

Print ISSN : 0972-8813  
e-ISSN : 2582-2780

[Vol. 20(2), May-August, 2022]

# Pantnagar Journal of Research

(Formerly International Journal of Basic and  
Applied Agricultural Research ISSN : 2349-8765)



G.B. Pant University of Agriculture & Technology, Pantnagar



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## Nucleocapsid Segment Sequence based phylogenetic analysis of different strains of Crimean Congo Haemorrhagic fever virus encountered in India over last decade

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**ABSTRACT:** Crimean Congo Haemorrhagic Fever (CCHF) is a potentially fatal tick born viral zoonotic disease affecting humans in a wide geographical area. In India, it is considered as an emerging viral zoonosis and repeated outbreaks are noticed especially in the Gujarat state since 2011. The current study is focussed on the phylogenetic relationship based on the genomic sequence of nucleocapsid segment of various strains of CCHF virus (CCHFV) circulated in India over past 10 years. It was found that there were considerable differences in the nucleotide sequences and the Indian strains are found to be related to two different phylogenetic groups i.e., Asia-1 and Asia-2. It suggests the transboundary nature of the disease which may be well coordinated with the travel history of the patients affected.

**Key words:** Crimean Congo Haemorrhagic Fever, nucleocapsid, phylogenetic analysis, sequence alignment, zoonosis

Viral Haemorrhagic Fevers are now becoming a severe threat to animal and human health due to their potential zoonotic nature and associated fatality (Mariappan *et al.*, 2021). Among these, Crimean Congo Haemorrhagic Fever (CCHF) is most widely distributed viral haemorrhagic fever and considered as an emerging zoonosis in India after its first outbreak occurred in Gujarat in 2011 (Kamboj and Pathak, 2013; Ergonul, 2006; Mani *et al.*, 2012). It is caused by Crimean Congo Haemorrhagic Fever Virus (CCHFV) which belongs to *Nairovirus* genus of *Bunyaviridae* family (Ergonul, 2006; Mardani and Jarhomi, 2007). CCHFV is a spherical, enveloped, negative sense single stranded RNA virus of approximately 100 nm diameter (Jakkula *et al.*, 2019). CCHF is distributed globally and its extensive geographical distribution can be well correlated with the distribution of its tick vector *Hyalomma* (Fillatre *et al.*, 2019). The virus has been isolated from various domestic and wild animals but there is no evidence of clinical disease in animals. However, a wide range of domestic and wild animals act as reservoir for CCHFV (Kamboj *et al.*, 2014). The disease is widely distributed in more than 30 countries of Africa, Europe, Middle East and Asia (Yadav *et al.*, 2013; Spengler *et al.*, 2019). In India, the first confirmed outbreak of CCHF occurred in 2011 in Gujarat in which few human deaths were reported including deaths of medical practitioners and nursing staff treating the infected cases (Mourya *et al.*, 2012). Prior to it, outbreaks of CCHF

were reported in our neighbouring countries Afghanistan and Pakistan in year 2009 and 2010 respectively (Zohaib *et al.*, 2020; Hussain *et al.*, 2016; Fillatre *et al.*, 2019). If we see the disease status in last two years the cases of CCHF and evidences of tick vector were reported from Bulgaria, Iran, India, Pakistan, Russia, Oman, Senegal, Kazakhstan, Mali, South Africa, Spain, Turkey and other neighbouring countries from 2019-2020 (Kuehnert *et al.*, 2021). CCHFV has a segmented genome of around 19.2 kb size which consist of three segments *viz.* Small (S), Medium (M) and Large (L) segment encoding nucleocapsid, virus polymerase and glycoprotein precursor respectively (Ergonul, 2006; Yadav *et al.*, 2013). The present study is focussed on the sequence based phylogenetic analysis of various strains isolated and sequenced in India targeting S segment encoding nucleoprotein which has a crucial role in establishment of viral infection and virion structure.

### MATERIALS AND METHODS

#### *Full length S Segment sequence retrieval*

The nucleotide sequences of full-length 'S' segment encoding nucleocapsid were retrieved from GenBank in FASTA format. A total of 20 sequences were retrieved including 13 sequences available in GenBank from India (Accession no. JX051650.1; MH396675.1; MH396672.1; MH396669.1; MH396666.1; MH396663.1; MH396660.1;



MH396657.1; MH396654.1; MH396651.1; MH396648.1; MH396645.1; MH396642.1) as well as a representative sequence from different phylogenetic groups present across the world including Oman (DQ211645.1), TAJ/HU8966 (AY049083.2), Hodzha (AY223475.1), Gaib (KX013446.1), Drosdov (DQ211643.1), Kashmanov (DQ211644.1) and SPU97/85 (DQ211646.1). The retrieved sequences from GenBank were first checked for sequence quality and length. The sequence of complete ORF of multifunctional nucleocapsid protein was selected for sequence alignment and phylogenetic analysis to ensure the uniform length of each sequence.

### Sequence alignment

The multiple sequence alignment (MSA) was performed using built in ClustalW programme of Alignment Explorer module of MEGA11 (Molecular Evolutionary Genetics Analysis version 11). The MSA parameters used were gap opening and gap extension penalty value of 15.00 and 6.6 respectively, IUB DNA weight matrix with transition weight of 0.50 and delay divergent cut-off of 30%. The variable and conserved sites were estimated at both nucleotide and amino acid level.

### Estimation of evolutionary divergence

For the estimation of evolutionary divergence between sequences, the number of base substitutions per site from between sequences were calculated. Analyses was conducted using the Maximum Composite Likelihood model (Tamura *et al.*, 2004). This analysis involved all 20 nucleotide sequences. Codon positions included were 1<sup>st</sup>+2<sup>nd</sup>+3<sup>rd</sup>+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option).

There were a total of 1449 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura *et al.*, 2021).

### Phylogenetic analysis

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993). The maximum likelihood phylogenetic tree with 500 bootstrap replicates was constructed using MEGA11 (Tamura *et al.*, 2021; Felsenstein, 1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.2362)). The tree was drawn to scale of 0.05 with branch lengths measured in the number of substitutions per site. This analysis also involved 20 nucleotide sequences and there were a total of 1449 positions in the final dataset.

## RESULTS AND DISCUSSION

### Conserved and variable sites in sequence alignment

In the nucleotide sequence of nucleoprotein coding segment of CCHF, out of total 1449 sites, 1088 conserved and 361 variable sites were detected using 100% coverage. So, the percentage of variable sites was approximately 25%. However, in translated amino acid sequence, out of total 482 positions, the conserved and variable sites were found to be 446 and 36. In this case the percentage of variable sites was approximately 7.5% which suggests the

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1																				
2	0.062																			
3	0.114	0.098																		
4	0.004	0.067	0.114																	
5	0.112	0.096	0.001	0.112																
6	0.003	0.064	0.114	0.003	0.112															
7	0.003	0.064	0.113	0.004	0.111	0.003														
8	0.003	0.064	0.115	0.006	0.113	0.005	0.005													
9	0.101	0.092	0.063	0.102	0.061	0.102	0.101	0.103												
10	0.001	0.063	0.112	0.004	0.111	0.003	0.003	0.003	0.102											
11	0.003	0.065	0.116	0.006	0.114	0.004	0.004	0.005	0.104	0.003										
12	0.003	0.065	0.116	0.006	0.114	0.004	0.004	0.005	0.104	0.003	0.004									
13	0.002	0.063	0.115	0.005	0.113	0.003	0.003	0.004	0.102	0.002	0.003	0.003								
14	0.021	0.064	0.116	0.024	0.114	0.023	0.023	0.023	0.104	0.021	0.023	0.023	0.022							
15	0.014	0.060	0.117	0.017	0.115	0.015	0.015	0.016	0.100	0.014	0.015	0.015	0.015	0.018						
16	0.100	0.100	0.061	0.103	0.060	0.101	0.101	0.102	0.048	0.101	0.103	0.103	0.101	0.103	0.102					
17	0.125	0.110	0.128	0.129	0.126	0.128	0.129	0.126	0.121	0.127	0.127	0.127	0.126	0.127	0.121	0.119				
18	0.127	0.115	0.133	0.131	0.131	0.130	0.130	0.128	0.125	0.129	0.129	0.129	0.128	0.130	0.122	0.119	0.009			
19	0.137	0.127	0.129	0.138	0.127	0.138	0.140	0.139	0.124	0.137	0.140	0.138	0.138	0.138	0.134	0.128	0.156	0.160		
20	0.020	0.058	0.114	0.023	0.113	0.021	0.021	0.022	0.096	0.020	0.021	0.020	0.020	0.023	0.018	0.099	0.124	0.123	0.136	

**Fig 1:** The number of base substitutions per site from between sequences is shown. The 20 sequences taken are given with accession numbers: 1. JX051650.1; 2. MH396675.1; 3. MH396672.1; 4. MH396669.1, 5. MH396666.1; 6. MH396663.1; 7. MH396660.1; 8. MH396657.1; 9. MH396654.1; 10. MH396651.1; 11. MH396648.1; 12. MH396645.1; 13. MH396642.1; 14. AY223475.1; 15. AY049083.2; 16. DQ211645.1; 17. DQ211644.1; 18. DQ211643.1; 19. DQ211646.1; 20. KX013446.1

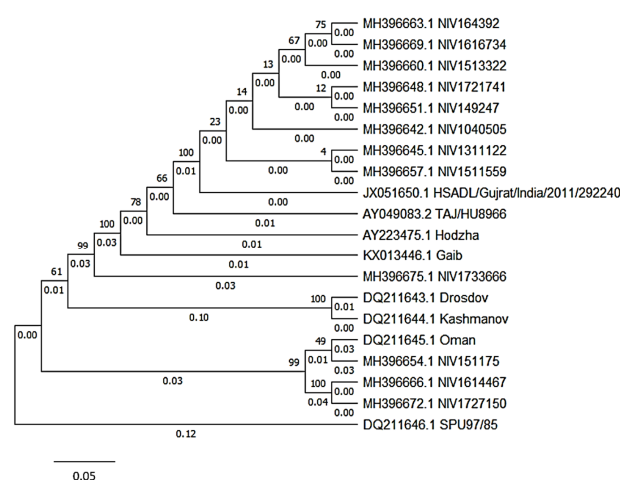
prevalence of silent mutations in the nucleocapsid segment.

### Evolutionary Divergence between Sequences

The evolutionary divergence or distance was simply estimated in terms of base substitutions per site between the 20 nucleotide sequences. Different values were obtained which signifies the variability in the evolutionary distance between CCHFV strains. More closely related or less divergent strains shows a lower value and more distantly related strains shows a higher value. The number of base substitutions per site from between sequences are shown in Fig. 1. The number of base substitutions per site from averaging over all sequence pairs was found to be 0.075.

### Phylogenetic analysis

Phylogenetic analysis was conducted by Maximum Likelihood method and Tamura-Nei model. The phylogenetic tree constructed using 500 bootstrap replicates with the highest log likelihood (-4417.95) is shown in Fig. 2. Various studies on phylogenetic analysis of CCHFV S segment have been already conducted which revealed that CCHFV strains are divided into seven phylogenetic groups namely Asia-1, Asia-2, Europe-1, Europe-2, Africa-1, Africa-2 and Africa-3 (Bente *et al.*, 2013). The strains prevalent in India belonged to both Asia-1 and Asia-2 groups. The Asia-1 group contain strains from Afghanistan, Pakistan, Oman and Iraq whereas Asia-2 group contain strains from China, Russia, Uzbekistan, Iran and Tajikistan. The phylogenetic nature of CCHF strains is highly correlated with the travel history of the patients.



**Fig 2:** The Phylogenetic tree with the highest log likelihood (-4417.95) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale which indicate the number of substitutions per site

It is an important finding because person-to-person transmission via exposure to virus-containing body fluids is a potential route of CCHF transmission (Spengler *et al.*, 2019). Many cases of CCHF were reported from India between 2011 and 2019 in Gujarat province and its adjoining state of Rajasthan (Patel *et al.*, 2021). It has also been reported in 2016 that a migrant returned from Oman was found positive for CCHF and the S segment phylogenetic analysis revealed that the isolated virus belongs to Asia-1 group to which Oman strain belongs (Yadav *et al.*, 2017). However, majority of Indian isolates sequenced and analyzed belongs to Asia-2 group. Many such diseases have been unknowingly imported by travelers from one country to another (Leblebicioglu *et al.*, 2016). It is therefore evident from studies that sequencing and phylogenetic studies serve as a powerful tool to predicting the pattern of disease spread across boundaries of countries.

### CONCLUSION

The increased international travel causes the movement and importation of diseases. CCHF is one of the most fatal viral hemorrhagic fevers which was prevalent in western part of India between 2011 to 2019. The phylogenetic studies of Indian strains revealed its similarity with two different phylogenetic groups Asia-1 and Asia-2 which suggests the migration of virus across the boundaries of country with the travelers. To control such fatal diseases, it is recommended to trace the travel history of patients and make persons aware of communicable diseases prevalent in the countries they visit.

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[/doi.org/10.3201/eid2604.191154](https://doi.org/10.3201/eid2604.191154)

*Received: May 3, 2022*

*Accepted: July 29, 2022*