Sequence analysis of the glycoprotein envelope gene of duck enteritis virus

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Abstract : Duck enteritis virus ul35 gene was amplified from two strains and the nucleotide sequence alignment was done . Many substitutions were seen between the two sequences which resulted in minimum changes in the amino acids denoting the silent mutations. Several deletion mutations also seen in both sequences. The deduced amino acid sequence showed that the conservative domain belongs to the fusion _gly_k protein superfamily which is essential in the viral attachment and assembly. Dot blot analysis of each gene against itself showed no inverted repeats but analysis of the genes against each other revealed the presence of difference at the 3' end. Antigenicity profile of each protein using Kolaskar and Tongaonkar revealed some difference at amino acid position 80 – 110 which more antigenic profile of the UL35 antigen of the local strain. In conclusion the production of a DNA vaccine against this protein from the local strain would persumply potentiate the immune system to prevent the virus from attachment and /or release?

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1. Introduction :

Duck viral enteritis, also known as duck plaque virus (DPV) is an acute, contagious, and lethal disease of birds from the order Anseriformes (ducks, geese, and swans) (Davison et al., 1993). DPV can result in heavy economic losses to the commercial duck industry due to its high mortality rate and decreased duck egg production (Marlier et al., 2001 and Malmarugan and Sulochana 2002). The causative agent of DPV is duck enteritis virus (DEV), also named Anatid herpesvirus 1 (AnHV-1), which is a member of the family Herpesviridae. Herpesviridae is a family of large DNA viruses which have as their natural hosts' humans, other mammals and vertebrates, and in one described case, an invertebrate (Le Deuff et al., 1994 and Fauquet et al., 2005).

The genomes of herpes viruses of mammals, invertebrates, and birds clearly descent from a common ancestor, but with a great range of variation in terms of nucleotide substitution, gene content, and genomic arrangement (McGeoch and Davison 1999) . On the basis of differences in the cellular tropism, genome organization, and gene content, herpes viruses have been classified into three subfamilies: Alpha-, Beta-, and Gammaherpes-virinae (Albà et al., 2007). Avian herpes viruses were grouped into the subfamily Alphaherpesvirinae except for DPV, which was assigned as an unclassified virus within the family Herpesviridae (Fauquet et al., 2005) . Lack of a genome sequence and genomic organization information are factors that limit DPV taxonomy and currently, DPV has not yet grouped into any genus according to the Eighth International Committee on Taxonomy of Viruses (Fauquet et al., 2005 and Ming-Sheng et al., 2010). DPV genome is a linear double-stranded DNA that contains approximately 180 kb with a G+C content of 64.3%, the highest report for any avian herpes virus, the typical genome of an alphaherpesvirus is divided into unique long (UL) and unique short (US) segment that are flanked by internal and terminal repeats (IR and TR) (Gardner et al., 1993).

DPV is a large, enveloped virus with 4 structural components including a linear, double-stranded DNA genome with 64.3% GC content, an icosahedral capsid, an amorphous tegument and a bilayer lipid envelope. During the infection cycle, many herpesvirus proteins are involved in the late step of viral maturation at the nucleocapsid, which include the ul35 protein of herpes simplex virus (Chi and Wilson 2000). With many similarities and a few differences, accumulating evidence indicates that the protein encoded by ul35 gene and its homologues of Alphaherpesvirus, Betaherpes virus and Gammaherpes virus play similar roles in viral assembly and maturation.

The ul35 gene encode for the viral nucleocapsid proteinVP26 protein antigen which is one of the glycoproteins localized in the virion envelope, which played a major role in virus entry by mediating attachment of virions to cell-surface receptors and fusion of the viral envelope with the plasma membrane during penetration. Previous research (Cai et al., 2009) has shown that there is no signal polypeptide cleavage site in VP26 and the polypeptide chain of VP26 may contain a

transmembrane domain. However, there are 9 potential phosphorylation sites in VP26, including 6 serine phosphorylation sites, 2 threonine phosphorylation sites and 1 tyrosine phosphorylation site, which may be modified to a certain degree after translation and may play an on/off control role in the process of playing its biological functions, as well as acting as an important step for regulating the activity of enzyme or protein, since phosphorylation is a widely existing chemical modification after protein synthesis (Kishimoto et al., 1985) . The aim of this study was to conduct genetic analysis ul35 gene of DEV strains for future use as potential DNA vaccine.

2. Material and Methods

Virus strains:

Duck enteritis virus strains (two vaccinal strains one prepared from local isolate L and the other was imported I) were used in the current study. The strains were propagated onto chicken embryo fibroblast cell culture till 90% cytopathic effect (CPE) (Catherine et al., 2006) and the virus was harvested by 2 cycles of freezing and thawing followed by centrifugation at 3500 rpm for 20 min at 4°C. The virus was then stored at -20°C till used.

DNA Extraction:

The genomic DNA was extracted using MegaDNA Extraction kit (Bioflux) according to the manufacture instruction. Briefly, 300µl of the virus suspension was lysed at 56°C/15 min in the presence of proteinase K (10µg/µl) and the released DNA was captured on the magnetic beads provided with the kit in the presence of binding buffer. The magnetic beads were washed twice with the washing solution and one with 80% isopropanol to remove all pretentious contaminants. The purified DNA was released from the magnetic beads using 50µl of the elution buffer and stored at -20°C till used.

PCR amplification:

The ul35 gene was amplified using set of primer that were designed using DNAstare software version 9 UL35-F (5'-CGGGATTGTTTACGTTCTGTCG-3') and UL35-R(5'-ATTTCGCCCACTCGCTATCCTCTT-3').

PCR was performed in 50-µl reaction mixtures containing 25µL of green Dream Taq master mix (fermentase cat # K1071) and 100 pmol of each oligonucleotide primer. DNA samples (100ng) were pipetted thoroughly into the mix. Thermal cycling was performed using T professional thermal cycler (Biometra, Germany), parameters for amplification were denaturation at 95^{o}C for 3 min for one cycle and then 30 cycles at 95^{o}C for 1 min (denature), 64^{o}C for 30 sec, and 72^{o}C for 45 min (extension). A final extension at 72 ^{o}C for 10 min was also included (Sambrook et al., 1989). The amplicon was then visualized under U.V. transelumination after electrophoresis on 1% agarose. The size of the amplicons was analyzed in comparison to gene ruler 100pb plus DNA ladder (Fremantase cat # SM0323).

Sequencing:

The complete nucleotide sequences of the ul35 gene of the two strains were performed in (Macrogen USA). For preparation of the gene for sequencing, the PCR product was separated on 1% low melting agarose and electrophoresed on low voltage (20 volt) at 4°C. The bands were sliced off and purified with the biospin PCR purification kit (Biobasic cat # BSC03S1) as described by the manufacture. Briefly, the gel slices were melted at 60°C for 5 min, mixed with 500 ml of gel extraction buffer and placed on the biospin column provided with the kit, centrifuged at 4000 rpm/2min/4°C and washed twice with the washing solution. Finally the amplicon was eluted in 50 µL of the elution buffer and stored at -20°C till sequenced. Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using ABI PRISM 3730XL Analyzer BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme Applied Biosystems), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using the primer used for PCR amplification. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Analysis:

The PCR analysis was performed with gel pro, gel documentation system. The sequence analysis including nucleotide alignments, deduced amino acid prediction and alignment and antigenicity profile were performed using CLC workbench Version 6 for Windows 7 platform developed by CLC bio A/S. Antigenicity profile was analyzed using either welling method (welling et al., 1985) that relays on the information about the relative occurrence of amino acids in antigenic regions to make a scale for prediction of antigenic regions or Kolaskar and Tongaonkar method (Kolaskar and Tongaonkar et al., 1990) which is a semi-empirical method for prediction of antigenic regions depending on information of surface accessibility and flexibility.

3. Results

PCR amplification:

The ul35 gene was amplified form all the tested strains **(L and I). A** very clear band with nearly the same intensity was observed migrating at ~ 380 bp (Photo 1).

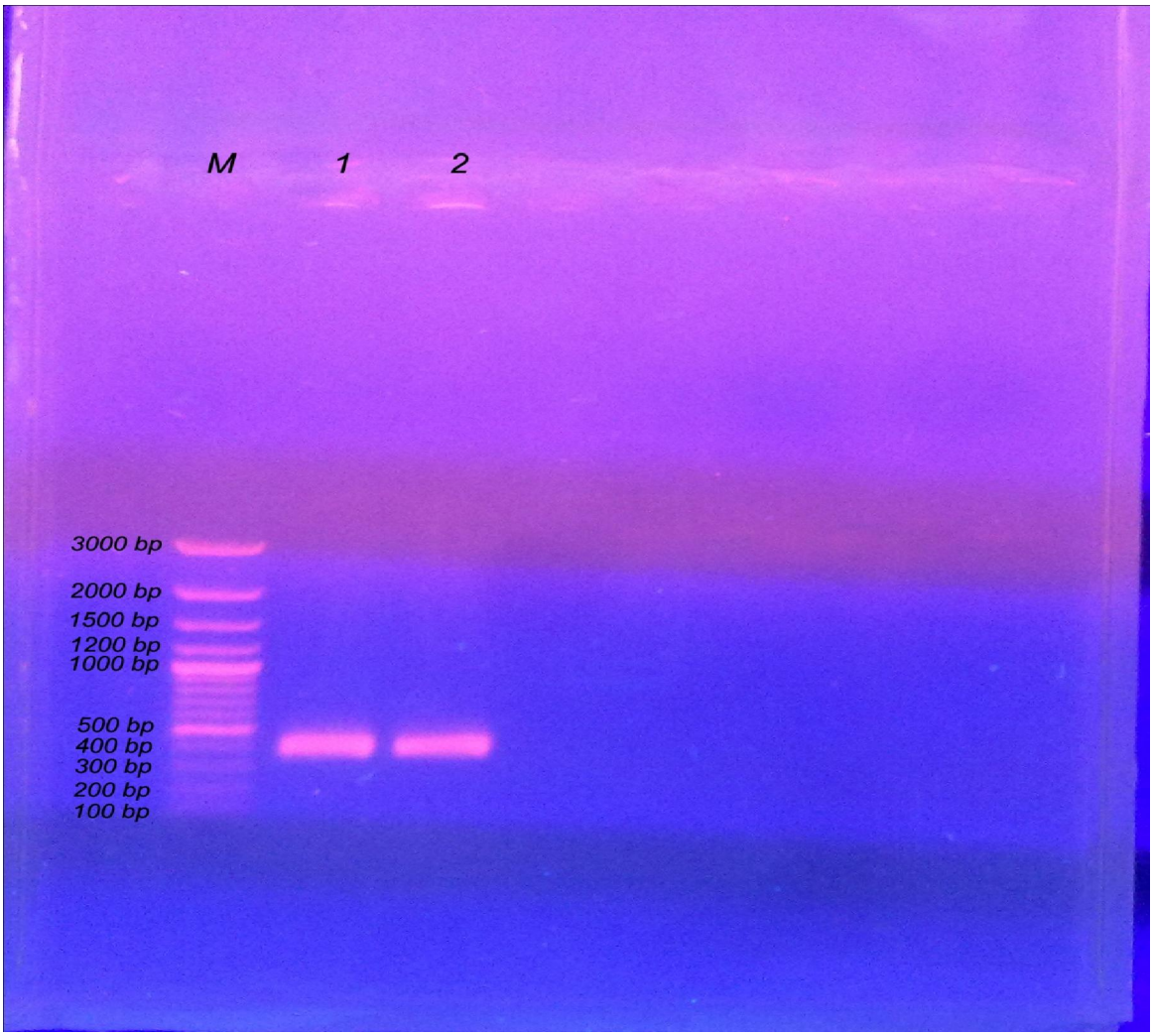

Photo (1) PCR results of amplification of *UL35* gene from ***DEV - L*** (lane 1), ***DEV-I*** (lane 2) note the clear band migrates about 380 bp (M is 100 bp DNA ladder)

Gene sequencing analysis:

The complete nucleotide sequence of the *ul35* gene amplified from the two strains was aligned using the CLC software - clustal *W*. As seen in Fig (1), many substitutions were seen which mostly accumulated at the 3' end of the gene these substitutions resulted in point mutation in the deduced amino acid sequence (fig 2). Deletion mutation also found in the sequence of both strains, at position 237, 238, 298, 361 and 362 for I strain and at 245, 288, 310, 321 and 322 for the L strain.

BLAST search (Table 1 and 2) revealed that the ul35 gene of both strains have several matches with identity of 93% with ul35 of *Anatid herpesvirus* 1 envelope glycoprotein K (UL53) gene and Duck enteritis virus strain VAC. BLAST n search revealed more identity (97%) with the same strains indicating that many substitutions resulted in silent mutations.

The conserved domain analysis of both strains revealed that the UL35 protein belongs to the fusion –gly-K superfamily (fig 3).

As shown in fig (4) no evidence of inverted repeats were found when each gene was analyzed against itself , on the other hand dot blot analysis of ul35 gene of L strain against I strain revealed the some differences at the 3' end of the genes.

Antigenicity profile using ***Welling*** methods could not explore any differences between the two protein antigens while using ***Kolaskar and Tongaonkar*** method could clarified the differences at position 80 – 110 (fig 5).

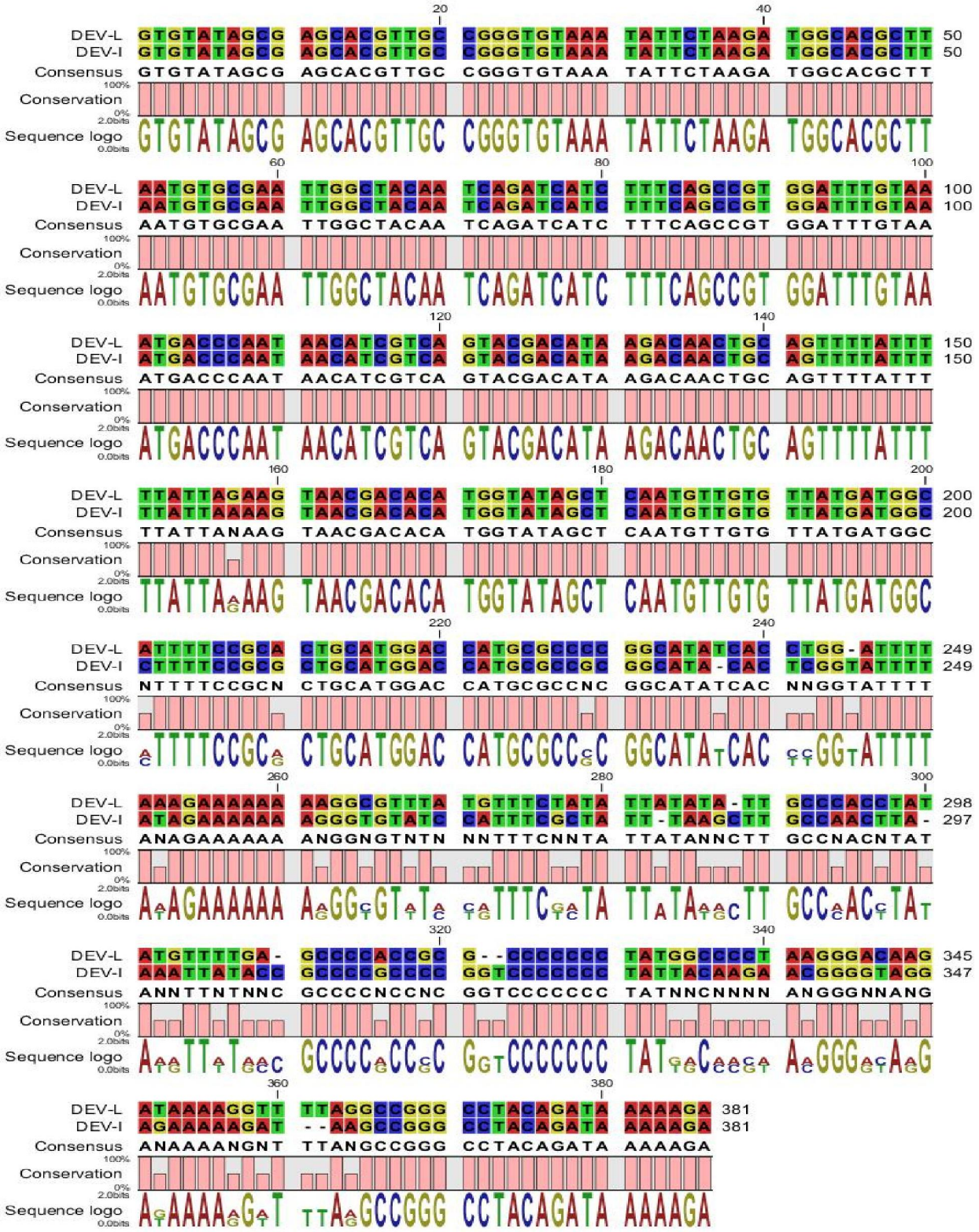

Fig (1) Sequence alignment of *UL35* gene of ***DEV – L and DEV-I*** and the nucleotide orthologs

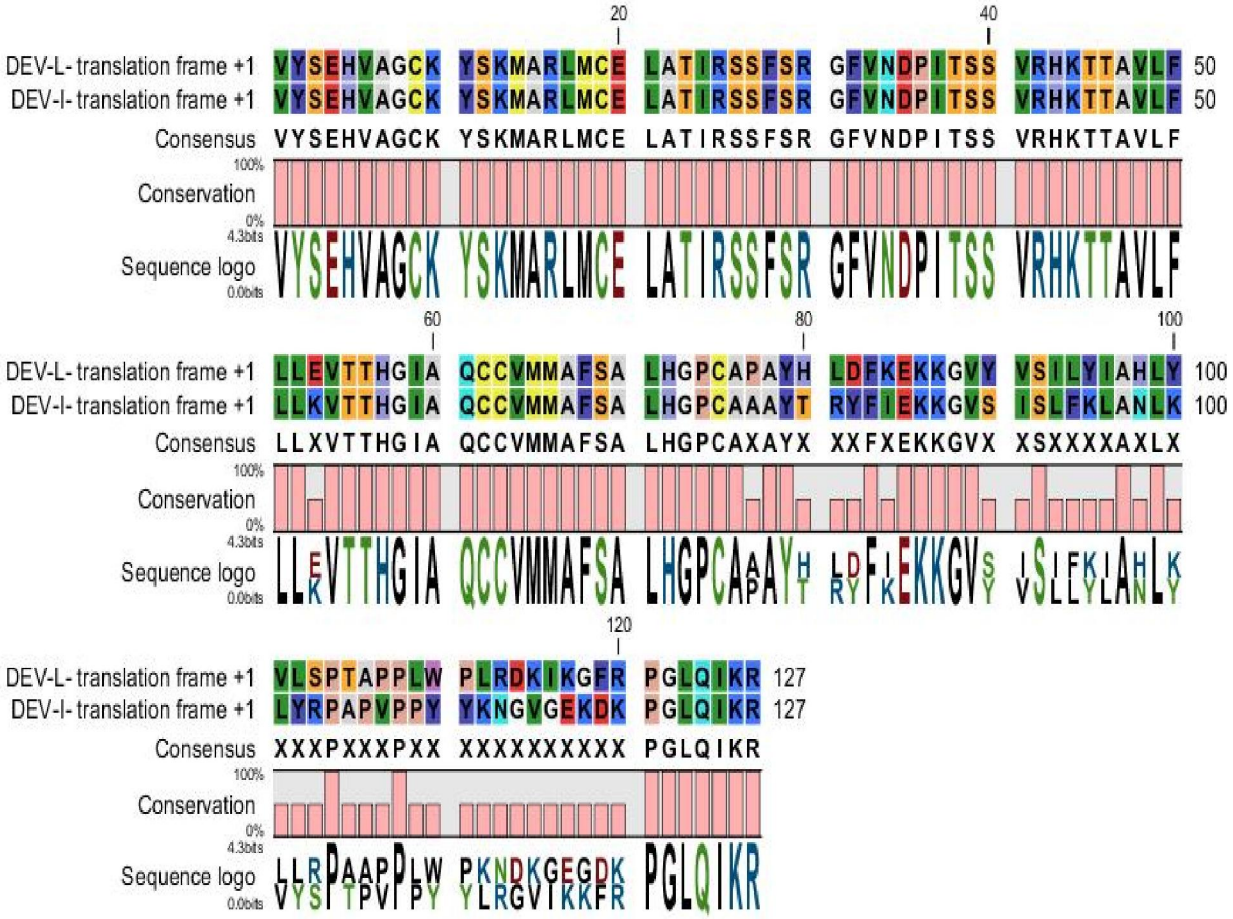

Fig (2) The deduced amino acid sequence alignment and the protein orthologs of the UL35 protein antigen of DEV-***L and DEV- I.***

Table (1) The BLAST-n search of *UL35* of ***DEV-L***

Sequences producing significant alignments:						
Accession	**Description**	**Max score**	**Total score**	**Query coverage**	**E value**	**Max ident**
EU071035.1	Anatid herpesvirus 1 envelope glycoprotein K (UL53) gene, complete cds	431	431	75%	2e-117	93%
EU082088.2	Duck enteritis virus strain VAC, complete genome	431	431	75%	2e-117	93%
EU294364.1	Duck enteritis virus strain DEV UL52 (UL52), virion glycoprotein K (UL53), UL54 (UL54), and UL55 (UL55) genes, complete cds	431	431	75%	2e-117	93%

Table (2) The BLAST-n search of *UL35* of ***DEV-I***

Sequences producing significant alignments:						
Accession	**Description**	**Max score**	**Total score**	**Query coverage**	**E value**	**Max ident**
EU071035.1	Anatid herpesvirus 1 envelope glycoprotein K (UL53) gene, complete cds	425	425	65%	8e-116	97%
EU082088.2	Duck enteritis virus strain VAC, complete genome	425	425	65%	8e-116	97%
EU294364.1	Duck enteritis virus strain DEV UL52 (UL52), virion glycoprotein K (UL53), UL54 (UL54), and UL55 (UL55) genes, complete cds	425	425	65%	8e-116	97%

Table (3) The protein statistics of both DEV-I and DEV-L translated protein of UL 35 gene.

strain	DEV –I	DEV - L
Sequence type	Protein	Protein
Length	127	127
Name	DEV-I- translation frame +1	DEV-L- translation frame +1
Weight	14.092	14.2
Isoelectric point	10	9.74
Aliphatic index	84.488	92.913

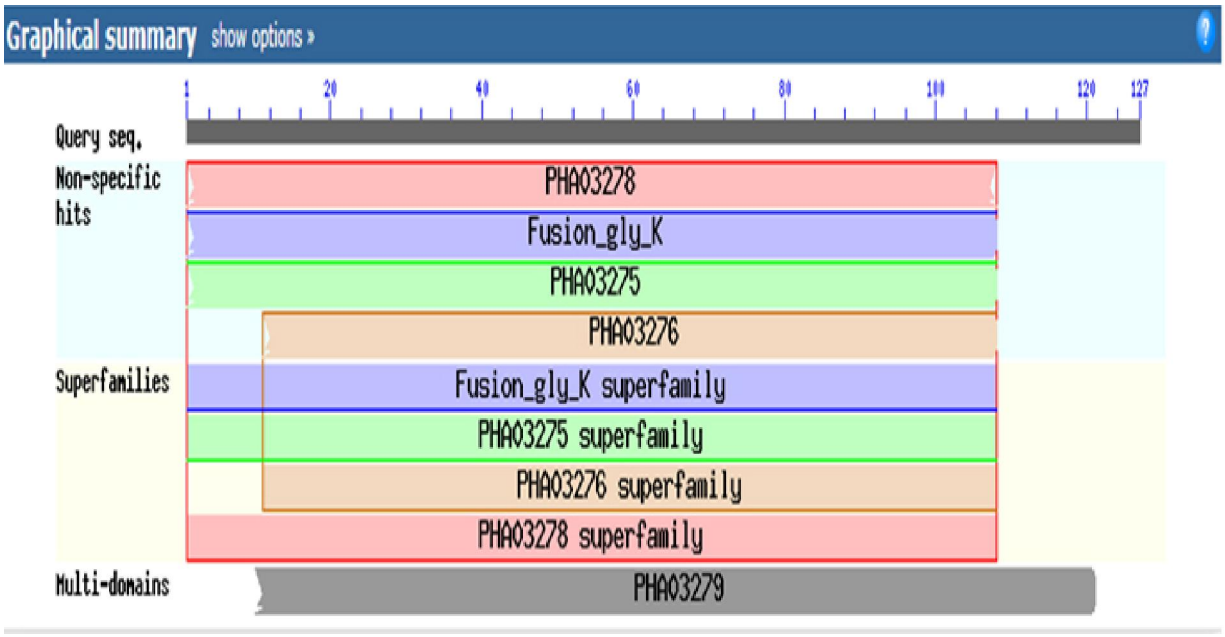

Fig (3) The conserved domain analysis of the UL35 gene which revealed that the UL35 protein antigen belong to the Fusion gly_K_ superfamily. This analysis was done using the online domain analysis (http://www.ncbi.nlm.nih.gov)

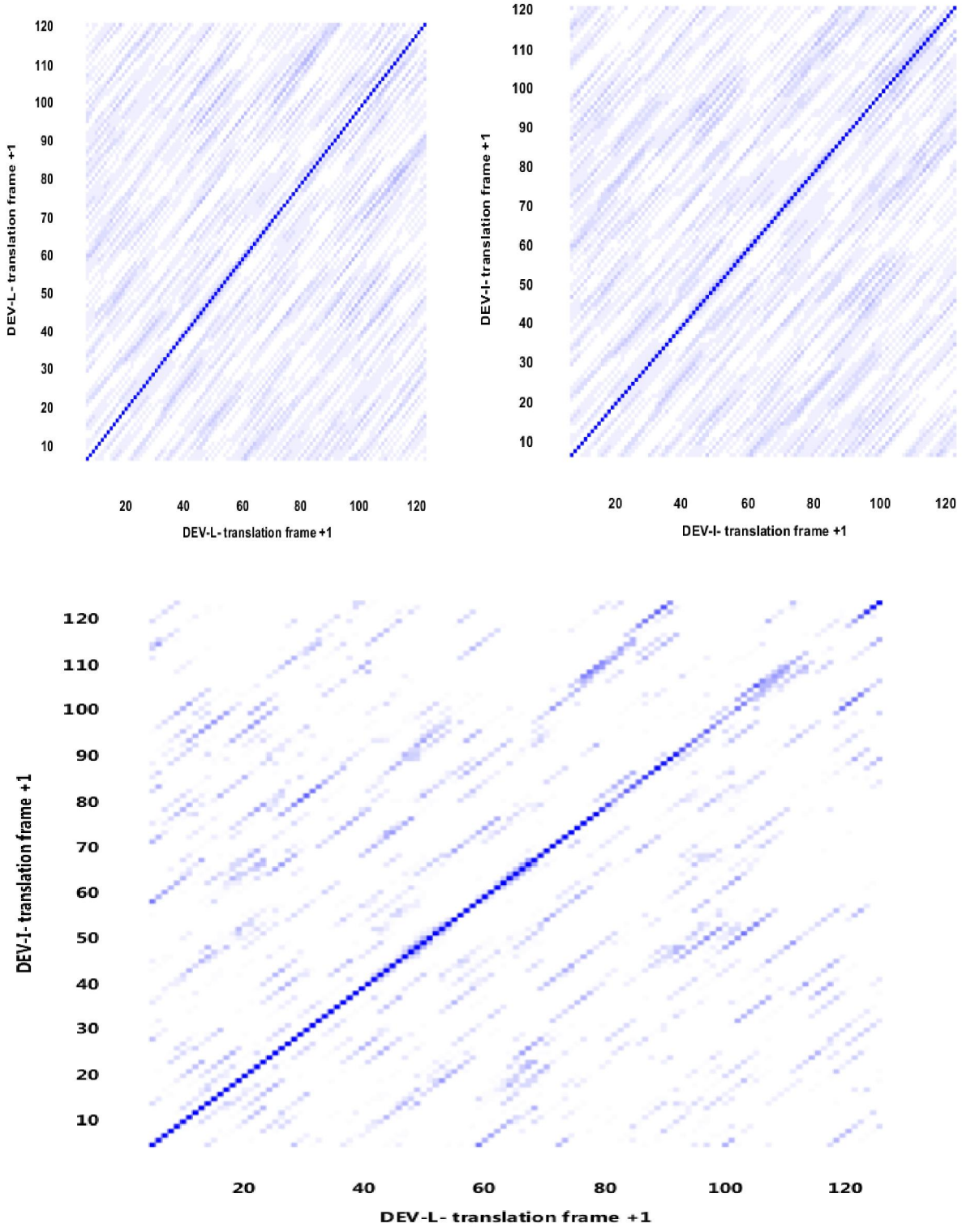

Fig (4) The Dot plot of the translated UL35 genes against itself of DEV-L (upper left) and DEV-I (upper right) and against each other (lower)

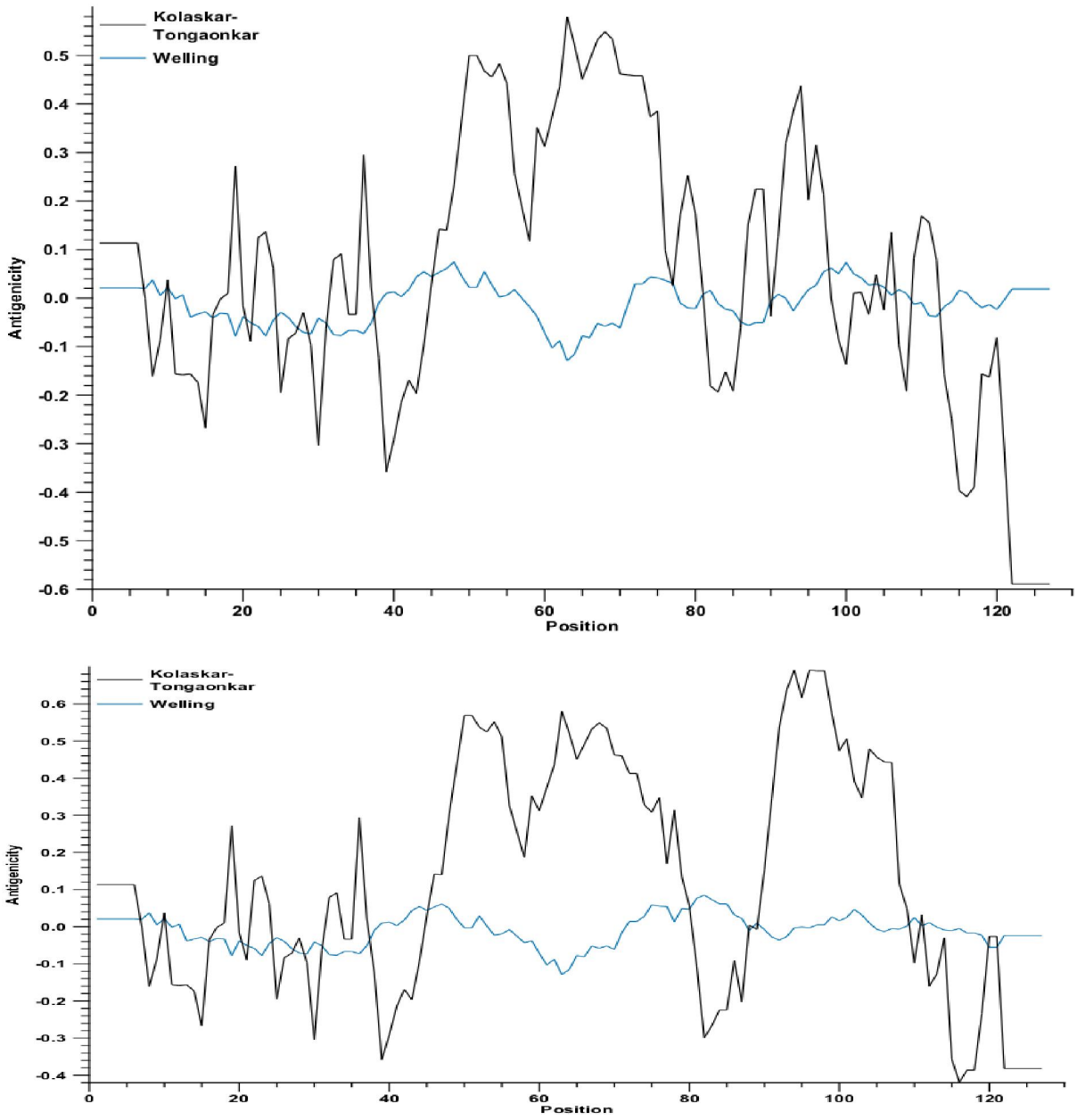

Fig (5) The antigenicity blot using both Kolaskar-Tongaonlar and welling methods of ul35 protein antigen of strain I (Upper) and strain L (lower)

4. Discussion

Viral structural proteins, especially the envelope proteins, are important, not only because they are involved in virion morphogenesis, but also because they are the first molecules to interact with the host. The structural proteins often play vital roles in cell targeting, virus entry, assembly, and budding (***Mettenleiter et al., 2006 and Reske et al., 2007***), as well as triggering host antiviral defenses. VP26 of DPV was implicated in the process of capsid transport by assembling capsids with a baculovirus expression system. Capsids assembled in the absence of VP26 show a reduced propensity to cluster around the nucleus after microinjection into cells, compared to capsids assembled in the presence of VP26 (***Douglas et al., 2004***). The delivery of viral DNA to the host cell nucleus is a critical step in the establishment of a herpes virus infection. This process is made efficient by the recruitment of the dynein motor complex to capsids deposited in the

cytosol following virus entry into cells (***Dohner et al., 2002***). Association of dynein with capsids is presumably essential for retrograde axonal transport and alphaherpes virus neurotropism. Thus recombinant vaccine based on the ul35 gene (that encode for the VP26 protein) would initially render the virus unable to establish a complete replicative cycle resulted in viral inhibition.

For that reason, the ul35 gene of the 2 commonly used vaccinal strains were subjected to extensive analysis on the molecular level.

The amplified 380bp amplicon of both viruses used in this study were subjected to sequencing and the deduced amino acid was established using the CLC software. although The two genes from the 2 viruses have only 93% identity on the nucleotide level, the deduced amino acid sequence were 97% identical with most strains when BLASTn was conducted , these because most substitutions were silent as it resulted in the same amino acid due to the degeneracity of the genetic code. This level of similarity between the genes comes from either strains explains the high cross protection rate of either vaccine against the field isolates although the high antigenicity profile of the vaccine prepared from the local isolate (fig 4) making it the choice from which the ul34gene is to be amplified for cloning and production of DNA vaccine.

Blast n search could also explore some differences between the VP26 proteins of both strains. As shown in table (3) the L strain gave more similarity with the Anatid herpes virus 1 and Duck enteritis virus strain VAC complete genome when compared with the VP26 protein of the I strain. This data supports the idea of using the L strain as a source for the DNA vaccine production

The conservative domain of the ul35 protein (antigen VP26) was found to be of fusion _gly_k superfamily (fig 3) which is very essential for the virus attachment and assembly, VP26 binds the VP5 major capsid protein at a 1:1 ratio on hexons, resulting in 900 copies of VP26 on the capsid surface and making VP26 an attractive candidate as a recruiter of dynein (***Booy et al., 1994; Trus et al., 1995 and Zhou et al., 1995***). This finding supports the idea why the DNA vaccine directed towards this antigen would prevent the virus from completing its replicative cycle and thus would be a vaccine candidate.

The antigenicity profiles of the deduced amino acid sequence of ul35 protein antigen of both strains were evaluated using either ***welling*** or ***Kolaskar and Tongaonkar*** methods. Using welling method, it was hardly to find significant difference between the two proteins as most of the mutations is silent, as a results the hydrophobicity profile was not affected greatly. On the other hand the using of Kolaskar ***and Tongaonkar methods*** could predict antigenic determinants with an accuracy of greater than 75% and it was found that some great differences at the carboxyl terminal of the ul35 gene of the Local strain these differences make the area around amino acid 90 – 110 more antigenic for the Local strain than the imported strain. These finding also supported the use of the local strain as a template for the cloning of ul35 gene for production of the DNA vaccine.

Detailed analysis of the deduced amino acids sequence of the 2 proteins revealed also difference in the isoelectric points *PI* (VP26 protein of the I strain had *PI* of 10 and 9.74 for the L strain) this difference attributed to the difference in the amino acid composition at the carboxyl terminal of the both proteins.

In conclusion, a great similarity was found between the ul35 gene amplified from either local or imported vaccinal strain but the antigenicity profile alone with the dote blot matrix revealed that the UL35 protein antigen (antigen VP26) from local isolate is more antigenic and thus the genome of the local strain would be a suitable templates for amplification of the ul35 gene which will be cloned and used as DNA vaccine.

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