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Sequence analysis of VP1, VP2 and VP3 genes of *Infectious Bursal Disease Virus* from a field outbreak in Kerala, India#

Akhila Joy[1*], Sreeja R. Nair[2], R. Ambily[2], M. Mini[3] and I. S. Sajitha[4]
Department of Veterinary Microbiology,
College of Veterinary and Animal Sciences, Mannuthy, Thrissur- 680 651,
Kerala Veterinary and Animal Sciences University, Kerala, India.

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Abstract

Infectious bursal disease (IBD), caused by infectious bursal disease virus (IBDV), is one among the top five infectious diseases of poultry that discernibly affects commercial poultry industry. The mutating viral genome of IBDV accounts for disease outbreaks in fields even after following stringent biosecurity measures and vaccination protocols. The present study is focussed on the characterisation of an IBDV field virus, IBD/CVAS/6, from a vaccinated flock in Kerala, based on the sequence analysis of VP1, VP2 and VP3 genes. Bursa of Fabricius samples collected from a 26 days-old chicken flock from a suspected outbreak in the Thiruvananthapuram district of Kerala formed the subject of the study. Reverse transcriptase-polymerase chain reaction (RT-PCR) targeting VP2 gene confirmed the presence of virus in the sample. The sequence analysis revealed that the deduced amino acid sequence of VP1 gene of IBD/CVAS/6 was 100 per cent homologous with an attenuated very virulent vaccine strain of Israel, mb and the VP2 gene was 100 per cent homologous with mb and Ventri IBDV plus vaccine strain of India. The analysis of VP3 gene also revealed the similarity with vaccine strains except for a single variation S745N in its deduced amino acid sequence. The phylogenetic analysis of IBD/CVAS/6 revealed it's close relation with mb, Ventri IBDV plus and a very virulent strain of Israel, ks. The characteristic virulent marker amino acid motifs 'SWSASGS' and 'TDN' were present in the VP2 and VP1 genes, respectively. Hence, the study revealed that the obtained virus has emerged from an attenuated very virulent vaccine strain and hence the present study is a report of involvement of the intermediate plus vaccine strain in field outbreaks in Kerala. The role of S745N in virulence cannot be accounted from the present study, however the involvement of IBD/CVAS/6 in the outbreak might be related to S745N variation or variations in other genes of the virus or due to the inefficiency of the vaccine or vaccination protocol followed, which can be defined only after further studies. The present report is the first

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1. *MVSc Scholar*
2. *Assistant Professor*
3. *Senior Professor and Head*
4. *Assistant Professor, Department of Veterinary Pathology*

**Corresponding author: akhilajoy.cps@gmail.com Ph: 994749196*

characterisation study in Kerala focussing on the analysis of VP1, VP2 and VP3 genes of IBDV.

Keywords: *Infectious bursal disease virus, RT-PCR, sequence analysis*

Infectious bursal disease (IBD) or Gumboro disease is an acute highly contagious immunosuppressive disease of young chicken caused by the infectious bursal disease virus (IBDV). The selective tropism of the virus towards the immature B-lymphocytes in the bursa of Fabricius of young chicken results in irreversible bursal damage and immunosuppression. Immunosuppression hinders the effectiveness of vaccination against other diseases and also increases the susceptibility of birds to other infections. Depending on the strain of virus, IBD can generate 10 to 90 per cent mortality and 100 per cent morbidity in a susceptible flock.

Infectious bursal disease virus is a non-enveloped, bisegmented double-stranded RNA virus belonging to the genus *Avibirnavirus* of the family *Birnaviridae* (Leong *et al*., 2000). Of the two segments, segment A (3.2 kbp) encodes the two structural proteins VP2 and VP3, the viral protease VP4 and the non-structural protein VP5. Segment B (2.8 kbp) encodes VP1, an RNA-dependent RNA polymerase involved in viral replication and genetic evolution (Gao *et al*., 2014). There are two serotypes of the virus, of which only serotype I is virulent in chicken and serotype I viruses were distinguished into classical virulent, antigenic variant, classical attenuated and very virulent IBDV (vvIBDV) based on the antigenicity and pathogenicity of the virus (Jackwood and Saif, 1987).

Molecular characterisation of the virus has been limited mostly to the hypervariable region of the VP2 gene, specified as *AccI-SpeI* region, since most of the amino acid alterations between antigenically distinct IBDVs were localised in this region. However, the role of other viral proteins as virulence determinants is currently under investigation. The present study is conceptualised to detect the genetic homogeneity/ heterogeneity of the field strains of IBDV from Kerala in comparison with the vaccine strains, based on sequence analysis of VP1, VP2 and VP3 genes. Thus, the investigation will help to gain better knowledge of mutations that are characteristic of virulence. The present study is the first characterisation study in Kerala based on the analysis of VP1, VP2 and VP3 genes.

Materials and methods

Sample collection

Bursa of Fabricius samples were collected from a flock of 26 days-old chicken, showing clinical signs and lesions suggestive of IBD, from a field outbreak (25 per cent mortality) in Thiruvananthapuram district of Kerala. The samples were pooled and collected aseptically in RNAlater and stored at -80°C until used.

Detection of IBDV by reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the sample and the vaccine (Infectious bursal disease living-intermediate plus strain) using TRIzol reagent (invitrogen) according to the manufacturer's protocol. The extracted RNA was treated with dimethyl sulfoxide (DMSO) and the DMSO-RNA mix (1:4) were incubated for five minutes at 95°C followed by snapchilling (Deorao *et al.,* 2021).

The DMSO treated RNA were reverse transcribed using Goscript™ reverse transcription system (Promega, USA) following the manufacturer's protocol. The reaction mix containing template RNA (2 µg), Random hexamer and oligo $(dt)_{15}$ primers (1µL) and nuclease-free water (to get a final volume of 5 µL) were incubated at 70ºC for five minutes and immediately transferred onto ice. The mix was added to reverse transcriptase mix containing 4 µL of Goscript 5X buffer, 2 µL of $MgCl_2$ (25m*M*), 2 µL of dNTP (10m*M*), 0.25 µL of Recombinant Rnasin Ribonuclease inhibitor, 1 µL of Reverse transcriptase and nuclease-free water to get a final volume of 20 µL. The total mixture was incubated at 25ºC for five minutes, 42°C for one hour and 70°C for 15 min. in the thermal cycler (MJ Mini Bio-Rad thermal cycler).

The cDNA synthesised were employed for PCR using primers targeting the *AccI- SpeI* region of VP2 gene (Eterradossi *et al.,* 1998) (table 1). The reaction was carried out

Table 1. Sequences of primers used for RT-PCR targeting VP2 gene

Primer name		Primer sequences 5'-3'	Product size	Reference
IBDV-VP2	Forward	GGTATGTGAGGCTTGGTGAC	556 bp	(Eterradossi *et al*.,1998)
	Reverse	GATCCTGTTGCCACTCTTTC		

by initial denaturation at 95°C for five minutes, followed by 35 cycles of denaturation at 95°C for 30 sec., annealing at 59.4°C for one minute and extension at 70°C for one minute, followed by final extension at 70°C for six minutes. The PCR product obtained were resolved electrophoretically on two per cent agarose gel and were visualised in the gel documentation system.

Amplification of VP1, VP2 and VP3 genes

The primers targeting VP1, VP2 and VP3 genes were designed and custom synthesised commercially (Sigma-Aldrich) as overlapping primer sequences (table 2). The coding nucleotide sequence of the three genes of UK661 were downloaded from NCBI for reference. The optimised reaction conditions for the amplification of the three genes are depicted in table 3. The amplified gene segments of the virus were detected by gel electrophoresis and the bands of respective amplicon size representing VP1, VP2 and VP3 genes were purified and extracted by QIAquick Gel Extraction Kit (Qiagen, Germany), as per the manufacturer's protocol. The purified DNA was sent for sequencing.

Sequencing and phylogenetic analysis of VP1, VP2 and VP3 genes

The sequencing was done by Sanger's dideoxy chain termination method at

Table 2. Details of primers targeting VP1, VP2 and VP3 genes

Sl. No.	Target gene	Primer name		Primer sequence (5'-3')	Product size
1.	VP1 (2640 bp)	VP1a	Forward	ACGGTAAGCGTCCCTCTTCT	537 bp
			Reverse	CCCACTCCCGTAGGCTTTAT	
2.		VP1b	Forward	CGAGGCCACAGATAACCTCA	549 bp
			Reverse	TACCTCTCAGCCTTGGGAAA	
3.		VP1c	Forward	AGGGTCGAACAAGAAGAAGC	494 bp
			Reverse	CAATGTTCATCGCAAAGGTG	
4.		VP1d	Forward	CCAGAGGATGGTCCGATAAC	492 bp
			Reverse	CTTTGGGATACGCAGCAGAG	
5.		VP1e	Forward	ACTCGGATGGTCTGCAACTT	537 bp
			Reverse	TTGACAGCGTCCTGTAGTCG	
6.		VP1f	Forward	CTAAGTGGCCTCGTCCTCCT	451 bp
			Reverse	GCTATTGGCGGCTCTCTTTT	
7.	VP2 (1332 bp)	VP2a	Forward	CGCAGCGATGACAAACCT	523 bp
			Reverse	TGATGTGGGTAAGCTGAGGA	
8.		VP2b	Forward	CAACAGCCAACATCAACGAC	522 bp
			Reverse	CCTGACCACCACTTTTGGAG	
9.		VP2c	Forward	TAACCCAGCCAATCACATCC	417 bp
			Reverse	CTGCAATCTTCAGGGGAGAG	
10.		VP2d	Forward	CCTCAACTCTCCCCTGAAGA	373 bp
			Reverse	ACCCTCTCTCAACACGCAGT	
11.	VP3 (705 bp)	VP3a	Forward	TACCTTCCACCCAATGCAG	490 bp
			Reverse	AGTCCTCGTTTGGATCAGGT	
12.		VP3b	Forward	CAACACCAGAATGGGTAGCA	435 bp
			Reverse	AGCCTCACTCAAGGTCCTCA	

Table 3. The PCR conditions optimised for the amplification of VP1, VP2 and VP3 genes

Sl. No.	Step	Temperature (°C)		Time	
1	Initial denaturation	95		5 min.	
2	Denaturation	95		30 sec.	
3	Annealing	VP1a	50.6	1 min.	35 cycles
		VP1b			
		VP1c			
		VP1d			
		VP1e			
		VP1f	62.8		
		VP2a	51.8		
		VP2b			
		VP2c			
		VP2d			
		VP3a	62.8		
		VP3b			
4	Extension	70		1 min.	
5	Final extension	70		6 min.	

Table 4. Strains of virus selected from GenBank, NCBI for phylogenetic analysis

Sl. No.	Sequence ID of strains	Accession number		Country	Strain of virus
		Segment B	Segment A		
1.	Cu-1wt	AF362748	AF362747	Germany	Classical virulent
2.	Edgar	AY918949	AY918950	USA	Classical virulent
3.	EDE14ABT/MVC/ India	KU558698	KU558697	India	Classical attenuated
4.	MB11	KU891987	KU891986	India	Classical virulent
5.	CEF94	AF194429	AF194428	Netherland	Classical virulent
6.	RPM14/ABT/MVC /India	KU578103	KU578102	India	Antigenic variant
7.	GLS Bursal derived	AY368654	KU578102	USA	Antigenic variant
8.	Variant E	AF133905	AF133904	USA	Antigenic variant
9.	UK661	NC_004179	NC_004178	Europe	vvIBDV
10.	HK 46	AF092944	AF092943	China	vvIBDV
11.	OKYM	D49707	D49706	Japan	vvIBDV
12.	Bpop/03	MH545935	MH545934	Poland	vvIBDV
13.	UPM08MF	KU516687	KU516686	Malaysia	vvIBDV
14.	UPM97/61	AF527040	AF247006	Malaysia	vvIBDV
15.	IBS536201	MT505344	MT505339	Malaysia	vvIBDV
16.	PY12	KX223750	-	India	vvIBDV
17.	SH 99	LM651366	LM651365	China	VvIBDV
18.	ks strain	DQ927043	DQ927042	Israel	vvIBDV
19.	AvvBvv	MG489893	MG489892	France	Mutant vvIBDV
20.	BGE/14/ABT2/India	KT884453	KT884452	India	Reassortantvv IBDV
21.	GX-NNL-IBDV	JX134486	JX134485	China	Reassortantvv IBDV
22.	mb strain	DQ927041	DQ927040	Israel	Attenuated vvIBDV
23.	Ventri IBDV Plus	-	KJ547670	India	Attenuated vvIBDV
24.	D78	EU162090	EU162087	USA	Classical attenuated
25.	OH	U20950	U30818	USA	Avirulent

Agrigenome, Cochin. The obtained sequences were merged using Emboss merger and analysed using BLASTn hit analysis in NCBI (http://blast.ncbi.nlm.nih.gov/Genbank). The deduced amino acid sequences were obtained using Transeq (EMBOSS). For phylogenetic analysis, sequences of other IBDV isolates were selected from GenBank, NCBI (table 4). The obtained sequences were aligned with downloaded sequences of different strains of virus using the Clustal W programme and the phylogenetic analysis was done using MEGA X software. The maximum likelihood method was used to interpret evolutionary history with 1000 bootstrap replications.

Results and discussion

Detection of IBDV by RT-PCR

On post-mortem examination, bursal enlargement with haemorrhage, thigh muscle haemorrhage (Fig.1) and haemorrhage at the proventriculus-gizzard junction were noticed which were in accordance with the lesions observed by Vengadabady and Sulochana (1995) and Akkara *et al.* (2018). The sample collected was confirmed positive by RT-PCR, detected a 556 bp amplicon on agarose gel electrophoresis (Fig.2) and was named as IBD/CVAS/6.

Amplification of VP1, VP2 and VP3 genes

The VP1, VP2 and VP3 genes of IBD/CVAS/6 were successfully amplified by RT-PCR using the primer pairs mentioned in table 2. The amplicons detected on two percent agarose gel by gel electrophoresis and visualised by ultraviolet illumination are depicted in Fig.3.

Sequencing and phylogenetic analysis of VP1 gene

The BLASTn hit analysis of the VP1 gene sequences revealed that IBD/CVAS/6 shared greatest nucleotide homology with an attenuated very virulent strain of Israel, mb strain. The nucleotide and deduced amino acid sequence analysis of IBD/CVAS/6 expressed 100 per cent homology with the mb strain. The presence of amino acid triplet 'TDN' at position 145, 146 and 147 in IBD/CVAS/6 indicates the virulence potential of the virus since TDN triplet had been identified as a characteristic of very virulent IBDV virus. Whereas, 'NEG' amino acid triplet is most commonly identified in non-virulent IBDV isolates (Kasanga *et al*., 2013). The phylogenetic analysis shows the clustering of IBD/CVAS/6 with the mb strain and both shares a common ancestor with ks strain, a very virulent virus strain of Israel. The phylogenetic tree is depicted in Fig 4.

According to Lazarus *et al*. (2008), mb strain is the attenuated form of a very virulent strain of Israel, ks. The author reported the change in amino acid from Threonine to Asparagine at position 96 (T96N) and Aspartic acid to Alanine at position 161 (D161A) as two novel changes in the deduced amino acid

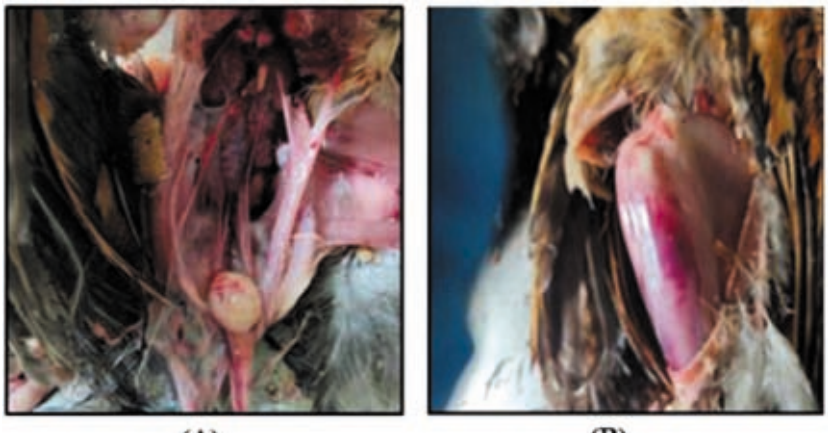

Fig.1. Post-mortem findings in the bird suggestive of IBD (A) enlarged bursa of Fabricius with haemorrhage (B) haemorrhages in the leg muscles

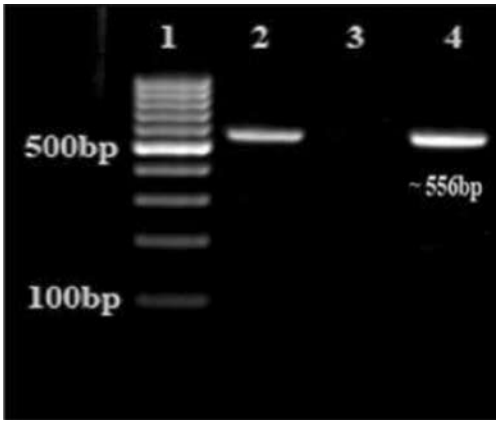

Fig.2. Agarose gel electrophoresis of RT-PCR amplified product of *AccI-SpeI* region of VP2 gene from bursal tissue, 556 bp

Lane 1: 100 bp DNA marker, Lane 2: Positive control, Lane 3: Negative control

Lane 4: bursal tissue sample

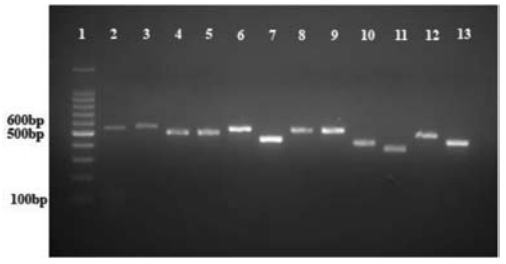

Fig. 3. Agarose gel electrophoresis of PCR amplified products of VP1, VP2 and VP3 genes of IBD/CVAS/6. Lane 1-100 bp DNA Ladder, Lane 2-VP1a:~537 bp, Lane 3- VP1b: ~549 bp, Lane 4- VP1c: ~494 bp, Lane 5- VP1d: ~492 bp, Lane 6- VP1e: ~537 bp, Lane 7- VP1f: ~451 bp, Lane 8- VP2a: ~523 bp, Lane 9- VP2b: ~522 bp, Lane 10- VP2c: ~417 bp

sequence of VP1 gene, responsible for the attenuation in mb strain. The presence of 96N and 161A in IBD/CVAS/6 and 100 per cent similarity with mb strain leads to the conclusion that IBD/CVAS/6 is having an attenuated very virulent genotype. So, the analysis suggests that the VP1 gene sequence of IBD/CVAS/6 is identical to the vaccine strain and has no identified role in the virulence expressed by the virus in the field condition.

Sequencing and phylogenetic analysis of VP2 gene

The BLASTn hit analysis of the VP2 gene sequence expressed greatest nucleotide homology of IBD/CVAS/6 with Ventri IBDV plus, a vaccine strain in India, and mb strain. The nucleotide and amino acid sequence analysis of IBD/CVAS/6 expressed 99.92 per cent nucleotide and 100 per cent amino acid homology with the Ventri IBDV plus and mb strains. The presence of characteristic serine rich heptapeptide sequence 'SWSASGS', a characteristic of very virulent virus, was identified in the sequence. The virulence hallmark amino acids 222A, 242I, 256I, 294I and 299S were also present indicating that the virus possess the characteristics of a very virulent virus. However, the presence of 279N amino acid, a characteristic of cell culture adapted or attenuated vaccine strain (Mittal *et al*., 2006), was noticed in IBD/CVAS/6 which confirms that IBD/CVAS/6 is diverged from an attenuated very virulent vaccine strain that has undergone attenuation in cell culture. According to Lazarus *et al.* (2008), the change in amino acid from Isoleucine to Threonine at position 272 (I272T) in the deduced amino acid sequence of VP2 gene was involved in the attenuation in mb strain. The 272T amino acid is conserved in IBD/CVAS/6 and thus, the VP2 gene characteristics also depict that IBD/CVAS/6 is an attenuated strain.

The analysis revealed that VP2 gene has no identified role in the virulence exhibited by the virus as the amino acid sequence of IBD/CVAS/6 is identical with the vaccine strain. The phylogenetic analysis shows that IBD/CVAS/6 shares a more recent common ancestor with mb, Ventri IBDV plus and ks strains. The phylogenetic tree is depicted in Fig 5.

Sequencing and phylogenetic analysis of VP3 gene

The BLASTn hit analysis of VP3 gene sequence revealed the greatest nucleotide homology of IBD/CVAS/6 with ks strain and on sequence analysis, there was 99.85 per cent nucleotide similarity and 99.15 per cent amino acid similarity with ks strain. On comparison with the Ventri IBDV plus and mb strain, IBD/CVAS/6 was found to have 99.7 per cent nucleotide homology and 99.15 per cent amino acid homology. The change in amino acid obtained, on comparison with Ventri IBDV plus, mb and ks strain, is S745N corresponding

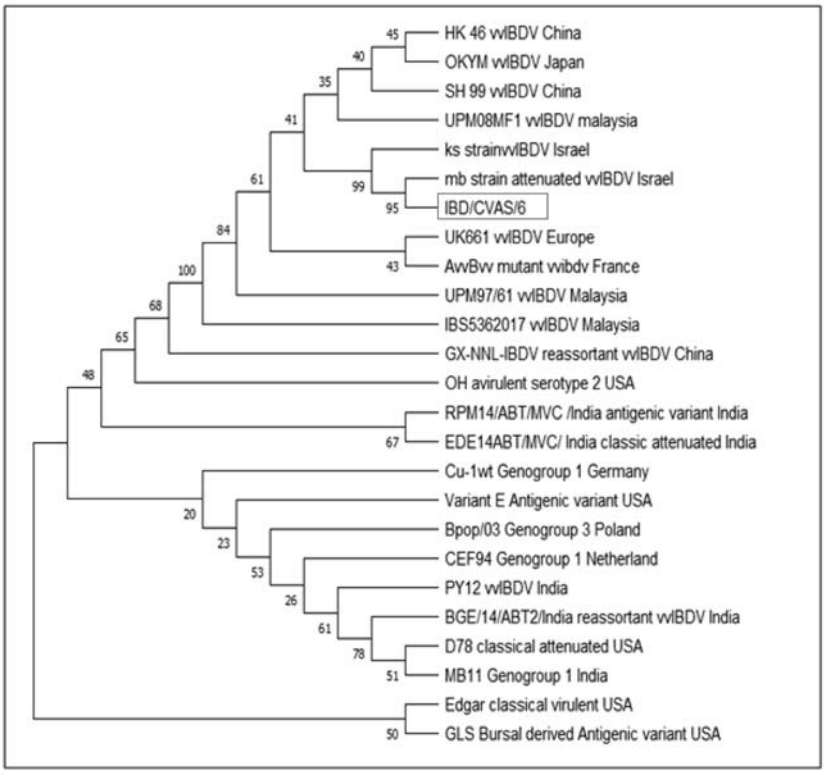

Fig.4. Phylogenetic tree based on the analysis of 2640bp nucleotides of VP1 gene

to the change in nucleotide G2303A (coding strand position) in the segment A (Fig. 6). On comparison with different strains of virus, 745N was noticed in very virulent, classical virulent, antigenic variant IBDVs and in attenuated very virulent IBDV, MB11. In the avirulent IBDV isolate, OH, 745T amino acid was noticed. Hence, from the obtained results of sequence analysis, S745N is the only variation noticed on comparison with the mb and Ventri IBDV plus, however it cannot be attributed for the change in virulence of the strain. The phylogenetic analysis of VP3 gene revealed that IBD/CVAS/6 shares a more recent common ancestor with ks strain than Ventri IBDV plus and mb strains. The phylogenetic tree is depicted in Fig.7.

The isolate IBD/CVAS/6 showed 100 per cent amino acid and nucleotide similarity with mb strain, an attenuated very virulent vaccine strain for VP1 gene. When analysed for VP2 gene, IBD/CVAS/6 showed almost 100 per cent amino acid similarity with mb strain as well as with Ventri IBDV plus, the intermediate plus vaccine strain used in India. For VP3 gene, IBD/CVAS/6 is having more than 99.15 per cent similarity with ks, mb and Ventri IBDV plus strains. This study reveals the close relation of IBD/CVAS/6 with intermediate plus vaccine virus as the sample was obtained from a vaccinated flock. The field isolates showing nucleotide and amino acid similarity wth intermediate plus vaccine strains were reported by Deorao *et al.,* (2021) and Nandhakumar *et al.* (2020). The authors opined that the use of intermediate plus vaccine may induce bursal lesions and immunosuppression in the flock.

Conclusion

Identifying and continuous monitoring of the circulating virus strains in a locality is always advisable as it could provide ample data for developing a local vaccine strain in areas where management and regular vaccination cannot bring the disease under control. Majority of virulence studies conducted so far were focused on the hypervariable VP2 region (hVP2) of the virus but recently, it has been demonstrated that molecular analysis of both the genome segments is equally important to study the characteristics of very virulent IBDV and for

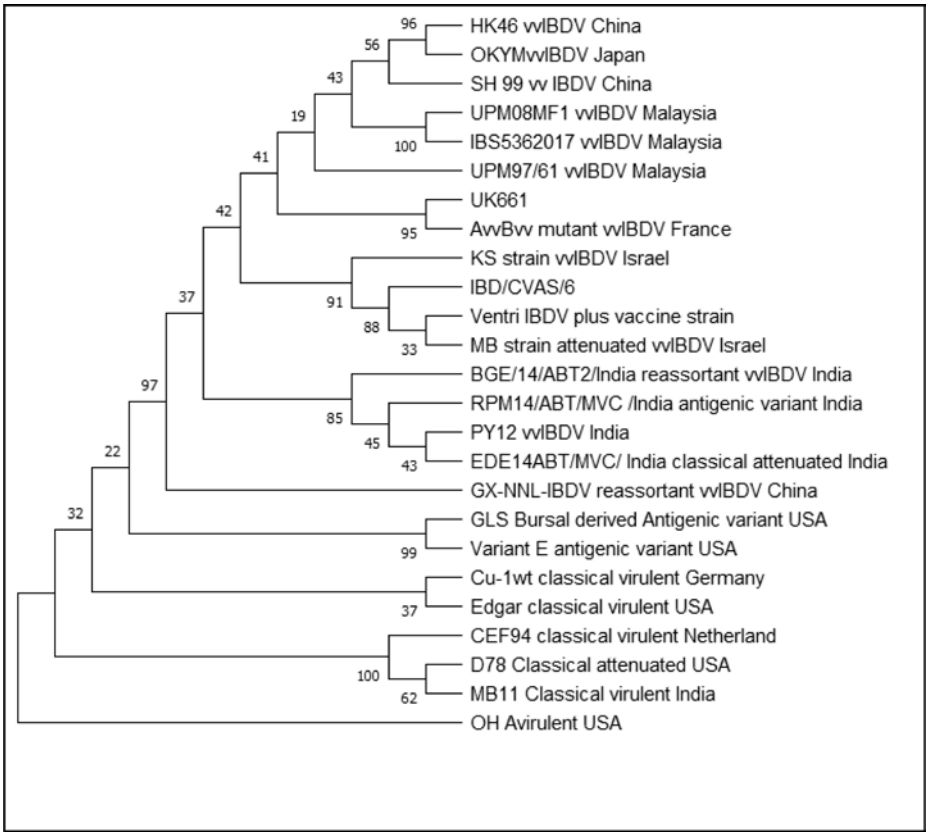

Fig.5. Phylogenetic tree based on the analysis of 1332 bp nucleotides of VP2 gene

genogrouping (Wang *et al*., 2019). The present study aimed at the molecular characterisation of the obtained IBDV with respect to VP1, VP2 and VP3 genes so as to identify any novel variations that may influence the virulence of the virus or may cause vaccination failure.

Following a complete sequence analysis of the coding strands of the three important genes, VP1, VP2, and VP3, it was found that the VP1 and VP2 genes are not responsible for the observed virulence of the virus studied. The only variation that differentiated the virus IBD/CVAS/6 from the attenuated very virulent vaccine strain was noticed in the VP3 gene. According to Lazarus *et al*. (2008), the amino acid variations responsible for the attenuation of ks (very virulent) strain to mb strain was T96N and D161A (VP1), I272T (VP2), and P527T (VP4). All these changes are conserved in IBD/CVAS/6 and the only variation noticed is the S745N variation in VP3 gene. But, with the limited study conducted, it is not possible to suggest that the single amino acid substitution at S745N could have a role in the change in virulence of the attenuated vaccine strain virus as 745N is not a novel variation and is noticed in other strains of IBDV. This shows the significance of characterisation of VP4 gene also. Hence, further characterisation studies involving the complete genome of the virus is necessary to substantiate the underlying condition. Moreover, the occurrence of outbreaks in vaccinated flocks is an important issue of concern and can be attributed to the failure of proper vaccination strategies as well as to the employment of inefficient vaccines. Untimely vaccination owing to the persistence of maternal antibodies in the vaccinated flocks can hinder the development of protective immunity in the vaccinated flocks. Furthermore, failure to administer adequate quantity of vaccine, as well as vaccine administration in immunocompromised or stressed birds, can result in vaccine virus induced disease. The use of vaccines developed with inadequately passed viruses may be another reason for vaccine failure. This study exacerbates the

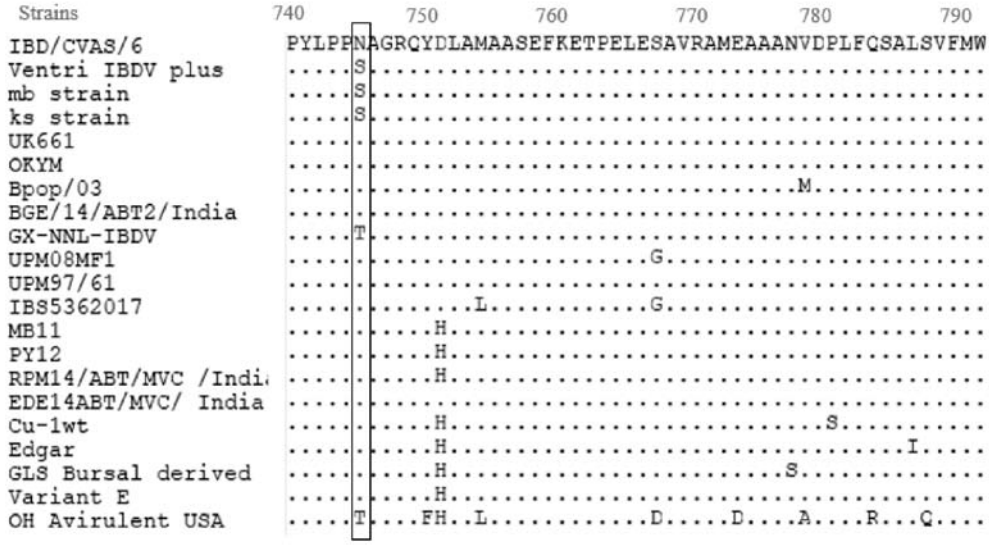

Fig. 6. Amino acid sequence analysis of VP3 gene showing variation 745N in IBD/CVAS/6 on comparison with different strains of virus (table 4)

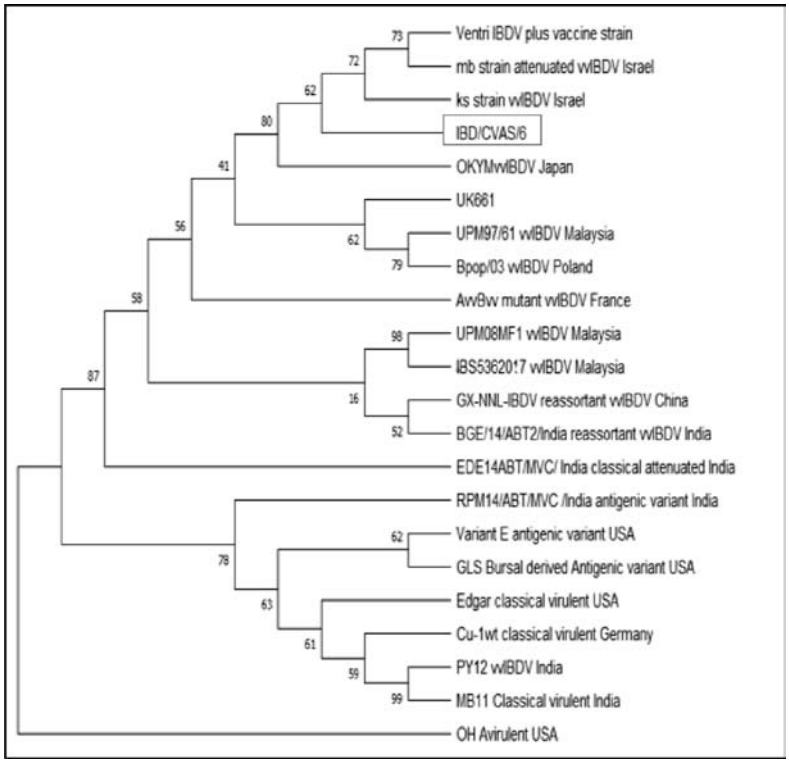

Fig.7. Phylogenetic tree based on the analysis of 705 bp nucleotides of VP3 gene

importance of considering a more advanced vaccination strategy, which could provide a fairly safe and efficient protective umbrella for the susceptible birds against IBD.

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Conflict of interest

The authors declare that they have no conflict of interest.

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