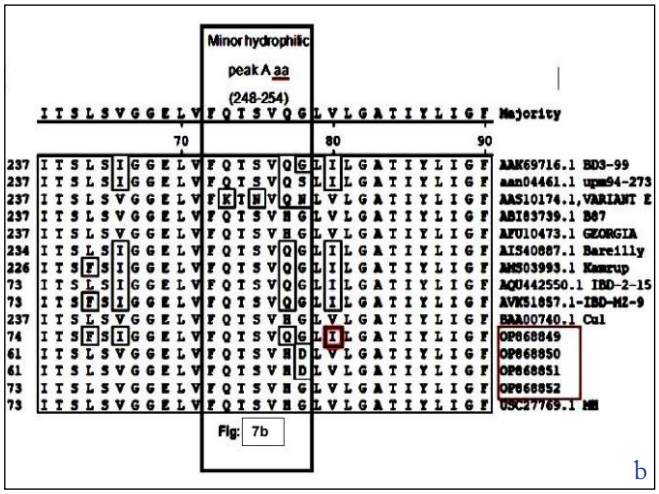
Presence of amino acid Alanine at position 222 was a genetic marker of virulent virus which belongs to genogroup-3, and most of virulent virus of Indian origin showed presence of unique amino acid marker at position 222-A, 256-I, 294-I and 299-S and serine rich heptapeptide (326SWSASGS332) (Kataria et al., 2001; Mittal et al., 2006). In these study the present field strain OP868849 showed above mentioned unique very virulent amino acid markers and heptapeptide, indicating VVIBDV (Table 2, Figure 7a, b, c, d, e), but there was substitution of one unique amino acid marker at position 294 where isoleucine substituted by Valine (Table 2, Figure 7c), it was similarly as reported earlier by by Morla et al., 2016 from Assam and Rajkhowa et al., 2018. from Meghalaya. Other 3 present field strains OP868851, OP868850 and OP868852 showed the same amino acid P-222, V-256, Q-253, L-294, N-299 with other classical genogroup-1 viruses, indicating classical IBDV. Present classical IBDV strains (OP868850 and OP868851) showed differences in amino acid at position 254 with the Georgia vaccine strain from D to G (Table 2, Figure 7b). Present

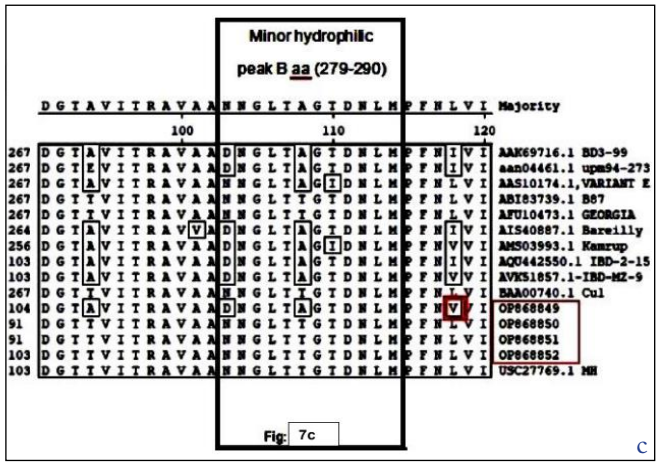
Table 2: Key amino acid comparison of present field samples with Georgia vaccine strain and previously reported isolates of India

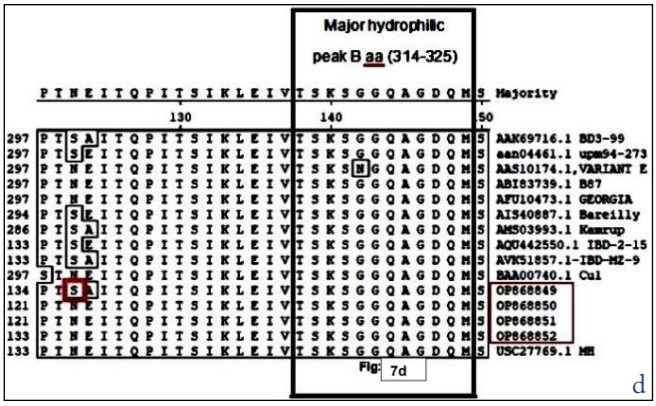
Accession No.	212	216	222	240	242	249	253	254	256	270	272	279	284	294	299	300	325	330
AJ249517.1 Georgia vaccine strain	D	F	P	L	V	Q	H	D	V	T	I	N	T	L	N	E	M	R
AIS40887 Bareilly-India	N	-	A	-	I	-	Q	G	I	A	-	D	A	I	S	-	-	S
AVK51857.1 Meghalaya	-	-	A	F	I	-	Q	G	I	A	-	D	A	V	S	A	-	S
AQU42550.1 Mizoram	N	-	A	-	I	-	Q	G	I	A	-	D	A	I	S	-	-	S
AMS03993.1 Kamrup/51 /2015	-	-	A	F	I	-	Q	G	I	A	-	D	A	V	S	A	-	S
USC27769.1 MH-HL-IVST1India	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-
OP868849 Present isolate	-	-	A	F	I	-	Q	G	I	A	-	D	A	V	S	A	-	S
OP868850 Present isolate	-	-	-	-	-	-	-	G	-	-	-							
OP868851 Present isolate	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-
OP868852 Present isolate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

VVIBDV strain (OP868849) showing 13 amino acid difference with Georgia vaccine strain (AFU10473.1) in hyper variable region of VP2 protein (Table 2, Figure 7a, b, c, d) and showing 4 key amino acids difference with previous Mizoram VVIBDV strain at position 212D-N, 240F-L, 294V-I and 300A-E, there was no substitution of unique virulent amino acid marker at position 294 in previous 2015 reported strain of Mizoram (AQU42550.1) (Table 2, Figure 7a, b, c, d). Indicating North-Eastern circulating VVIBDV









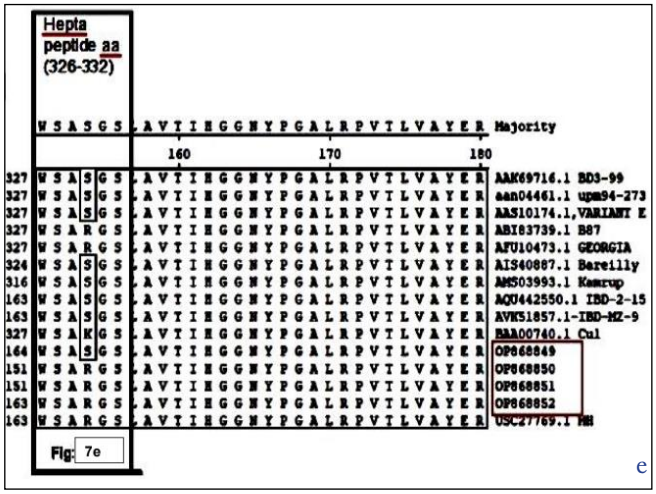


Figure 7a,b,c,d,e: Deduced amino acid sequence of present field samples comparison with previous isolates sequences using BioEdit sequence alignment Editor software. Two hydrophilic regions- major peakA (212- 224), peak B (314-325) and minor peak 1(248-252), peak 2 (279-290). Unique virulent marker amino acid A-222, I-256, I-294, S-299 and heptapeptide ser-trp-ser ala-ser-gly-ser(326-332)

differs from other parts of the country. One of the primary reasons is that viral RNA polymerase lacks proof reading activity. As a result it led to genetic diversification, resulting in the emergence of viruses with new properties and their persistence in the poultry population. A serine instead of glycine mutation at amino acid position 254 (PDE loop) caused vaccination failure (Negash et al., 2013) as result presented classical field IBDV causing field outbreak.

Four neutralizing epitopes were identified at position aa (211-225) epitope-1, aa (245–256) epitope-2, aa (277–289) epitope-3, aa (313–331) epitope-4 (Islam, 2015). Present VVIBDV field strain (OP868849) shows 1 amino acid substitution at position 222P-A and present classical field IBDV strain (OP868850) showed 1 amino acid substitution at position 213D-G in epitope-1 antigenic site (Figure 8a), In epitope-2 present study VVIBDV field strain (OP868849) shows 2 amino acid substitution at position

253H-Q, 256V-I and present classical IBDV strains (OP868850, OP868851) showed 1 amino acid substitution at position 254G-D (Figure 8b), In epitope-3 present study VVIBDV strain (OP868849) showed 2 amino acid changes at position 279N-D and 284T-A (Figure 8c) and one amino acid change in epitope-4 330 R-S (Figure 8d). In the present study, present field VVIBDV strain (OP868849) showed 6 amino acid differences with Georgia vaccine strain in 4 antigenic sites epitope region of VP2 capsid protein. The present classical field IBDV strain (OP868850 and OP868851) showed 2 amino acid differences in 4 antigenic sites. In a present study, present field VVIBDV strain (OP868849) showed a total 13 amino acid difference with Georgia vaccine strains in hypervariable regions of VP2 protein. Yamaguchi et al., 1996. had reported that the virus-neutralizing epitope was within the hypervariable region of VP2 protein from amino acid 204–359 (Yamaguchi et al., 1996). Mutations in this variable region resulted in virus immune evasion. Single or combined mutations in the hVP2 region affect the virus's virulence pattern, leading to outbreaks of disease and failure of vaccination (Dey et al., 2019).

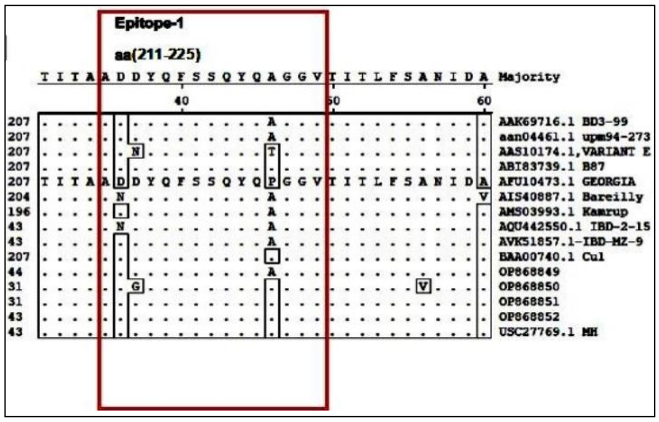


Figure 8a: Epitope-1 antigenic sites amino acid 211-225

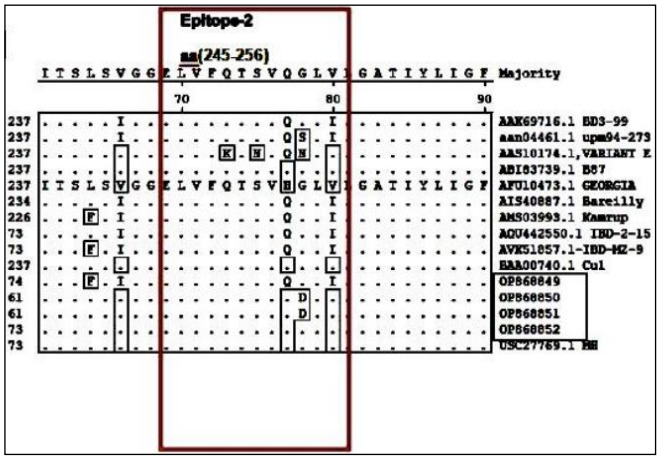


Figure 8b: Epitope-2 antigenic sites amino acid 245-256

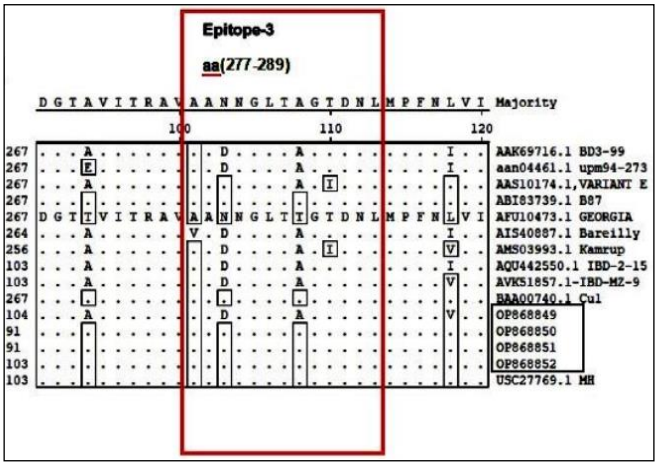


Figure 8c: Epitope-3 antigenic sites amino acid 277-289

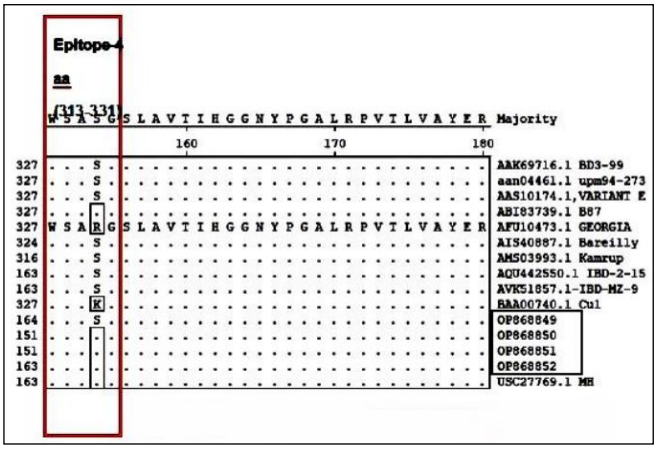


Figure 8d: Epitope-4 antigenic sites amino acid 313-331

5. CONCLUSION

Both Classical IBDV and Very Virulent IBDV belonging to serotype-1 were associated in the outbreaks of Mizoram. Very virulent IBDV of the present field sample showed substitution in one of the unique virulent amino acid markers at position 294 where isoleucine substituted by Valine which was not seen in previously reported isolates of Mizoram. Classical IBDV of present samples showed single amino acid substitution at position 254 from aspartic acid to glutamic acid with Georgia vaccine strain; this might lead to vaccination failure.

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