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Isolation, Cloning and *In silico* Study of Hexon Gene of Fowl Adenovirus 4 (FAV4) Isolates Associated with Hydro Pericardium Syndrome in Domestic Fowl

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Abstract

Hydropericardium syndrome is an important emerging disease of domestic fowl caused by fowl adenovirus serotype 4 (FAV-4). The full length hexon gene was isolated by PCR from the three virus isolates and it has been possible to clone hexon gene from all the three isolates into pGEM-T Easy vector, sequenced and analyzed using online bioinformatics tools (ExPASy server). The sequences were submitted to NCBI Gen Bank. These sequences showed significant homology with FAV-4 hexon gene sequences available in the NCBI Gen Bank and also found to be clustered with FAV-9 and FAV-10 serotypes. Probable secondary structures were also studied and compared using on line ExPASy servers. Possible B-cell epitopes were also predicted using on line ABCpred server and their position on the 3D structure of the proteins were also studied.

Introduction

Poultry keeping in India has emerged as one of the fastest growing sector of Indian agriculture. Consequent to intensification, many non-existent diseases have emerged and some of the existing has appeared in altered form, *viz.*, very virulent infectious bursal disease (vvIBD), hydropericardium syndrome (HPS), chicken infectious anaemia (CIA), egg-drop syndrome 76 (EDS-76), reoviral infections and infectious bronchitis. Of these, HPS is an important emerging disease occurring in specific areas of world where broilers are reared under intensive conditions. Hydropericardium syndrome (HPS) is caused by fowl adenovirus 4 (FAV-4), which belongs to genus *Aviadenovirus* of family *Adenoviridae* [14]. The unique features of the disease are sudden onset of high mortality (>70%), hydropericardium, hepatitis with basophilic intra-nuclear inclusion bodies in the hepatocytes. Initially reported from the Angara Goth, Pakistan [9,10], the disease was first noticed in India during 1994 in poultry belt of Jammu and Kashmir [16] and thereafter, outbreaks of the disease have been reported from almost all part of the country [3,11] causing heavy economic losses to the poultry industry. FAV-4 is non-enveloped and icosahedral in shape measuring 70-90 nm in size. The virion is composed of an outer protein shell and the viral core that contains a double stranded, linear DNA of 33 to 45 kb in size, as genome. The icosahedron viral core comprises of 252 capsomeres with 12 pentons and penton attached fibers located at the vertices and 240 hexons on 20 faces and 30 edges.

In spite of considerable research conducted on HPS, it is not enough to meet the requirements to control the disease. The identification and characterization of structural proteins of FAV-4 prevalent in a geographical area is a necessary prerequisite to investigate possible variation in polypeptide composition and to understand their immunogenic property, which may in turn help in the development of suitable immunodiagnostic and immunoprophylaxis. The major structural proteins are the hexons and pentons. Hexon, being the major immunogenic protein and harbouring the type, group and sub-group specific determinants, is of prime importance in molecular epidemiology of the virus. Hence, the present study was performed to characterize the hexon gene from Indian isolates of FAV-4 after PCR amplification, cloning of the amplicons into the suitable vectors followed by phylogenetic analysis, primary and secondary structure analysis of the hexon protein.

Materials and Methods

Three virus isolates, used in the present study, were originally isolated from the natural outbreaks of hydropericardium syndrome and maintained in the Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Pantnagar. The virus isolates were designated as HPS-G, HPS-K and HPS-R. The details of outbreaks are given in Table 1.

All the three virus isolates were propagated in chicken embryo liver cell (CEL) culture, prepared from 14 to 16-days-old chicken embryos [1] and typed as FAV-4 by micro serum neutralization assay [6].

Viral DNA was extracted from the infected cell culture showing 70-80% CPE using Qiagen DNAeasy Kit as per manufacturer's recommendations. The primer sequence used for amplification of hexon gene from all the three isolates was MA1F 5' ATGTCAGCAGTAGGCGATTGTGT3' as forward primer and MA3R 5'CCCGTCATGCCGTCGCTCTAA3' as reverse primer having Tm of 56°C. The amplified products were analyzed on agarose gel and gel eluted by using QIAquick Gel Extraction Kit, Qiagen, Germany. The amplified products were cloned in pGEM-T Easy vector (Promega, USA) as per the manufacturer's recommendations. The recombinant clones were screened by PCR and confirmed by restriction digestion with *Pst*I enzyme. Each clone was sequenced using T7 and SP6 primers

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at the DNA sequencing facility of University of Delhi South Campus, New Delhi.

The sequenced hexon genes were subjected to homology search using BLAST and FASTA tools of NCBI (http://www.ncbi.nlm.nih.gov) [13]. The nucleotide sequences were submitted to NCBI GenBank database and assigned accession numbers EU177544, EU177545 and EU177546 to HPS-G, HPS-K and HPS-R, respectively. The amino acid sequences along with the other hexon sequences present in the Database (Table 2) were aligned using clustalW [18] and phylogenetic tree was constructed using MEGA4.0 [17].

The amino acid sequences were imported to ExPAsy server (http://www.expasy.org/tool/) for primary structure analysis. These sequences further analyzed by Pfam [7] (http://www.pfam.janelia.org/cgi.bin/) for functional domain and protein family study and PDBsum server (http://www.ebi.ac.uk/pdbsum) for secondary structure prediction and analysis. For structural comparison, all the three protein sequences were modelled using homology modeling tool, SWISSMODEL. Further the protein sequences were analyzed for the prediction of B-cell epitopes using ABCpred server (www.imtech.res.in/raghava/abcpred) using neural network. The vaccine candidate characterization of hexon was done using Vaxijen computational tool.

Result and Discussion

Efforts were made to isolate and clone full length hexon gene from the three FAV-4 field isolates by using specific primers. A single amplicon of 3kb was observed from all the isolates (Figure 1). All the three amplicons were gel eluted, purified and cloned.

S. No.	Isolate	Year and Location	flock size	% mortality	Age
1.	HPS-R	1999,Haldwani, Uttarakhand, India	3000	66	6 weeks
2.	HPS-K	2002,Haldwani, Uttarakhand, India	2000	60	1 week
3.	HPS-G	1998,Gurgaon, Haryana, India	40000	50	3 weeks

Table 1: Details of outbreaks from where isolates were recovered.

Virus serotypes	Accession number
FAV-1(CELO)	Z67970
FAV-2 strain 685	AF508947
FAV-3 strain SR49	AF508948
FAV-4 strain KR-5	AF508951
FAV-4 strain VR-829	AF339917
FAV-4 Banglore	AF154246
FAV-4 Izatnagar	AJ459805
FAV-4	AJ431719
FAV-5 strain TR-22	AF508953
FAV-6 strain VR-831	AF339921
FAV-7 strain VR-832	AF339922
FAV-8 strain 58	AF508956
FAV-9 strain VR-834	AF339923
FAV-10	U26221
FAV-11 strain C2B	AF508959
FAV-11 strain XII	AF339920
FAV-12 strain 380	AF339925
EDS-76	ESU63515
HAV-2	EU128938
HAV-5	AB601020
HAV-40	EU099395

Table 2: Accession numbers of the virus serotypes used in the study.

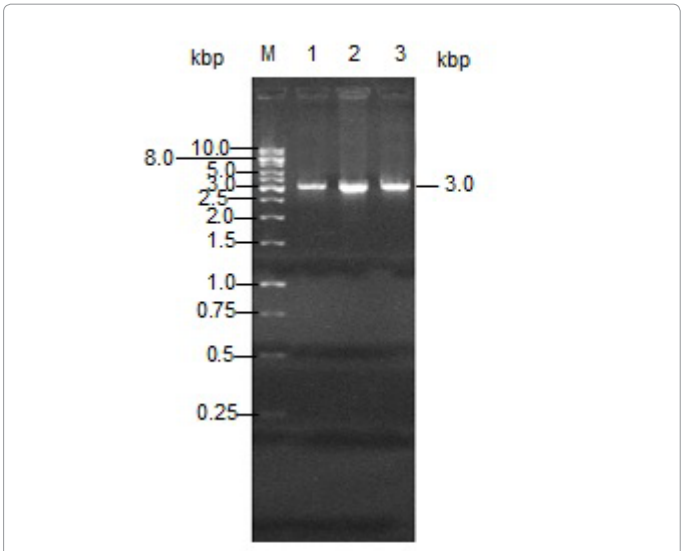


Figure 1: Amplification of hexon gene, Lane 1: HPS-K; Lane 2: HPS.R; Lane 3: HPS-G; M: 1kb DNA ladder.

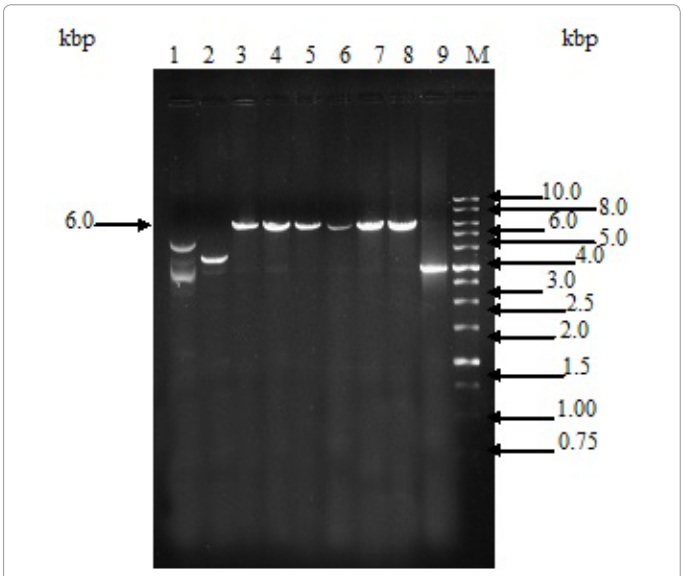


Figure 2: Showing Pst I digested plasmids, Lane 1: Undigested control plasmid, Lane 2: control plasmid; Lane 3 & 4: Digested plasmids from isolate G colony; Lane 5&6 Digested plasmids from isolate K colony; Lane 7&8 Digested plasmids from isolate R colony; Lane 9: 3kb PCR product, M: 1kb DNA ladder.

The recombinant colonies were screened for the amplification of the desired inserts by PCR using the same primer pair that was used to amplify the gene. Single amplified product of expected size was obtained with all the selected colonies. Plasmid DNA was isolated from each of the PCR positive colony and the recombinant plasmids were subjected to single digestion with *Pst* I enzyme as the size of the cloned gene (3kb) and that of the vector (3.015kb) was almost equal so it was not possible to detect the insert by using double digestion. All the recombinant plasmids showed a band of 6 kb in size, while control plasmid was showing a single band of 3 kb, which confirmed the insertion of the desired hexon gene amplicon into the vector (Figure 2).

The cloned hexon gene from all isolates were sequenced and

subjected to BLAST which showed more than 97% identity with FAV-4 strain VR-829. These sequences were submitted to NCBI and assigned the accession numbers EU177544, EU177545 and EU177546 for the isolates HPS-G, HPS-R and HPS-K, respectively. Sequencing results revealed a complete open reading frame (ORF) of 2914 bp from HPS-G and 2910 bp from HPS-K and HPS-R. The corresponding amino acid sequences of these three isolates were subjected to multiple sequence alignment and phylogenetic tree construction revealed clustering of all FAV-4 strains together along with FAV-10 and FAV-9 except FAV-4 Izatnagar isolate, which was grouped with FAV-11 strain C2B (Figure 3). The HPS isolates showed more than 96 % identity with FAV-10 and only 29.3 % with FAV-9 and were found to be clustered with FAV-10 and FAV-9. Sequence identity/divergence analysis based on nucleotide and deduced amino acid sequences of these isolates also supported the above conclusions. These facts indicate that FAV-4 is more closely related to FAV-10 but clustering of FAV-9 indicated that FAV-4 might have evolved by a recombination of FAV-9 and FAV-10, as also reported by Barua and Rai [2]. This may be due to the possible mechanism of illegitimate recombination, as reported by Crawford-Crawford-Miksza and Schnurr [4]. They also reported that the hypervariable regions of hexon gene are 'hotspots' for both, *i.e.*, single base mutation and illegitimate recombination.

Human adenoviruses initially clustered together but after some distance HAV-2 gets separated and HAV-5 isolate Ys/382/02 and HAV-40 Dhaka-731 was formed one cluster. EDS-76 formed a distant lineage.

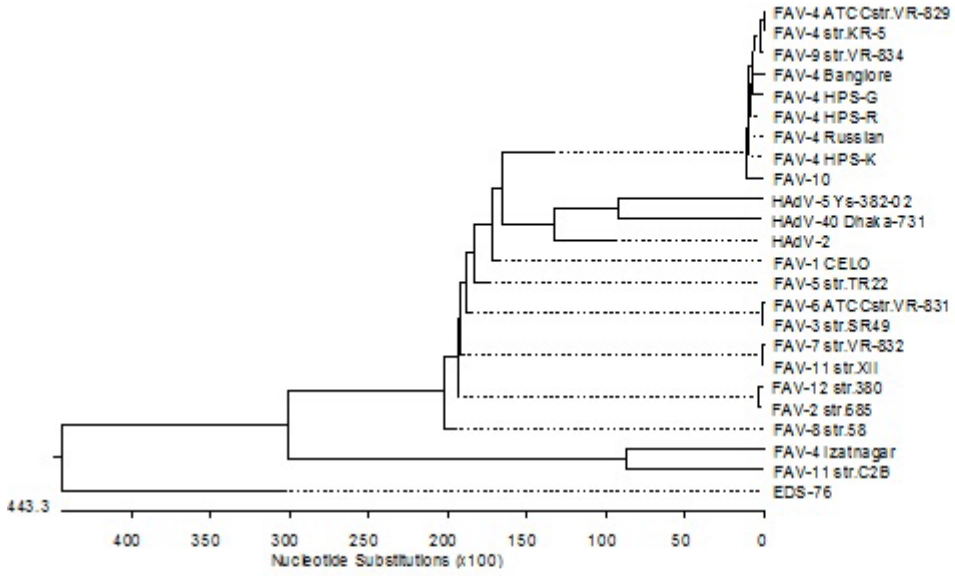


Figure 3: Shows phylogenetic tree based on amino acid sequence revealed clustering of all FAV-4 strains together along with FAV-10 and FAV-9 except FAV-4 Izatnagar isolate, which was grouped with FAV-11 strain C2B.

Virus isolate	Amino Acid	Mol. Wt (kDa)	Theoretical pI	Aliphatic Index	GRAVY
HPS-G	962	108.669	5.63	68.04	-0.408
HPS-R	962	108.689	5.53	67.53	-0.416
HPS-K	962	108.776	5.53	67.22	-0.428

Table 3:

[962 residues]

Domain	Start	End	Evalue
Adeno_hexon	75	567	0
Adeno_hexon_C	626	872	1.1e-181

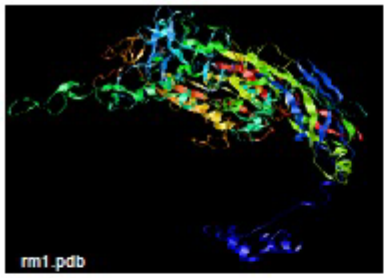
Figure 4: Showing the conserved domains of deduced hexon protein sequence.

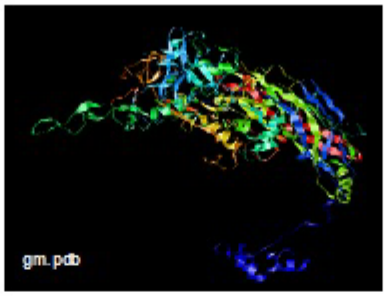
Virus isolate	TNF		Scytalone dh		PagP	
	Start	End	Start	End	Start	End
HPS-G	253	275	630	644	-	-
HPS-R	253	275	630	644	-	-
HPS-K	253	275	630	644	496	625

Table 4:

Virus isolate	Strand	α-helix	3-10 helix	Other	Total residues
HPS-G	213 (23.2 %)	102 (11.1 %)	42 (4.6 %)	563 (61.2 %)	920
HPS-R	213 (23.2 %)	102 (11.1 %)	39 (4.2 %)	566 (61.5 %)	920
HPS-K	213 (23.2 %)	102 (11.1 %)	39 (4.2 %)	566 (61.5 %)	920

Table 5:





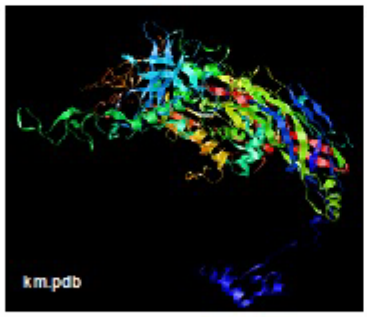


Figure 5: 3D structure of hexon proteins of the three isolates shown is Ras Windows.

Rank	Epitopes		
	HPS-G	HPS-R	HPS-K
1	AGEGYGPDLSQIKLYT QGPGRNPLRQVENANT	DLVSYTPTTDNSGQQP	DLVSYTPTTDNSGQQP QGPGRNPLRRVENANT
2	DLVSYTLTTDNSGQQP FAKSQYNYAYGAYVKP	AGEGYGPDLSQIKLYT QGPGRNPLRQVENANT	AGEGYGPDLSQIKLYT
3	NFHIQVPQKFFAIKNL TSVAPTTYEYMNKRVP QVTKISGVFPNPNQGP	FAKSQYNYAYGAYVKP	FAKSQYNYAYGAYVKP

Table 6: B-cell epitopes selected for the study.

Hexon protein analysis was done using computational biology. Amino acid sequences were deduced from the nucleotide sequences and imported to expasy server (http://www.expasy.org/tool/) for primary analysis, Pfam (http://www.pfam.janelia.org/cgi.bin/) for functional domain and protein family study and PDBsum server (http://www.ebi.ac.uk/pubsum) for secondary structure prediction and analysis. *In silico* translation and primary structure of the hexon protein were done using on line bioinformatics tools [8]. Theoretical pI, Aliphatic index and Grand average of hydropathicity (GRAVY) of hexon protein from isolate G, R and K are given in Table 3.

The instability index was computed to be 32.59 for isolate HPS-G, 31.93 for isolate HPS-R and 32.77 for isolate HPS-K and this classified the hexon protein as stable. The half-life of predicted hexon protein from all the three isolates was estimated to be 30 hours (in mammalian reticulocytes, *in vitro*), > 20 hours (in Yeast, *in vivo*) and > 10 hours

(in *E.coli, in vivo*). Information on primary structure supported the possibility of expression of hexon gene in *E.coli*.

Pfam analysis showed that the protein belong to Pfam-A family PD002815. Two major functional domains were found in the sequences under study: these are Adenohexon and Adenohexon C by trusted matches. The detailed results of the study are given in the Figure 4. The Pfam study also showed more domains other than these two major domains based on the potential match of the sequences. All the 3 entries showed two common domains: TNF and Scytalone dh, while isolate HPS-K showed one extra domain PagP. The details of the results are given in Table 4.

The results of the PDBsum and PROMOTIF studies provide information about the secondary structure of the deduced hexon proteins (Table 5). HPS-G isolate shows one extra helix than HPS-K and HPS-R due to the change of single amino acid at position 813 from proline to leucine, as proline acts as helix breaker. Homology Modeling of hexon protein from all the three different isolates was done by using SWISSMODEL. The software used human adenovirus 2 (HAd 2) hexon protein structure as template to generate the 3D structure of hexon proteins from the three different isolates of fowl adenovirus 4. The 3D structure of all the three isolates is shown in Ras windows (Figure 5).

Hexon is one of the major immunogenic proteins of the FAV-4 and produce type, group and sub-group specific antibodies. Therefore, it can be a good candidate for subunit vaccine. Keeping this point in mind the hexon was predicted as a subunit vaccine candidate using Vaxijen server with a score of 0.4865, 0.48590 and 0.4871 for HPS-G, HPS-R and HPS-K respectively showing it as probable antigen.The antigenic regions of protein that are recognized by the binding site of immunoglobulin molecules are called B-cell epitopes. Identification of B-cell epitopes on proteins is of vital importance for developing synthetic peptide vaccines, immunodiagnostic tests and antibody production. Pichla-Gollon et al. [12] recently mapped epitopes recognized by seven hexon-specific monoclonal antibodies in case of chimpanzee adenovirus 68 (AdC68). The prediction of promiscuous B-cell epitopes was done by using ABCpred server [15]. The prediction accuracy of this programme was 65.93%. The results were taken in tabular forms. The protein was analysed at a threshold setting of 0.5. Isolates HPS-G and HPS-R were showing 36 epitopes, while HPS-K isolate showed 37 epitopes. The highest scoring B-cell epitopes were chosen for the further study (Table 6) of their position onto the 3D structure of the protein, which was made by using SWISSMODEL server (Figure 6, 7). All the epitopes were found to be on the surface and hydrophilic in nature, which is the characteristic of the B-cell epitopes. The position of each epitope and its score was found to vary in each protein. This may be due to the difference in their secondary structure as discussed above.

Conclusion

Hydropericardium syndrome is an important emerging disease of domestic fowl caused by fowl adenovirus serotype 4 (FAV-4) with high mortality rate causing heavy economic loss to the poultry industry. In the present study, the major structural and immunogenic polypeptide *i.e.* hexon of the Indian isolates of FAV-4 was amplified,

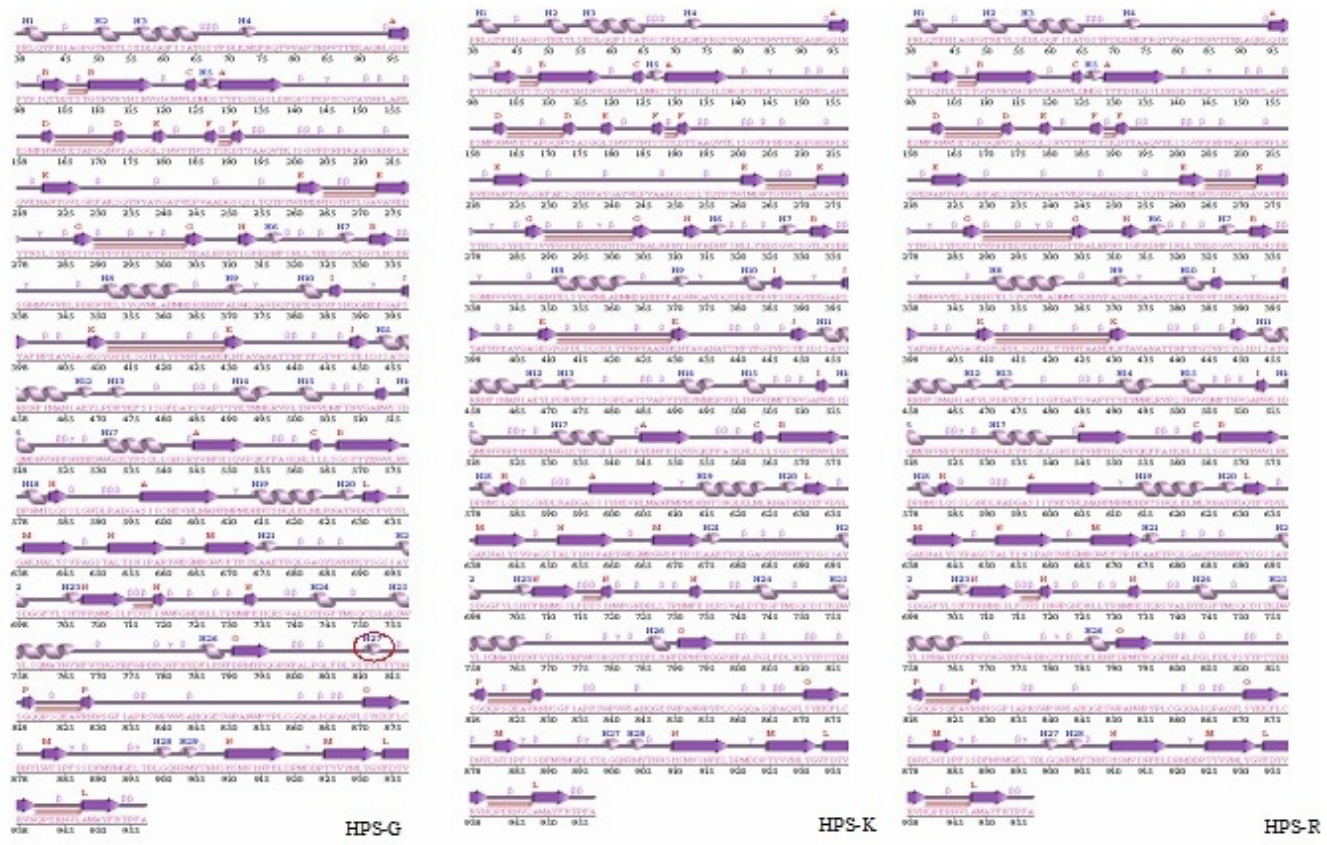


Figure 6: Shows comparative secondary structures after PROMOTIF study. Red circle shows the extra helix formed in HPS-G isolate.

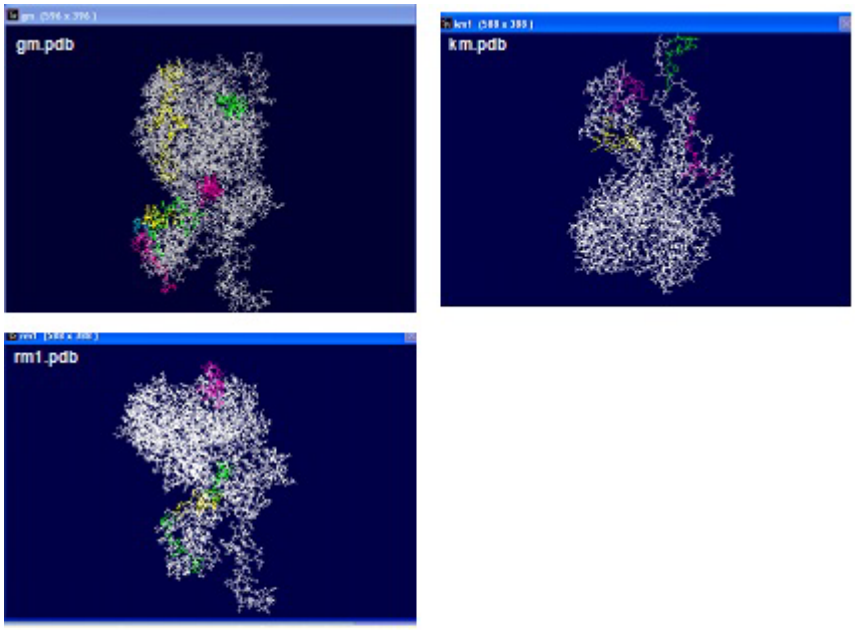


Figure 7: Shows the location of B-cell epitope in their respective protein. The epitopes having rank 1,2 and 3 are shown in pink, green and yellow colour, respectively. The parts of epitope shared by2 epitopes were shown in blue colour.

cloned, sequenced and studied by using various bioinformatics tools. Sequencing results showed an ORF of approximately 2.9kb. Phylogenetic analysis revealed clustering of these isolates with other FAV-4 Indian isolates. Primary and secondary structural studied showed that hexon protein is the member of Pfam-A family PD002815. From perusal of available literature, it appears that the highest scoring predicted epitopes using ABCpred server are reported first time for FAV-4 and could eventually be proposed as a component of a peptide-based vaccine. However, further validation is required through in-vitro synthesis of the determined peptides. Besides this, in-vivo experimental study has to be done, in order to, finally test the efficacy of the vaccine.

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