

## Structure prediction of ORF3 encoded protein of a novel Pakistani avian hepatitis E virus strain

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### ABSTRACT

Avian HEV (aHEV) has been considered as causative agent of hepatitis-splenomegaly syndrome (HSS) in chickens. Like other HEV strains, it is single-stranded positive sense RNA virus belonging to *Hepeviridae*. In the present study we report partial structural homology of ORF3 encoded protein (ORF3P) of Pakistani novel aHEV (Pak naHEV) strain with a kinase 2-amino-4-hydroxy-6-hydroxymethylidihydropteridine pyrophosphokinase (HPPK) which participates in folate biosynthesis in bacteria. This finding is in agreement with the role of ORF3P as multifunctional protein modulating host cell signaling and gene expression to promote viral replication during infection.

**Keywords:** Avian HEV, ORF3, Kinase, Pakistan

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### INTRODUCTION

Avian HEV, like other HEV strains, is single stranded-positive RNA virus which belongs to genus *Orthohepevirus*, which is placed in family *Hepeviridae* as a separate species *Orthohepevirus B* (Smith *et al.*, 2014; Smith *et al.*, 2015; Purdy *et al.*, 2017). The length of aHEV genome is approximately 6.6 kb which is 600 bp shorter than those of mammalian HEV (mHEV) genotypes. The shorter portion has a putative missing region between MeT and Hel (Reuter *et al.*, 2016a). The genome organization is almost same as mHEV with three open reading frames (ORFs); ORF1, ORF2 and ORF3. ORF1 encodes single non-structural polypeptide, with multiple functional domains. ORF2 encodes capsid protein, while ORF3 codes for a small multifunctional regulatory protein (Huang *et al.*, 2004).

The aHEV is associated with HSS and Big Liver and Spleen (BLS) disease in chickens (Haqshenas *et al.*, 2001; Huang *et al.*, 2002; Marek *et al.*, 2010; Payne *et al.*, 1999). Decreased egg production, high mortality rate, hepatitis and splenomegaly have been identified as common clinical symptoms of this infection (Jhone *et al.*, 2014). At present, worldwide four different

genotypes (Gt) of aHEV isolated from chickens have been reported. In Australia, United States of America (USA), China and Taiwan only a single genotype (Gt1, Gt2, Gt3 and Gt4, respectively) is circulating in chicken population but in Korea (Gt1, Gt2) and Hungary (Gt3, Gt4) two different genotypes are co-circulating among chickens (Zhao *et al.*, 2015; Moon *et al.*, 2016). Prevalence of HEV in other bird species has been reported in many studies. A novel avian-like HEV was reported in wild little egret (*Egretta garzetta*) (Reuter *et al.*, 2016a). But interestingly in carnivorous wild prey bird kestrel (*Falco tinnunculus*) and red-footed falcon (*F. vespertinus*) novel HEV strains related to mammalian HEV (mHEV) from ferret and rat, were identified (Reuter *et al.*, 2016b).

The ORF3P is a multifunctional protein which is transcribed from bicistronic subgenomic RNA (Graff *et al.*, 2006). Its overall structure presented to have hydrophobic domains towards the N-terminal and proline-rich domain (s) (PRD) in C-terminal (Kannan *et al.*, 2009; Holla *et al.*, 2013). A single PRD (PREPSAPP) with a PSAP motif has been identified in aHEV ORF3P. This PRD is known to be a binding site for SH3-domain proteins (SDP) and vacuolar sorting proteins (VSP). The interaction of PSAP motif with components of

endosomal sorting complex (ESC) and host tumor suppressor gene 101 (TSG101) suggests role of ORF3P in triggering release of enveloped virions from cellular membrane of host cell (Jouvenet *et al.*, 2011; Kenney *et al.*, 2012; Nan *et al.*, 2014, 2015). The role of ORF3P as a viroporin is elaborated in a recent study which facilitates virus egress from host cell (Ding *et al.*, 2017). The other important function of ORF3P is modulation of host cell signaling and genes expression; for instance MAP kinase activation, down regulation STAT3 mediated genes, inhibition of mitochondrial apoptosis pathway and interferon- $\alpha$  mediated signaling inhibition (Kar-Roy *et al.*, 2004; Chandra *et al.*, 2010; Chandra *et al.*, 2011; Dong *et al.*, 2012). In a recent study a wide range of host proteins were found to which ORF3P interact in combination of other HEV proteins (Subramani *et al.*, 2018).

The role of ORF3P in modulation of host cell signaling and gene expression may build an assumption of its function as a kinase and may be participating, directly or indirectly, in host proteins phosphorylation which is an important regulatory mechanism (Ardito *et al.*, 2017). In connection to this in the present study our data based on ORF3P structure prediction and modeling analysis of Pak naHEV strain revealed homology with 2-amino-4-hydroxy-6-hydroxymethylidihydropteridine pyrophosphokinase (HPPK) which is an enzyme of folate biosynthetic pathway (Yang *et al.*, 2005). The potential function of ORF3P as a kinase needs further investigation.

## MATERIALS AND METHODS

### Sampling

The samples of liver tissue, bile fluid and feces were collected from 19 layer chickens (dead before less than 12 hours) aged 30 – 90 weeks, from different poultry forms situated in Pattoki Punjab, Pakistan (31° 1' 0" North, 73° 51' 0" East). The mortality was not simultaneous and no outbreak was reported in the area. The sterilized 1.5 ml microfuge tubes were used of bile fluid and fecal samples collection, transported to Molecular Biology lab of Department of Zoology, University of Gujrat on ice packets and stored at -80°C in ultralow freezer for future analyses. The bile and fecal samples were designated as PT1B – PT19B and PT1F – PT19F, respectively.

### Detection of Viral RNA and Complete ORF3 Amplification

Total RNA was isolated from bile and fecal suspension through Trizol (Invitrogen) method following manufacturer instruction. The cDNA synthesis was done through Reverse Transcription PCR (RT-PCR), using both gene specific primers as well as random hexamer primers. For random hexamer-primers VILO 2X master mix (Invitrogen) was used. To detect viral RNA in samples, partial helicase (ORF1) 186 bp and partial capsid protein (ORF2) 280 bp fragments were amplified (Kwon *et al.*, 2012) using PerfeCta SYBR Green FastMix 2X (Quanta Biosciences). The primer set for helicase used; forward (RPHF) 5'-TGGCGCACY-GTWTTCYCACCG-3' and reverse (RPHR) 5'-CCTCRTGGACCGTWATCGACCC-3' and primer set for capsid protein used was; forward (RPO2F) 5'-GGTATGGTTGATTTTCCATAAAG-3' and reverse (RPO2R) 5'-GCTGCNCGNARCAGTG-TCGA-3'. The PCR was carried at; initial denaturation 95 °C for 2 min followed by 40 cycles of 95 °C for 30 sec, 50 °C for 30 sec, 72 °C for 1 min and final extension 72 °C for 10 min. PerfeCta SYBR Green FastMix 2X (Quanta Biosciences) was used for amplification of complete ORF3 using primer set; forward (APO31S) 5'-ACCATCC-AGCTTGTGGCGG-3' and reverse (APO31A) 5'-CACAAACCATGAGCATGCCGGACG-3'. The PCR conditions followed were; initial denaturation 95 °C for 2 min followed by 40 cycles of 95 °C for 45 sec, 55 °C for 45 sec, 72 °C for 3 min and final extension 72 °C for 10 min

### Sequence analysis and phylogeny

The obtained sequences were compiled through DNASTAR software (Lasergene). Then these sequences were used for homology analysis through NCBI Basic Local Alignment Search Tool (BLAST) (Zhang *et al.*, 2000; Morgulis *et al.*, 2008). Further, percent identity (PI) multiple sequence alignment (MSA) and phylogenetic analysis was done by MEGA 6 (Tamura *et al.*, 2013) and ClustalX2.1 (Larkin *et al.*, 2007) by comparing with other aHEV and mHEV strains.

### Protein structure prediction and modeling

Protein structure prediction and modeling was carried out through SWISS-MODEL (Biasini *et al.*, 2014). The deduced amino acids sequence was

used as input data and templates from SWISS-MODEL Template Library (SMTL) were obtained through BLAST and HHBlits and the highest quality templates were selected. On the basis of template data, structure modeling was done using ProMod3 (Guex *et al.*, 2009) and model quality, at global and per-residue level, was evaluated by QMEAN scoring function (Benkert *et al.*, 2008; 2011). Moreover, the quaternary structure of the model in its oligomeric form was estimated according to Bertoni *et al.* (2017).

### Complete ORF3 cloning

The complete ORF3, along with flanking regions from ORF1 and ORF2, was cloned through TOPO TA cloning kit (Invitrogen) in TOPO XL pCR cloning vector following manufacturer instructions.

## RESULTS

Two novel strains of Pakistani aHEV (PT12B, PT16B) were isolated from the bile fluid of two layer chickens aged 70 weeks. Sequence analysis on the basis of ORF3 revealed that they shared 100% identity which suggests that it is a single strain circulating in layer chickens population in the area.

### Molecular detection of Pak naHEV

The bile and fecal samples collected from layer chickens were processed for molecular detection through PCR. No fecal sample was found positive while only two (10.5%) bile samples, PT12B and PT16B, were found positive for aHEV RNA (Fig. 1).

### ORF3 amplification and sequence analysis

A fragment of 902 bp was amplified from aHEV RNA positive bile samples PT12B and PT16B (Fig. 2). This fragment contained complete ORF3 (264 bp) with some portion of ORF1 at 5' end and oORF2 at 3' end. So, it represents typically the junction of three ORFs. Complete nucleotides sequence of 902 bp fragment was obtained from TA clones by sequencing using vectors primers (M13). Sequences were submitted to GenBank under accession numbers MH018052 and MH018053.

Table I presents summary of sequence identity analysis of aHEV strains, including Pak naHEV strains, with mHEV strains from different genotypes showing the highest sequence identity

(95%) with sequences AM943647 and EF206691. It was observed that all aHEV strains analyzed, including Pak naHEV, showed highest sequence identity (45%, 46%) with mHEV strains from Gt4 (FJ763142, AJ272108). Restriction analysis of complete ORF3 nucleotides sequence of Pak naHEV and other aHEV strains for commonly used restriction enzymes, demonstrated a conserved restriction site GGTACC of *KpnI* at 255 nt position in all strain (data not shown). Multiple sequence alignment (MSA) of Pak naHEV strains complete ORF3 deduced amino acids sequence with other aHEV strains identified conserved amino acids sequence motifs at positions; aa 1-12, aa 16-28, aa 36-44, aa 55-58, aa 60-64, aa 66-72 and aa 76-87 (Fig. 3). Distant clustering of Pak naHEV strains was shown as result of phylogenetic analysis based on complete ORF3 deduced amino acids sequence with other aHEV strains (Fig. 4).

Comparison of complete ORF3 deduced amino acids sequence of aHEV strains, including Pak naHEV, with mHEV strains identified sequence gaps in aHEV ORF3 at following positions (reference AAA45726); 1-10 aa, 25-28 aa, 52-54 aa, 60-63 aa, 87-94 aa and 108 aa. Similarly, some conserved amino acids were also identified at positions (according to AM943647); C(9), L(10), C(12), G(33), G(36), P(38), Q(42), P(43), P(78) and R(84) (data not shown).

### ORF3 protein structure and modeling

As both Pak naHEV strains showed 100% sequence identity (nucleotides and amino acids) of complete ORF3 with each other, so the identical structure models were obtained on the basis of complete ORF3 deduced amino acids sequence (Fig. 5,6) The Pak naHEV ORF3P structure model built with ProMod3 on the basis of homology modeling showed that C-terminal region, from aa 47-86, is structurally homologous to a kinase HPPK (aa 35-74) (1rtz.1.A) (Fig. 5, 6 and Table II) . The Pak naHEV ORF3P model presented 3  $\beta$ -sheets followed by single  $\alpha$ -helix at the extreme C-terminus and loop structures connect  $\beta$ -sheets and  $\alpha$ -helix with each other. The superimposition of Pak naHEV ORF3P model on its template homologous region is also shown (Fig. 5B, 6d). Fig. 6 demonstrates different structural parameters of Pak naHEV ORF3P structure model. The comparison with non-redundant PDB structures identified that over all Z-score of the model is less than 2 and greater than 1 which suggests good quality of the model (Fig. 6).

## DISCUSSION

The Pak naHEV strains shared 100% sequences identity of complete ORF3 with each other which suggests that a single novel aHEV is circulating in the layer chickens population of the area. The overall sequence identity range presented was 93 – 95% for other aHEV strains and 41 – 45% for mHEV strains of different genotypes. The above mentioned sequence identity range is almost in agreement with earlier studies (Haqshenas *et al.*, 2001; Huang *et al.*, 2002). At present only 4 aHEV genotypes are reported (Zhao *et al.*, 2015) but phylogenetic analysis based on ORF3 deduced amino acids sequence presented distant clustering of Pak naHEV which suggests that it belongs to a previously unknown genotype.

The role of ORF3P is as multifunctional protein is quite evident and it plays with host cell signaling and gene expression during course of infection (Ding *et al.*, 2017; Nan *et al.*, 2016). Our data showed that the C-terminal (aa 46-86) of ORF3 is structural homology to 2-amino-4-hydroxy-6-hydroxymethyl dihydropteridine pyrophosphokinase (HPPK) which is a kinase and plays important role in folate biosynthesis.

The corresponding homologous portion of HPPK (aa 35 – 74) represents loop 2 and adjacent area which is a component of active site. The active site of HPPK is palm like structure in which loop 2 and 3 are flexible and used for ligand recognition and probably catalysis (Yang *et al.*, 2005; Pemble *et al.*, 2010). These findings may reinforce role ORF3P to interact host cellular signaling and hence modulation of host immune responses and other functions which promote viral replication (Tong *et al.*, 2016). The function of ORF3P as a kinase to phosphorylate host proteins for cell signaling and host gene expression modulation is not reported so far. Moreover, the ORF3P homologous region represents only a part of HPPK active site, so further investigation is needed in this regard.

## ACKNOWLEDGEMENT

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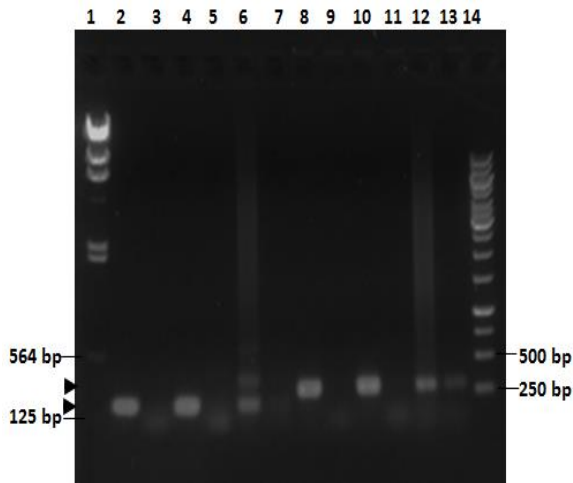
**Table I: Pak naHEV strains complete ORF3 nucleotides sequence based PI Matrix with other aHEV and mHEV strains**

Host	HEV strains	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
aHEV	<b>1: GU954430</b>	100	99	95	96	94	94	94	94	93	94	94	94	43	43	42	42	41	42	44	44
	<b>2: AM943646</b>	99	100	95	96	94	93	94	94	93	94	94	94	44	44	43	42	42	42	45	45
	<b>3: KF511797</b>	95	95	100	97	94	93	92	92	92	92	92	93	45	45	45	44	44	44	45	45
	<b>4: JN997392</b>	96	96	97	100	94	94	93	93	93	93	94	93	44	44	44	43	43	43	45	45
	<b>5: AM943647</b>	94	94	94	94	100	97	95	95	95	95	95	95	43	43	43	42	41	42	46	44
	<b>6: JN597006</b>	94	93	93	94	97	100	95	95	95	95	94	94	42	42	42	42	41	41	45	44
	<b>7: AY535004</b>	94	94	92	93	95	95	100	100	97	97	94	94	43	43	42	43	42	42	46	44
	<b>8: AY043166</b>	94	94	92	93	95	95	100	100	97	97	94	94	43	43	42	43	42	42	46	44
	<b>9: KM377618</b>	93	93	92	93	95	95	97	97	100	97	93	93	44	44	45	43	42	43	45	45
	<b>10: EF206691</b>	94	94	92	94	95	95	97	97	97	100	95	95	43	43	44	42	41	42	46	45
<b>Pak naHEV</b>	<b>11: MH018052</b>	94	94	93	93	<b>95</b>	94	94	94	93	<b>95</b>	100	100	42	42	42	42	41	42	<b>45</b>	43
<b>12: MH018053</b>	94	94	93	93	<b>95</b>	94	94	94	93	<b>95</b>	100	100	42	42	42	42	41	42	<b>45</b>	43	
mHEV	<b>13: D11092</b>	43	44	45	44	43	42	43	43	44	43	42	42	100	100	99	91	85	85	83	84
	<b>14: M80581</b>	43	44	45	44	43	42	43	43	44	43	42	42	100	100	99	91	86	85	83	84
	<b>15: M73218</b>	42	43	45	44	43	42	42	42	45	44	42	42	99	99	100	90	85	86	83	85
	<b>16: M74506</b>	42	42	44	43	42	42	43	43	43	42	42	42	91	91	90	100	85	86	84	84
	<b>17: AF082843</b>	41	42	44	43	41	41	42	42	42	41	41	41	85	86	85	85	100	96	85	85
	<b>18: FJ527832</b>	42	42	44	43	42	41	42	42	43	42	42	42	85	85	86	86	96	100	87	87
	<b>19: AJ272108</b>	44	45	45	45	46	45	46	46	45	46	45	45	83	83	83	84	85	87	100	95
	<b>20: FJ763142</b>	44	45	45	45	44	44	44	44	45	45	43	43	84	84	85	84	85	87	95	100

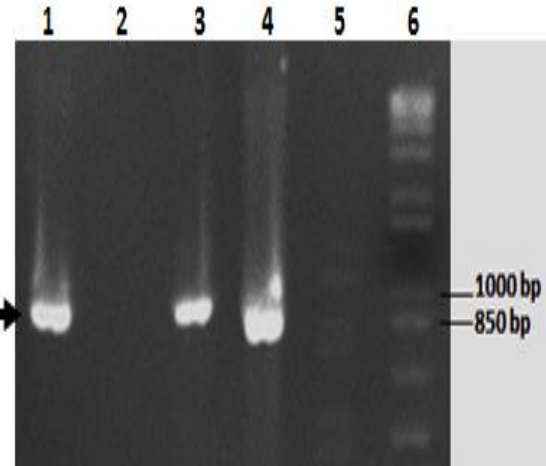
Over all PI of Pak naHEV strains with each other = 100%, with other aHEV strains = 93 – 95%, with mHEV strains = 41 – 45%. Highest PI in bold

**Table II: Pak naHEV strain ORF3P homology modeling template information**

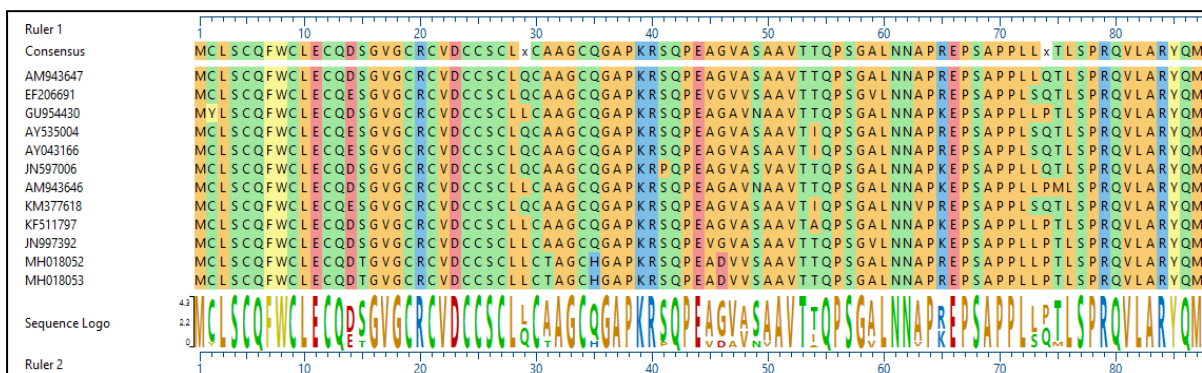
Template	Seq Identity	Oligo-state	Found by	Method	Resolution	Seq Similarity	Range	Coverage	Description
1rtz.1.A	27.50	monomer	HHblits	X-ray	1.33Å	0.31	47 - 86	0.46	2-amino-4-hydroxy-6-hydroxymethyl dihydropteridine pyrophosphokinase



**Fig. 1:** Detection of aHEV genome in bile and fecal suspension samples of PT12 and PT16 chicken (0.8% Agarose Gel). Lane 2 – 7 helicase (186 bp, arrow head) and lane 8 – 13 ORF2 amplification (280 bp, arrow head); lane 2 = PT12B, lane 3 = PT12F, lane 4 = PT16B, lane 5 = PT16F, lane 6 = positive control, lane 7 = negative control, lane 8 = PT12B, lane 9 = PT12F, lane 10 = PT16B, lane 11 = PT16F, lane 12 = positive control, lane 13 = negative control, lane 1 = λ-Hind III DNA marker, lane 14 = 1 kb DNA marker (B = ble sample, F = fecal sample)

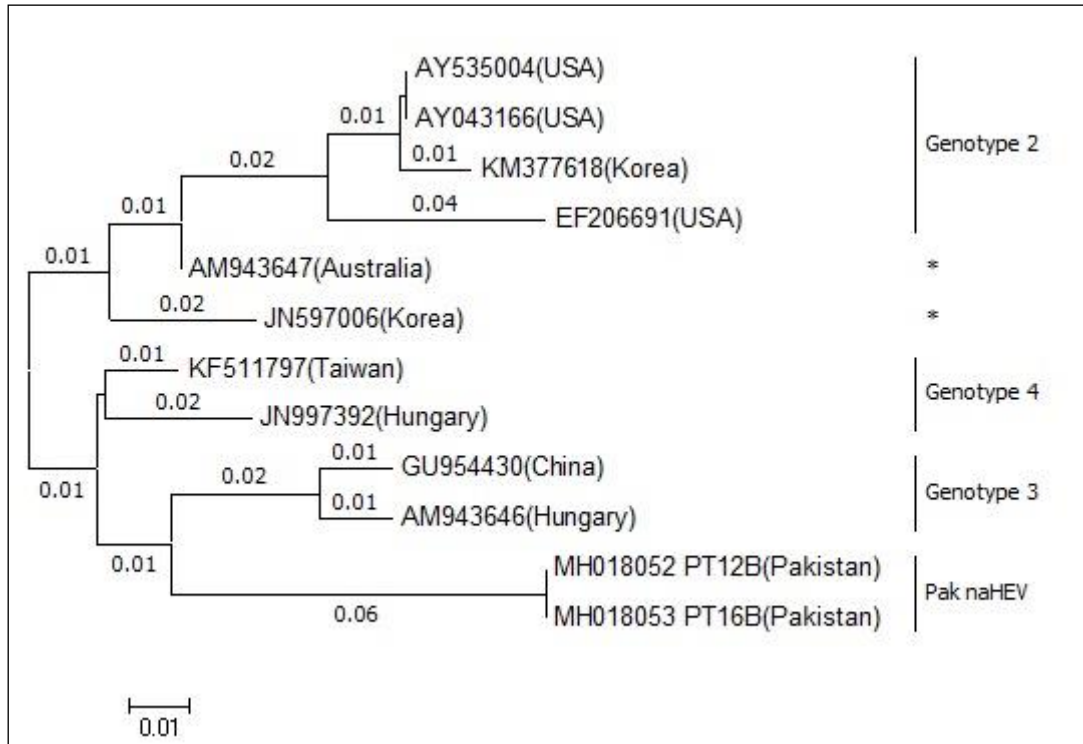


**Fig. 2:** Amplification of complete ORF3 (902 bp, arrow head) from Pak naHEV strains bile samples (2% Agarose Gel). Lane 1 = PT12B, lane 2 = PT14B, lane 3 = PT16B, lane 4 = positive control, lane 5 = negative control, lane 6 = 1 kb plus DNA marker

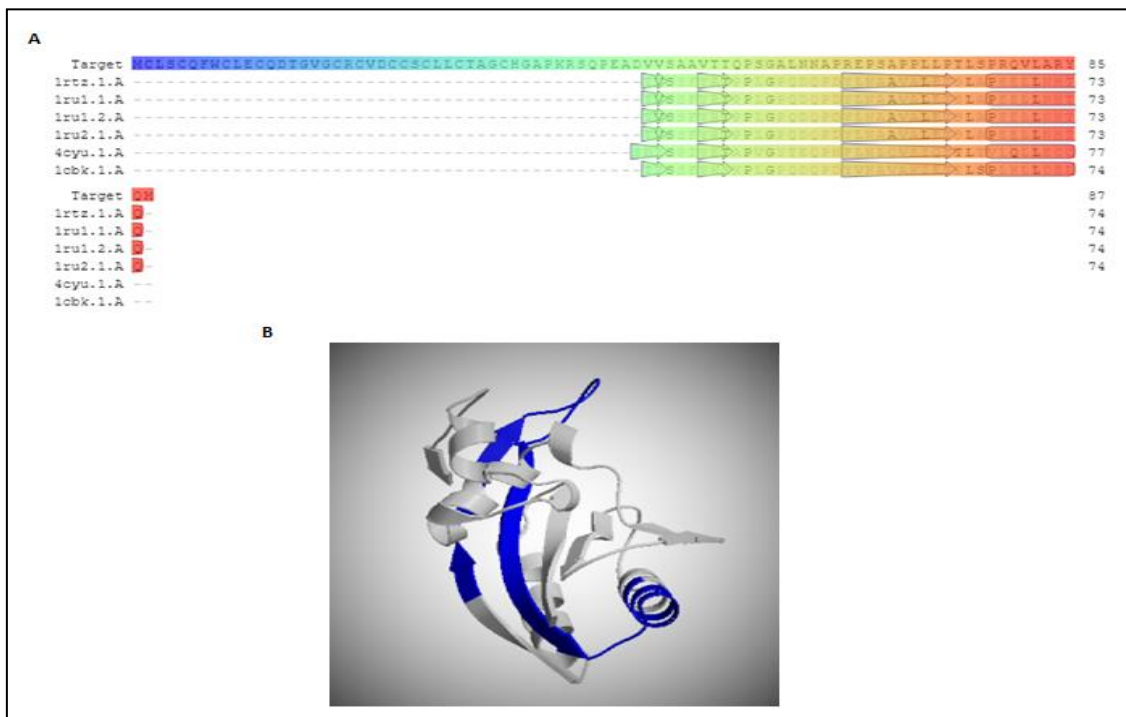


**Fig. 3:** Multiple sequence alignment of complete ORF3 deduced amino acids sequence of Pak naHEV strains (MH018052, MH018053) with other aHEV strains.

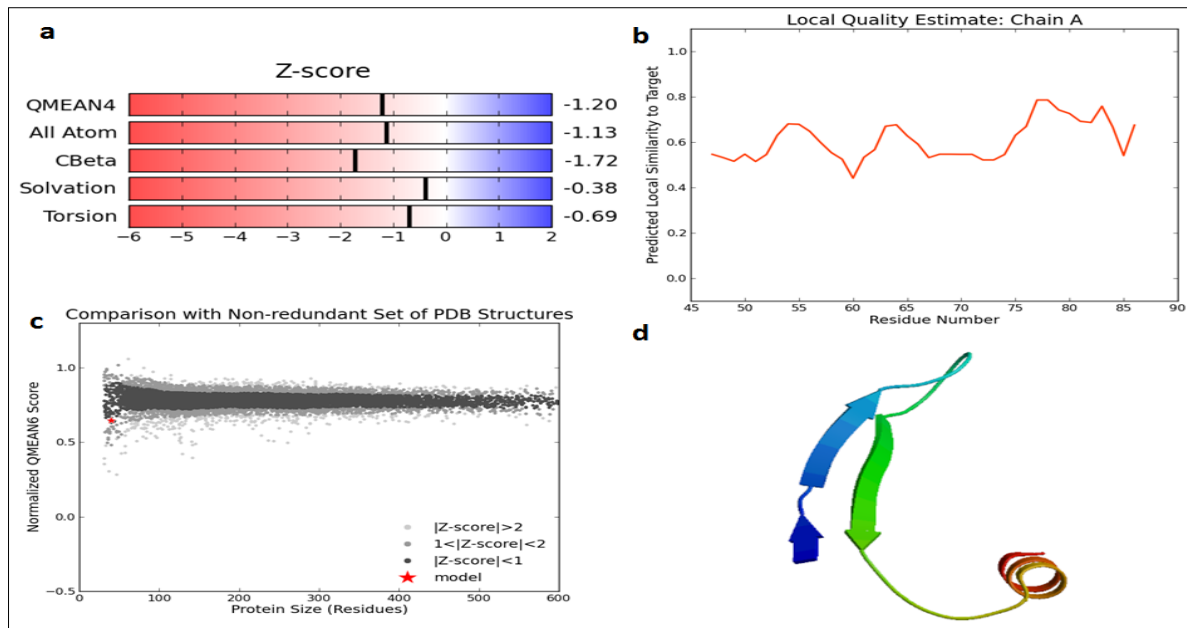




**Fig. 4:** Phylogenetic relationship of Pak naHEV with other aHEV strains on the basis of complete ORF3 deduced amino acids sequence. Asterisk shows genotype 1 strains which are distantly clustered in this case. Numbers shows branch length.



**Fig. 5:** Pak naHEV strain ORF3P homology modeling, A) alignment with template structure (1rtz.1.A), B) structure model showing identical (in blue colour) portion in template structure (1rtz.1.A)



**Fig. 6:** Pak naHEV strain ORF3P structure model, a) Z-score of different structure parameters, b) local quality estimate of each amino residue, c) comparison of built model with non-redundant set of PDB structures, d) final Pak naHEV ORF3P model

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