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Molecular characterization and phylogenetic analysis of vIL8 oncogene of Marek's disease

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Abstract
The present study was carried out with the objective to detect and analyse one the important viral oncogenes (vIL8) sequences of field Marek's Disease Viruses. Despite vaccination, field isolates of MDV-1 tends to increase in virulence, developing symptoms of MD with obvious visceral lymphoma making Marek's Disease an economically important viral disease of chickens. vIL8 gene being one of the important oncogenic genes of MDV-1 was amplified and sequenced. Homology comparison of the nucleotide sequences 5 isolates and other reference was found to be 99.1 – 100%. High nucleotides and amino acids identities between isolated MDVs and the reference MDVs were observed, indicating the genetic stability of this gene. The present study indicated that virulent oncogenic MDV-1 is circulating in this region and the point mutation which occurs in the vIL8 gene may be one of the factors responsible for increasing the virulence of MDV and thereby vaccine failure. Few point mutations were observed and these mutations could be considered as the unique markers of the MDVs circulating presently in Punjab.

Keywords: Marek's disease virus, field isolates, vIL8 gene, Punjab India, chicken, PCR

Introduction
Marek's disease virus (MDV: family, Herpesviridae; subfamily, a-Herpesvirinae; genus, Mardivirus; species, Gallid herpes virus 2) is the causative agent of Marek's disease (MD), characterized by lymph proliferative and neuropathic syndromes. Despite vaccination at the hatchery level, MD outbreaks have occasionally been reported in various Indian states (Orissa, Andhra Pradesh, Punjab, Assam, Uttar Pradesh, Arunachal Pradesh, Tripura, and Gujarat (Ramasamy *et al.* 2021; Arulmozhi *et al.* 2011) [1-2]. Marek's disease presents a serious threat to poultry production, responsible for a huge economic loss in the poultry industry, not only in India but all over the world Song *et al.* 2022 [3].

Marek's disease virus (MDV) is an oncogenic cell-associated herpes virus with 3 serotypes, among which serotype 1 (MDV-1) is the oncogenic strain causing MD, whereas serotype 2 (MDV-2) and serotype 3 (MDV-3) are considered non-oncogenic. Several genes unique to MDVs have been identified (Afonso *et al.* 2001; Izumiya *et al.* 2001; Lupiani *et al.* 2001; Lee *et al.* 2000) [4-7] which include Meq (Marek's EcoQ), latency-associated transcripts(LATs), vIL-8, viral lipase, pp38/pp24, telomerase RNA(vTR), MDV-encoded microRNAs, and the 1.8 kb gene family (Saif and Barnes 2008) [8]. While some of these genes are unique to MDV serotype 1, others may also be found in HVT and/or MDV serotype 2. Meq, vIL-8, and pp38 genes were shown to be the ones with the highest likelihood of being linked to both viral oncogenicity and pathogenicity (Bertzbach *et al.* 2020; Tian *et al.* 2011) [9, 10].

Viral interleukin-8 (vIL8), a spliced gene with broad similarity to IL-8, is present in the MDV genome. MDV-encoded vIL8 plays an important role as a potent chemo attractant and may promote the recruitment of activated T cells to the site of infection (Liu *et al.* 1999; Parcells *et al.* 2001; Cui *et al.* 2004) [11-13], facilitating the switch of infection from B cells to T cells. This viral chemokine has the power to draw immune cells to the infection site. Tumour incidence and MDV pathogenesis are both significantly impacted by the deletion of vIL8 from open reading frames (Parcells *et al.* 2001; Cui *et al.* 2004; Engel *et al.* 2012; Liao *et al.* 2021) [12-15]. During the catalytic phase, no cell-free virus is produced; however, the interaction between infected cells facilitates MDV spread from cell to cell, with virus replication peaking between 4 and 7 dpi (Yunis *et al.* 2004) [16]. The host response mounts after initial virus infection and replication and plays a significant role in the induction of the latent phase from the productive catalytic phase around 7-8 d.p.i. (Yunis *et al.* 2004) [17]. Therefore, finding field MDVs and analysing the gene sequences in those MDVs can be very helpful in illuminating their molecular characteristics.

Material and Method

Tissue samples were collected from suspected cases of Marek's disease in adult vaccinated poultry birds brought for disease diagnosis to the Department of Veterinary Pathology, GADVASU, Ludhiana, and Punjab. Tissue samples of the liver, spleen, heart, kidneys, lungs, brain, proventriculus, and peripheral nerves were collected from 116 morbid birds of different age groups from 21 vaccinated poultry farms located in Punjab. These birds were diagnosed on necropsy as having neoplastic lesions in different visceral organs.

DNA was isolated from all the samples using the Wizard® Genomic DNA Purification Kit (Promega, USA). The isolated DNA was stored at -20 °C until further use.

PCR amplification of vIL8 oncogene

Conventional PCR was performed using the vIL8 gene primer. Forward: 5'-GAGACCCAATAACAGGGAAATC-3' and reverse: 5'-TAGACCGTATCCGTGCTCCATC-3', targeting 886 bp as per Tian *et al.* 2011 [10].

A 25µlreaction mixture of 12.5µl of 2X Master Mix (Qiagen), 1µl each Forward and Reverse primers (10 pmol), NFW 5.5µl, template DNA 5 µl (150 ng/µl) and thermal cycling conditions were used as follows 94 °C for 4 mins, 35 cycles at 94 °C for 1 min, 56 °C for 1 min, 72 °C for 1.5 mins and final elongation at 72 °C for 10 mins. The PCR product was analyzed in 1% ultra-pure agarose in Tris base-acetic acid-EDTA (TAE) buffer gel containing 0.5 mg/ml ethidium bromide.

DNA cloning and sequencing

The PCR products were gel-purified (Wizard SV Gel and PCR clean-up System) and cloned into pGEM-T easy vector (Promega, USA) according to the manufacturer's protocol. The ligation mixture was transformed into One Shot TOP10-competent *E.coli* cells (Invitrogen) and grown overnight on agar plates containing ampicillin/ IPTG, and Xgal. The positive clones were identified using blue-white screening and then screened by colony PCR. The selected clones were grown overnight in Luria Bertani (LB) broth in a shaker incubator kept at 37 °C. The plasmid containing the insert was purified by alkaline lysis method (Sambrook and Russel 2001) [18] and the full length oncogenic of 5 field isolates of MDV were sent for sequencing to Eurofins Genomics India PVT Ltd, Bangalore. The sequences obtained were analyzed by using different bioinformatics tools like DNASTAR, ClustalW, and Mega6 to determine the nucleotide and amino acid sequence similarity as well as phylogenetic analysis of the sequences in relation to Genbank available MDV vIL8 gene sequences

Sequence analysis of oncogenic genes

Only a few researchers had done a study on vIL8. The reference sequences used for comparison were retrieved from the Gen Bank database, and accession numbers are shown: KT272877, SD2012-1(vvMDV) (KC511812), CVI988 (attMDV) (DQ530348), RB1B (vvMDV) (EF523390), CU-2 (vMDV) (EU499381), 584 (vv+MDV) (DQ534532), 648 (vv+MDV) (DQ534534), Md5 (vvMDV) (AF243438)

Results and Discussion

PCR amplification for vIL8 gene:

A PCR based on the primer vIL8 gene as per Tian *et al.* 2011 [10] was used. The complete sequence of the vIL8 gene was successfully amplified (Figure. 1).

Sequence analysis:

The nucleotide sequence and their deduced amino acid sequences of the 5 vIL8 genes were obtained and submitted to the GenBank database with accession numbers KY651241, KY651242, KY651243, KY651244, and KY651245. They were named GADVASU-M1, M2, M3, M4, and M5.

Nucleotide Sequence Distance Analysis

Comparison of the nucleotide sequence of the vIL8 genes of the 5 GADVASU with the 8 reference strains (KT272877, SD2012, CVI988, RB1B, CU2, 584, 648, and Md5) showed nucleotide homology between 99.1% and 100%. GADVASU isolates have the highest homology with KT272877 (100%) from a south Indian isolate, followed by SD2012 (99.7%) from a Chinese isolate. The lowest homology (99.1%) was with isolates from the USA, i.e., 584 (vv+MDV), 648 (vv+MDV), and Md5 (vvMDV) (Figure 2).

Alignment Analysis/ Report of nucleotide

The overall length of vIL8 for all 5 GADVASU isolates was 678 bp (nucleotide). As for the vIL8 gene, the nucleotide sequence alignment of the 5 isolates along with the 8 reference strains showed few mutations at positions 11 (T to C), 228 (T to C), and 267 (A to G), which were also found in the other Indian isolate (KT372877) and the China isolate (SD2012-vvMDV). GADVASU-M1 showed mutations at positions 595 (G to C) and 616 (G to A) (Table 1).

Phylogenetic Analysis

A phylogenetic tree based on the comparison of vIL8 gene sequences of the 5 GADVASU isolates with KT272877, SD2012, CVI988, RB1B, CU2, 584, 648, and Md5 showed that the first clade or cluster comprises the Indian isolates, and the second cluster showed that there was a close resemblance with the China isolate (SD2012-vvMDV). The isolates from the USA, i.e., 584A and 648 (vv+ MDV), as well as CU2 (vMDV) and RB1B (vvMDV), formed a different cluster (Figure 3).

Viral interleukin-8 (vIL8) is a spliced gene harboured in the MDV genome and has general homology to IL-8. MDV-encoded vIL8 plays an important role as a potent chemo-attractant and may promote the recruitment of activated T cells to the site of infection (Liu *et al.* 1999)[11], facilitating the switch of infection from B cells to T cells. MDV pathogenesis and cancer incidence are significantly impacted by the deletion of vIL8 from open reading frames ((Parcells *et al.* 2001; Cui *et al.* 2004; Engel *et al.* 2012; Liao *et al.* 2021) [12-15]

Although MD vaccines of both HVT and HVT+SB1 have been widely used in India for a long time, failures of MD vaccination and outbreaks occur frequently. The emergence of Marek's disease virus with increasing virulence is a significant problem for the poultry industry. In this study, homology comparisons of the nucleotide and deduced amino acid sequences of the 5 vIL8 genes with other reference strains were conducted, and high nucleotide and amino acid identities were observed, which indicates the genetic stability of the gene of study, the vIL8 gene (Liao *et al*. 2021; Pratt *et al*. 1994; Yamaguchi *et al*. 2000; Gimeno *et al*. 2005; You *et al*. 2021) [15, 19-22].

Table 1: Comparison of Amino Acid substitutions in vIL8 nucleotide sequences

vIL8 (Nucleotide sequence)	Origin	Virulence	Position					
			11	164	228	267	595	616
GADVASU-M1	India		C	G	C	G	C	A
GADVASU-M2	India		C	G	C	G	G	G
GADVASU-M3	India		C	G	C	G	G	G
GADVASU-M4	India		C	G	C	G	G	G
GADVASU-M5	India		C	G	C	G	G	G
KT272877	India	N/A	C	G	C	G	G	G
SD2012-1	China	vvMDV	C	G	T	G	G	G
584A	USA	vv+MDV	T	C	T	A	G	G
648A	USA	vv+MDV	T	C	T	A	G	G
CU-2	USA	vMDV	T	G	T	A	G	G
CVI988/Rispens	Netherlands	attMDV	T	G	T	A	G	G
Md5	USA	vvMDV	T	C	T	A	G	G
RB1B	USA	vvMDV	T	G	T	A	G	G

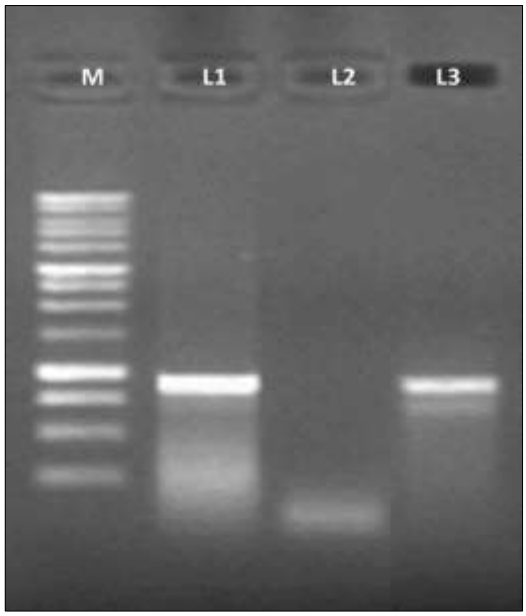

Fig 1: Agarose gel electrophoresis of PCR-amplified vIL8 gene (886 bp) M: DNA 1KB ladder, L1: Positive control, L2: Negative control, L3: positive sample

Conclusion

The study indicates that vIL8 does play a role in viral reactivation in the latent period and cell transformation. Few mutations have occurred in the oncogenic vIL8 genes, which could be one of the many reasons for the outbreak in vaccinated flocks. This study does provide additional information on Marek's disease. A future study on molecular epidemiological studies of the MDV isolates from different regions is required, which will then help in choosing a better immunization protocol so as to prevent the MDV infection in the field.

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