AN INSILCO APPROACH TO ANALYZE EPIDEMIOLOGICAL ASSOCIATION BETWEEN ALL ASIAN STRAINS OF CRIMEAN-CONGO HEMORRHAGIC FEVER VIRUS ACCENTUATING PAKISTANI STRAINS THROUGH BIOSTATISTICAL APPROACH TO DESIGN VACCINE

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ABSTRACT: *Crimean-congo hemorrhagic virus (CCHFV) is a severe hemorrhagic, zoonotic disease spreading around the globe ultimately leading to the death. Preferentially in Asia, favorable climatic conditions, common ritual practices customs and trouble-free migration between countries are triggering CCHFV spread. The deadly life threatening outcomes spanning many countries of Asia have averted the attention of researchers to understand its emergence, its genomic analysis, association between strains and vaccine designing. Considering these factors, we have analyzed all Asian strain's segments (L, M and S) relationship and highlighted Pakistani strain's association with all other Asian strains. Following MSA (MUSCLE), phylogenetic tree construction software and principle component analysis, there is unambiguous evidence of strong association between Pakistani strain's L segment (FJ435383, FJ435384P) with Afghanistani strain's L segment, Pakistani strain's M segment (ACK58333, ACK58346) with Afghanistani and turkey strains, Pakistani strain's segment (JX227939,KC869988, KC869989, KC869992, KC869993, KC869990) with turkey, Afghanistani and Irani strains. Our results are logical manifestation of previous reported research. As going towards vaccine deign, a number of Insilco analysis have performed on each segment of all Asian strains to find out a conserve region which may act as antigen/epitope. Up to our knowledge except in L segment, no conserve region is found in any other segment. After checking the Antigenicity score (0.6253) we purposed a sequence representative of all Asian strain's L segments. After all findings, we eagerly expect that it would be worthwhile in management of this deadly disease and in constructive course of vaccine design.*

Keywords: crimean-congo hemorrhagic fever virus, zoonotic disease, phylogenetic, genomic analysis, association

INTRODUCTION:

As the world moves into lager dimensions in terms of disease prevention, management and treatment, new diseases keep surfacing with the same pace. Many emerging diseases, epidemics included, are triggered by zoonosis. Infectious diseases like viral hemorrhagic fevers (VHFs) are the focal point of studies now a day owning to their zoonotic nature and associated with higher mortality rates. Out of 700 hard tick species around the world, only 269 are capable of causing disease in humans [1-3]. VHFs are threating to both humans and animals alike [4]. Crimean Congo hemorrhagic fever (CCHF) is one of the widespread VHFs. It is brought about by *Crimean-Congo hemorrhagic fever virus* (CCHFV) [5-8]. Mortality rates for different CCHF outbreaks vary markedly. Average fatality rate is often 30–50% [9].

It has been found throughout Central Asia, South Eastern Europe, Africa, and the Middle East, the disease is perfectly capable of causing an epidemic. CCHF has affected more than 30 countries worldwide [10] making itself a public health threat globally. CCHF is prevalent in Asia, extending from the XinJiang region of China to the Middle East. Kosovo, Bulgaria, Turkey, Iran, Kuwait, China, Albania, Pakistan are some of the countries in this region with reported CCHF cases (view table no.1) [11,12]. CCHFV is distributed throughout the world, spanning much of Asia, from China to the Middle East and Southern Russia. It is more endemic in Africa and southern Europe, including Kosovo and Turkey [13]. In Iran, from 1999–2008, 528 cases were reported. From 1998–2002, Afghanistan witnessed some more than 100 cases of CCHFV, Where as in Pakistan, 108 cases were reported from year 1976–2002 [11]

Climatic changes such as droughts, warm temperatures and fewer rains made the Asian countries most favorable for the infection. Cross border migrations also seems to be a major reason of disease spread in the region [11,14,15]. Pakistan had its first case of CCHF reported in 1970. Since then it keeps springing its head up at various times during the past years. From 1976-2002 and 2002-2004, 108 and more than 50 cases were reported, respectively [16]. Rawalpindi had its first CCHF case in1976 with three reported deaths at central general hospital [17,18]. After this, an outburst of CCHFV caused a lot of deaths with a wave unbroken. In December 1994, one person died in Quetta (Baluchistan), December 2000, another person died in Islamabad, December 2005; 32 years old healthy person died in Abottabad, February 2002; 25 year old women died in Kashmir, October 2010; WHO reported 26 cases by national focal point, in 2012 sixty one cases were suspected and 17 deaths, 1st January to 9th June 2013; 16 suspected cases with 6 deaths, 2015; 25 cases was reported in critical situation in Peshawar and nearby areas. Seven of them died [5,19-21].

CCHFV genome consists of three single-stranded negative-sense RNA segments, namely small (S), medium (M), and large (L) segments [22]. CCHFV S segment encodes Nucleoprotein [23] , M segment codes a precursor polyprotein for the two envelope glycoproteins [24] , whereas L segment of CCHFV encodes for the RNA-dependent RNA polymerase. Ixodid ticks are the agents of transmission to humans (mostly of the *Hyalomma* genus), or it can spread by contact with blood or tissues from Infected humans or diseased livestock [5-8]. The disease progresses through four distinct phases including incubation, prehemorrhagic,

hemorrhagic, and convalescence [9,25]. Following incubation, there is a pre-hemorrhagic period that is characterized by a sudden onset of fever, chills, and many serious manifestations [25-28]. In severe cases, hemorrhagic symptoms rapidly manifest themselves 3–6 days after the inception of disease [29,30].

Treatment is majorly supportive in case of CCHFV. Ribavarin was found effective against CCHFV since CCHFV is susceptible to Ribavarin *in vitro*. Most of the countries do not have vaccine available against CCHF [4]. Bulgaria and former USSR have used formalin inactivated vaccine derived from suckling mouse [31,32]. However there is not yet valuable work has been done regarding vaccine formation suitable for all the Asian strains. Our focus is to check if any association is present between the Asian strains and a step towards development of a vaccine which may cover all those strain.

Table no 1: A detailed report of CCHFV cases in different countries and their fatality rate.

Year/Period	Country	No. of cases	Case Fatality Rate/Death count	Maiden case reported	References
1995-2003	Kosovo	228	25.5%[i]	1954	[33,34]
1999-2008	Iran	528	8 deaths	1970	[35]
1965-1994	China	260	21%	1965	[36]
1998	Afghanistan	19	12	1950	[37]
1979-1982	Kuwait	20		1979	[38]
1953-1974	Bulgaria	1,105	17%	1952	[39]
2000	Russia	83	8	1940s	[40]
2010	Pakistan	<100	>10%	1970	[41]
2002-2007	Turkey	1820	5%	2002	[42,43]

MATERIAL AND METHOD:

DATA INPUT/SOURCE OF DATA:

The datasets and tools used in this study were obtained online from the NIAID Virus Pathogen Database and Analysis Resource (ViPR) through the web site http://www.viprbrc.org. The Acknowledgement is as follows: (ViPR) has been funded with funds from the National Institutes of Health, National Institute of Allergy and Infectious Diseases, Department of Health and Human Services. From VIPR, all the reported Asian strain's segments (L, M, S) nucleotide sequences (FASTA) of crimean-congo hemorrhagic virus from 1995 till date were collected as a input data for further in silico analysis.

PHYLOGENETIC TREE:

All virus strains of same segment are subjected to multiple sequence alignment using java based application. 10 reported strains of L segment (Pakistan, Oman, Afghanistan, Turkey, and India) 62 strains of M segment (Iran, Pakistan, Turkey, India, Afghanistan, Oman) and 226 reported Asian strains of S segment as described above were subjected to MSA using MUSCLE (Multiple Sequence Comparison by Log-Expectation) algorithm [44], a fast distance estimation approach (Results are given as supplementary data). For deep insight into evolutionary relationship and transmission of life threatening contagion, MSA of all three described segments, distance based approach was used for phylogenetic tree construction (Fig. 1, 2 & 3). Tree visualization is supported by java based visualization software tool (Archaeopteryx).

PRINCIPLE COMPONENT ANALYSIS (PCA):

Variance analysis of all MSA of three segments was performed following the method outlined and described by Steele & Torrie [45]. PCA results are highly appreciable for the indication of differences and similarities of all strain's genotypes and reduction of bulk amount of variable data into smaller sets of linear composites for further processing [46].

VACCINE DESIGNING:

No vaccine of CCHF has been yet reported. In our study we have found an antigenic region (CAAAAGGTTAG) of L segment of all Asianstrains using bioedit followed by MSA (multiple sequence alignment) using MUSCLE [44]. According to Bioedit scale above mentioned region lies from 2815 to 2825.Antigenicityscore (0.6253) was checked using VEXIJEN [47] which was found significant as compared to threshold value (0.4). No such antigenic region has been found in M and S segments of all Asian strains.

PRINCIPLE COMPONENT ANALYSIS:

Principle component analysis (PCA) displaying more appropriate visualization of large amount of data. Verification of results produced by phylogram is further achieved by PCA for L, M, and S segment shown in figures 4, 5 & 6.Where each white square represents a single or cluster of strains closely packed or far apart on the basis of similarity or differences respectively.

RESULTS AND DISCUSSION:

As previously discussed based upon sequence analysis of M,L, and S segments we can interpret the relatedness of all the CCHF strains from various countries. Taking Pakistan on high risk in regard of CCHFV infection, our work illustrates the relationship of all Asian strains highlighting specifically Pakistani strain's phylogenetic relationship with other Asian strains. We purpose a conserved sequence (epitope) representing L segment of all Asian strains with high Antigenicity score. So we can target our specific area for future management of CCHFV in context of vaccine development and preventive management. Initially CCHF viruses from different geographic regions were thought to be antigenically indistinguishable. However, later it became evident that these viruses possess extensive genetic diversity; thanks to the nucleic acid sequence analysis. S segment of the genome is most widely studied segment using these nucleic acid sequence techniques. However, most recent studies have focused on M RNA segment of the genome [48]. Livestock forming, served as a major source of income for majority of population in Pakistan. 93% of rangeland in provisional area cannot be supported to vegetation through the whole year, leading seasonal migration in different areas of Baluchistan and area bordering the Afghanistan of

nomadic people with their livestock. As a carrier of CCHFV, these cattles and sheep might be the major cause of disease transmission across migratory areas [49,50]. Illegal animal transportation come about through Quetta (city of Pakistan) to Afghanistan due to cultural and tribal civilization similarities. Baluchistan is a centre for receiving animal's skin and hides from Iranian blochistnan and southern Afghanistan through Taftan gateway [51].

Interestingly many strains , with similar genetic makeup but in different geographical areas is a manifestation of constant cross bordering of virus source in Asian countries especially on religious occasions7. A report from Afghanistan shows close transmission association between Pakistan and Afghanistan due to 100% similarity shared among their S segment [52]. Another report supports the close linkage between pak-Matin strain (U75678) and similarly, Iranian strains imply the transmission between Pakistan and Iran [53]. All CCHFV strains from Hamadan (province of iran) was assembled with Pakistani strains providing an evidence of constant and continuous transmission of virus source between two countries [54]. According to Burt et al from a tick species (*Boophilus microplus)*, Madagascar CCHFV strain was isolated and thought to be from Asia as mentioned tick species were primarily in Pakistan and india [55].

On our part MSA (Multiple sequence alignment) of all sequences separately (10 sequence of L, 65 M and 226 S) performed using MUSCLE program. Following MSA of all segments, distance based approach was used for phylogenetic tree construction and visualized using java based program Archaeopteryx. Our findings were further confirmed by performing variance analysis, PCA (principle component analysis) using MSA results as an input data. Described in figure 1, phylogram clearly depicts the division of all strain's L segments into five groups. Pakistani strains with accession no. FJ435383, FJ435384P fall under group 1 clearly shows close association with Afghanistani strains (accession no. JX999734) confirming the previous report published. The same association was further confirmed statistically by using PCA analysis as shown in figure 4. Eigen values of all strains display much similarity (supplementary files are attached).

As illustrated in figure 2, phylogram of M segments holding all Asian strains. Out of 3 groups, Pakistani strains with accession no ACK58333, ACK58346 hold its position in group 1 closely related with Turkey and Afghanistani strains. Further manifestation of M segments analysis results are explained by PCA as shown in figure 5.As in figure 3, phylogram of S segment of all Asian strains shows 3 groups. Pakistani strains JX227939 being in group 1 and KC869988, KC869989, KC869992, KC869993, KC869990 strains in group 2 show close association with turkey Afghanistan and Iran respectively. PCA of same strains depicts confirmation of above results as shown in figure 6.Eigen values of all analysis are attached. Our findings became more valuable as it correlate the previously published studies, a strong relation between Pakistan and Afghanistan in case of L and M segment. While S segment is found distantly related, in context of origin of strains, cross border transfer of either animals, high migration of humans and many other factors described previously in literature.

Development of vaccine, a hot issue, is till now in very early phase of processing due to substantial genetic variation in almost all strains of CCHFV [56]. However some conserved regions, derived from various strains, are found as epitopes in vaccine production being immunogenic [57] As making our participation in early Insilco step for vaccine development, we made our best efforts of finding conserved region in all three segments of Asian strain by performing various analysis but only one conserved region in L segment leaded to success. CAAAAGGTTAG was predicted as an epitope. Its

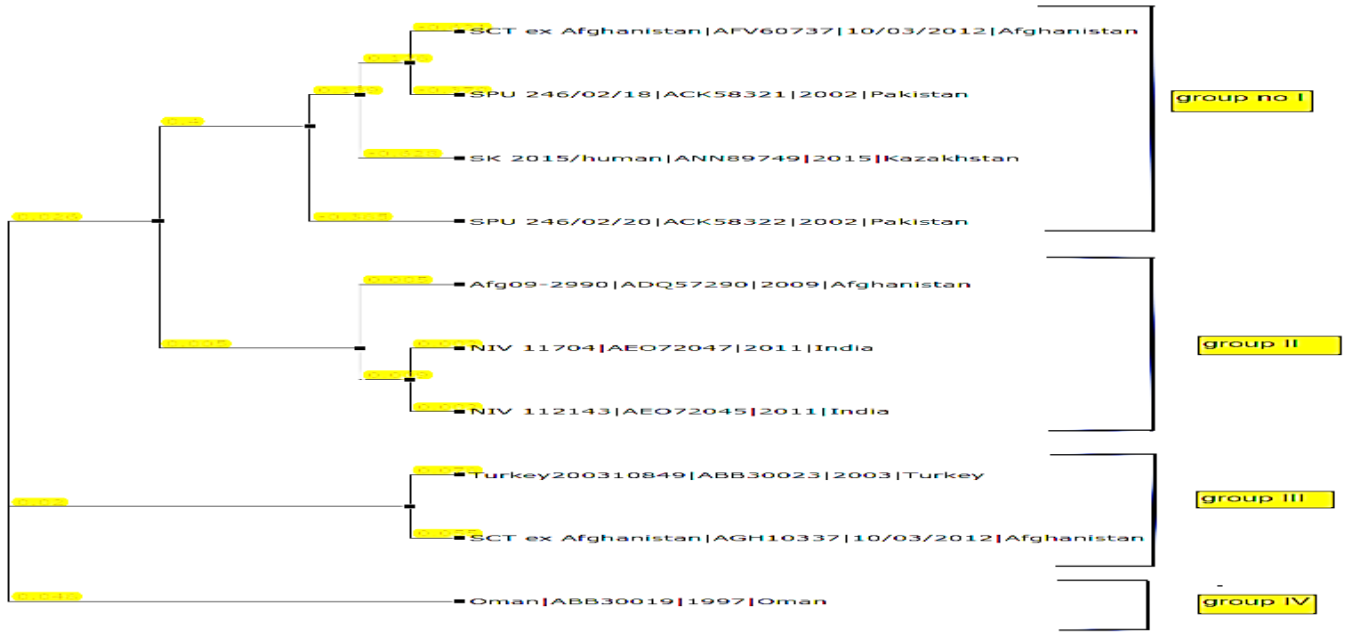

Fig. 1: Phylogram depicts relationship between Crimean-Congo hemorrhagic virus (CCHFV) Asian strains (previously published sequences) of L segment. Each leaf is labeled with branch length, strain name, accession number, date/year of isolation and location. Group I represents Pakistani strain's origin and close similarity with Afghanistan

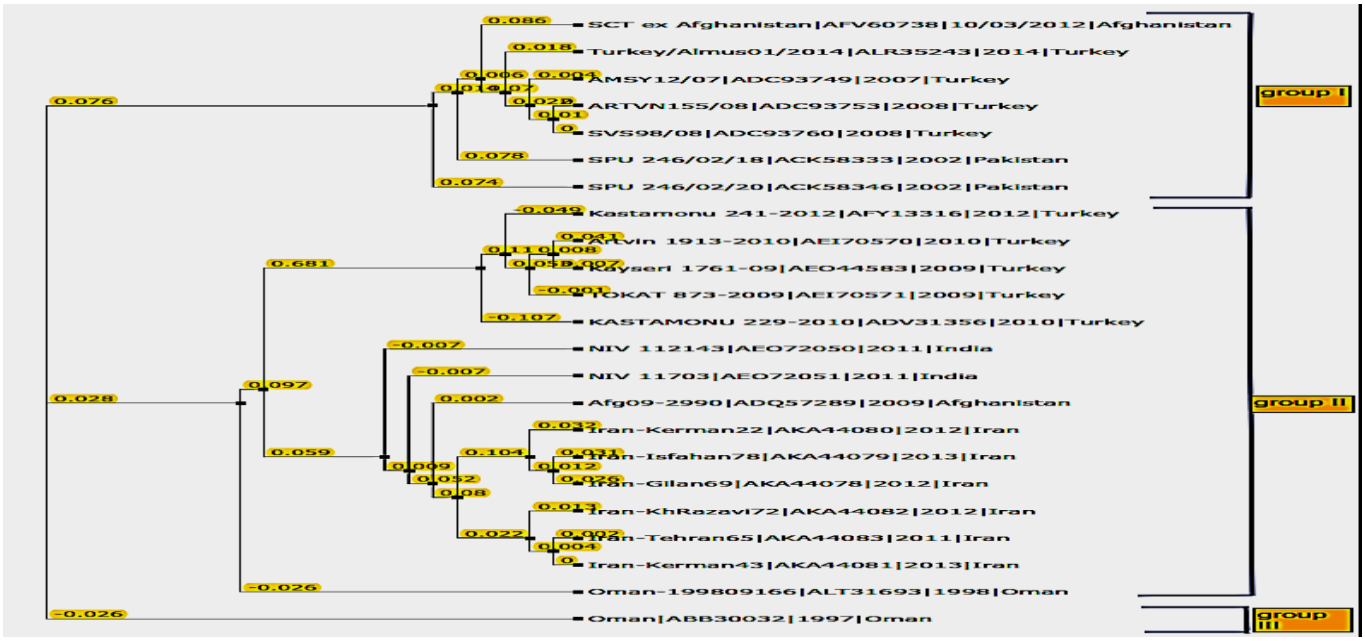

Fig. 2: Phylogram depicts relationship between Crimean-Congo hemorrhagic virus (CCHFV) Asian strains (previously published sequences) of M segment. Each leaf is labeled with branch length, (shows association of all strains with each other) strain name, accession number, date/year of isolation and location. Group I represents Pakistani strain's origin and close similarity with Afghanistan and Turkey

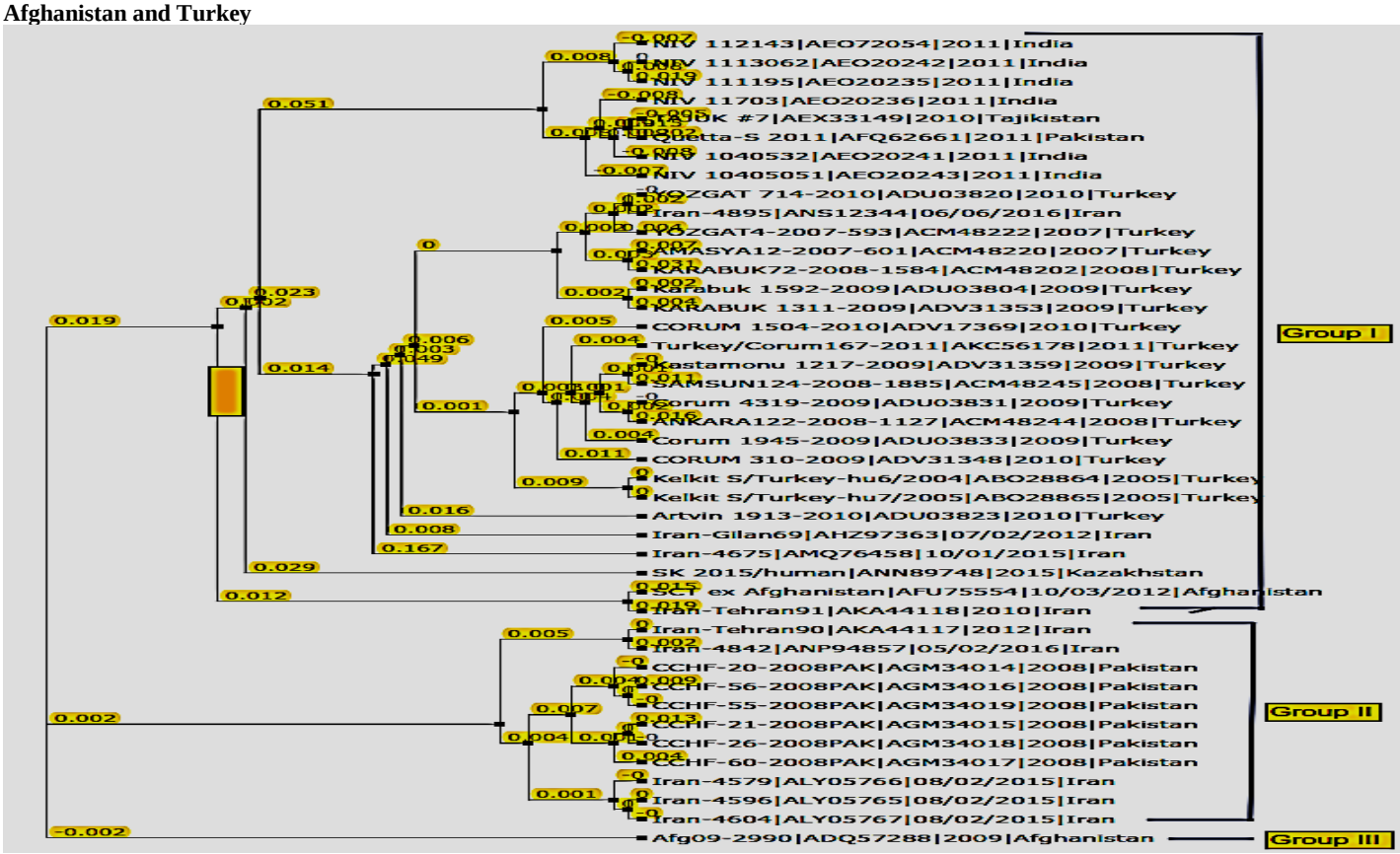

Fig no 3: Phylogram depicts relationship between Crimean-Congo hemorrhagic virus (CCHFV) Asian strains (previously published sequences) of S segment. Each leaf is labeled with branch length, (shows association of all strains with each other) strain name, accession number, date/year of isolation and location. Group I represents Pakistani strain's origin and close similarity with Iran and indirectly with Afghanistan.

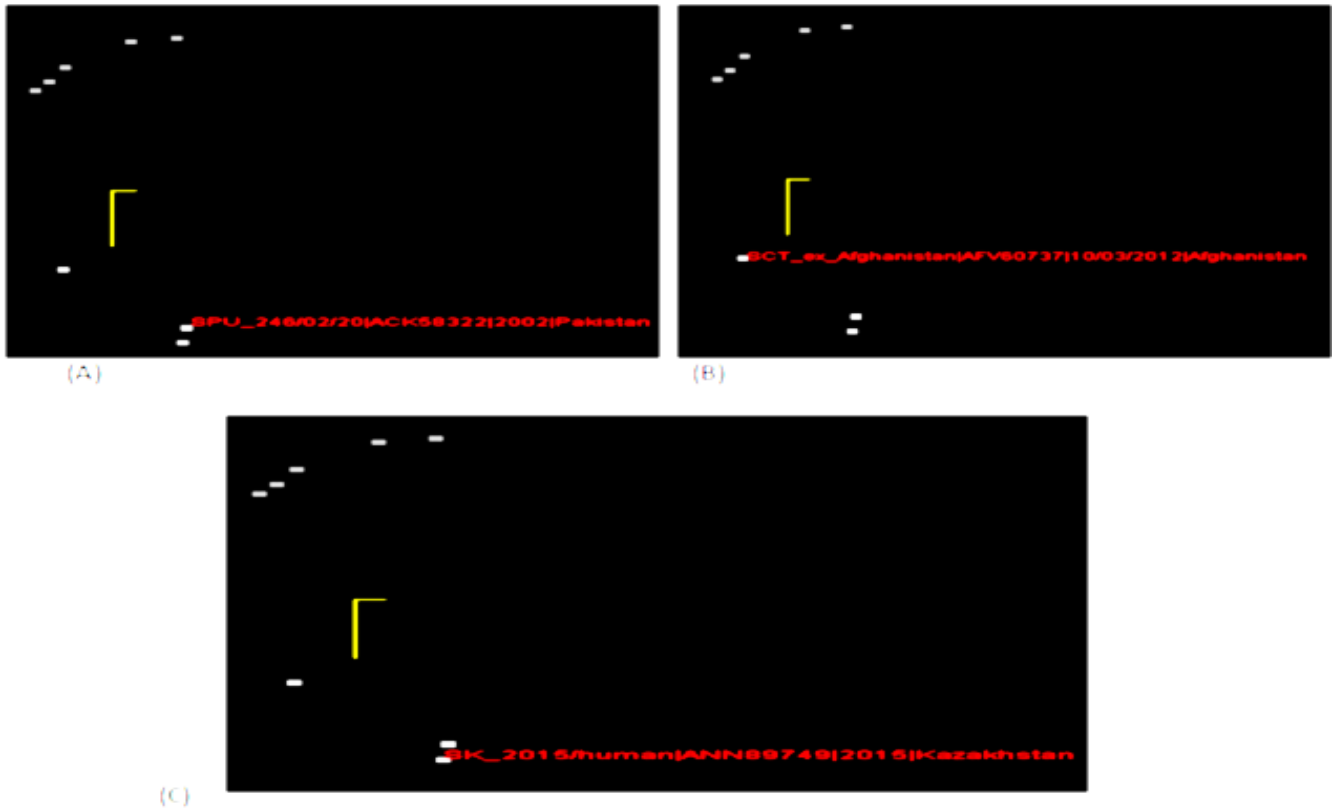

Fig. 4: PCA of L segment off all selected Asian strains, (A) Shows association of all strains highlighting Pakistani strain, (B) Highlighting Afghanistan, (C) Highlighting Kazakhstan

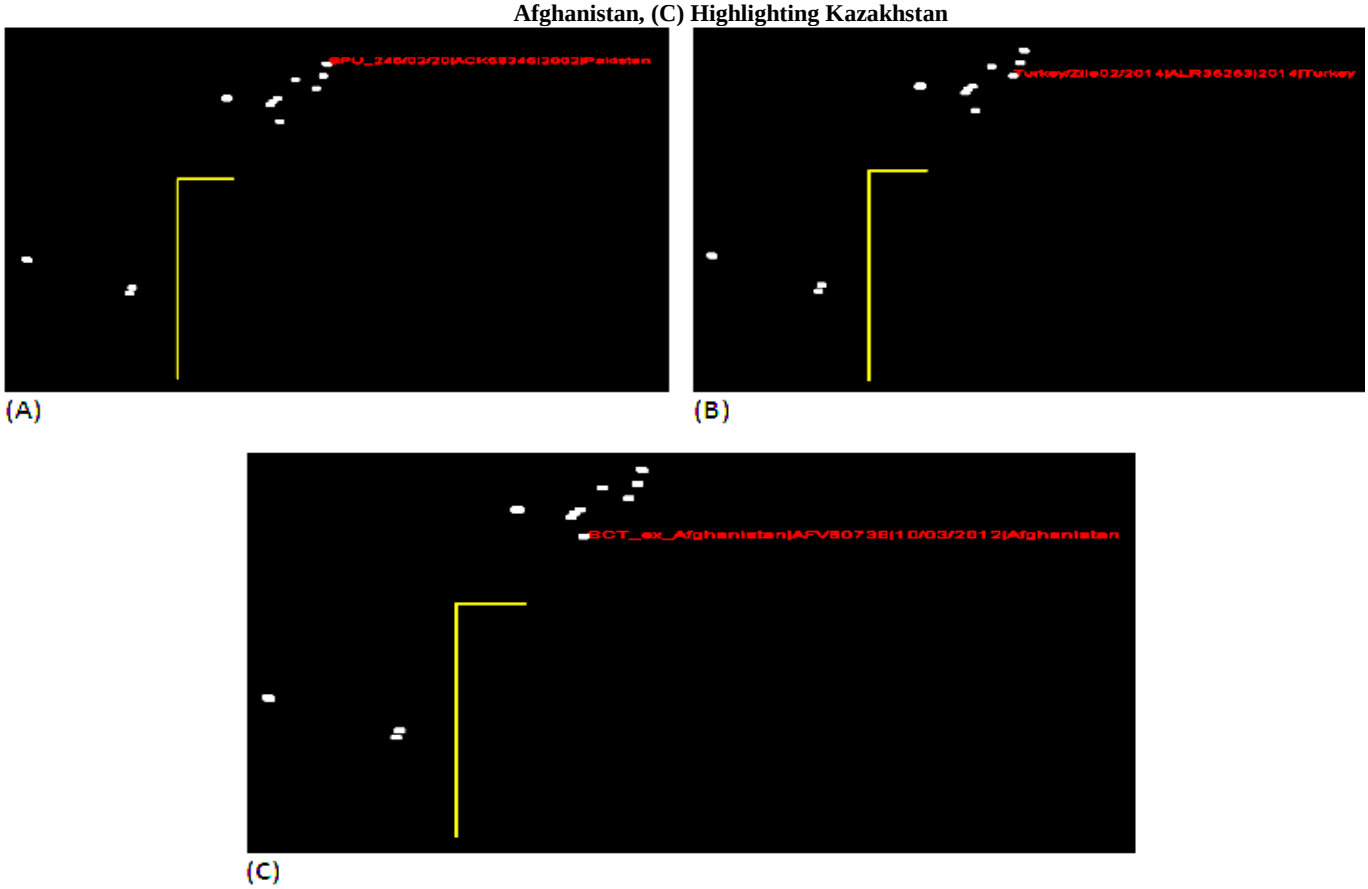

Fig. 5: PCA of M segment Each white square is a single or cluster of strains closely packed or far apart on the basis of similarity or differences respectively, (A) Shows Pakistani strain, (B) Highlighting turkey strain, (C) Shows Afghanistan strain

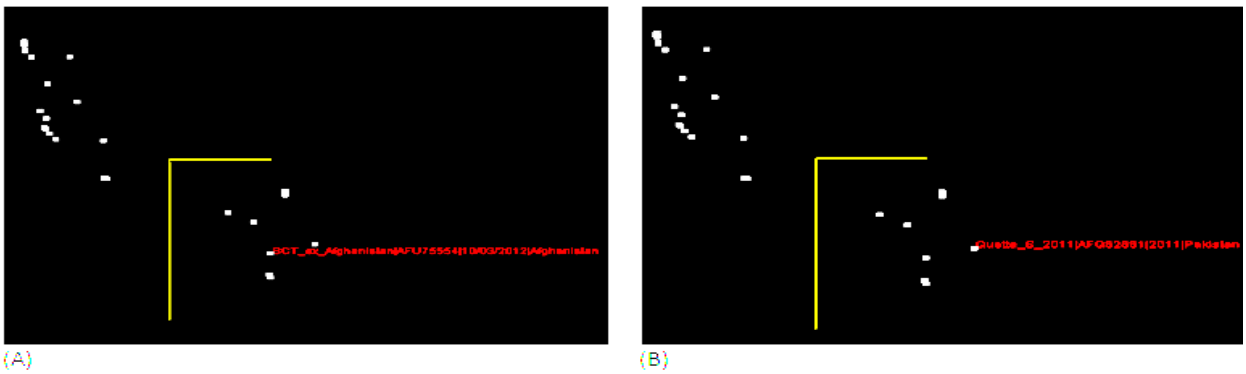

Fig. 6: principle component analysis (PCA) of S segment. Each white square is a single or cluster of strains closely packed or far apart on the basis of similarity or differences respectively, (A) Highlighting SCT_OX_afghanistan strain in three dimensional axis, (B) Highlighting Quetta_S-2011 Pakistani strain.

Antigenicity was checked using VAXIJEN, the Antigenicity score was 0.6253 which is valuable enough being above the cut off limit. We purpose this epitope will be able of provoking striking immunogenic response as a vaccine. Moreover, our effort would be a significant success in regard of vaccine production in near future.

CONCLUSION:

We conclude from our phylogenetic analysis and further variance analysis (PCA) of all Asian strains of CCHFV that Pakistani strains have close association with Afghanistani and turkey strains. Where L segment has direct association with Afghanistan while M segment with Afghanistan and turkey and S segment with Afghanistan, Turkey and Iran. Further insight analysis of all Asian strain's segments separately have enabled us to purpose a worthwhile prediction of an antigenic region (CAAAAGGTTAG) with antigenicity score being (0.6253). We are fully hoping it might be a blastic input for vaccine designing for all the Asian strains of CCHFV.

REFERENCES

1. Guglielmone AA, Robbins RG, Apanaskevich DA, Petney TN, Estrada-Peña A, et al. The hard ticks of the world. Springer, Dordrecht doi, (2014); 10(1007): 978-994.
2. Apanaskevich DA, Apanaskevich MA. Description of a new Dermacentor (Acari: Ixodidae) species from Thailand and Vietnam. Journal of medical entomology, (2015); 52(5): 806-812.
3. Apanaskevich MA, Apanaskevich DA. Reinstatement of Dermacentor bellulus (Acari: Ixodidae) as a valid species previously confused with D. taiwanensis and comparison of all parasitic stages. Journal of medical entomology, (2015); 52(4): 573-595.
4. Kamboj A, Pathak H. Crimean-Congo hemorrhagic fever: a comprehensive review. Veterinary World, (2013); 6(10).
5. Khan J, Rehman S, Fisher-Hoch S, Mirza S, Khurshid M, et al. Crimean Congo-haemorrhagic fever treated with oral ribavirin. The Lancet, (1995); 346(8973): 472-475.
6. Garcia S, Chinikar S, Coudrier D, Billecocq A, Hooshmand B, et al. Evaluation of a Crimean-Congo hemorrhagic fever virus recombinant antigen expressed by Semliki Forest suicide virus for IgM and IgG antibody detection in human and animal sera collected in Iran. Journal of clinical virology, (2006); 35(2): 154-159.
7. Suleiman MNEH, Muscat-Baron J, Harries J, Satti AGO, Platt G, et al. Congo/Crimean haemorrhagic fever in Dubai: an outbreak at the Rashid Hospital. The Lancet, (1980); 316(8201): 939-941.
8. Van Eeden P, Joubert J, Van de Wal B. A nosocomial outbreak of Crimean-Congo haemorrhagic fever at Tygerberg Hospital. Part I. Clinical features. (1985).
9. Ergonul O, Whitehouse CA Crimean-Congo Hemorrhagic Fever. Chapter: Book Name. 2007 of publication; Springer.
10. Flick R, Flick K, Feldmann H, Elgh F. Reverse genetics for Crimean-Congo hemorrhagic fever virus. Journal of virology, (2003); 77(10): 5997-6006.
11. Chinikar S, Ghiasi SM, Hewson R, Moradi M, Haeri A. Crimean-Congo hemorrhagic fever in Iran and neighboring countries. Journal of clinical virology, (2010); 47(2): 110-114.
12. Ozkaya E, Dincer E, Carhan A, Uyar Y, Ertek M, et al. Molecular epidemiology of Crimean-Congo hemorrhagic fever virus in Turkey: occurrence of local topotype. Virus research, (2010); 149(1): 64-70.
13. Vatansever Z, Uzun R, Estrada-Pena A, Ergonul O (2007) Crimean-Congo hemorrhagic fever in Turkey. Crimean-Congo Hemorrhagic Fever: Springer. pp. 59-74.
14. Nazir S, Faraz A, Shahzad N, Ali N, Khan MA, et al. Prevalence of HCV in β-thalassemia major patients visiting tertiary care hospitals in Lahore–Pakistan. Advancements in Life Sciences, (2014); 1(4): 197-201.
15. Javed Z, Iqbal MZ, Latif MU, Yaqub HMF, Qadri QR. Potent Implications of miRNA in Cancer Biology–A Brief Review. Advancements in Life Sciences, (2015); 2(3): 106-109.
16. DARVISH M, Hoogstraal H, Roberts T, Ghazi R, Amer T. A SERO-EPIDEMIOLOGICAL SURVEY FOR BUNYAVIRIDAE AND CERTAIN OTHER ARBOVIRUSES IN PAKISTAN. (1983).
17. Burney M, Ghafoor A, Saleen M, Webb P, Casals J. Nosocomial outbreak of viral hemorrhagic fever caused by Crimean Hemorrhagic fever-Congo virus in Pakistan, January 1976. The American journal of tropical medicine and hygiene, (1980); 29(5): 941-947.

18. Athar MN, Baqai HZ, Ahmad M, Khalid MA, Bashir N, et al. Short report: crimean-congo hemorrhagic fever outbreak in Rawalpindi, Pakistan, February 2002. The American journal of tropical medicine and hygiene, (2003); 69(3): 284-287.
19. Bangash S, Khan E. Treatment and prophylaxis with ribavirin for Crimean-Congo Hemorrhagic Fever--is it effective? JPMA The Journal of the Pakistan Medical Association, (2003); 53(1): 39-41.
20. Saleem J, Usman M, Nadeem A, Sethi SA, Salman M. Crimean–Congo hemorrhagic fever: a first case from Abbottabad, Pakistan. International Journal of Infectious Diseases, (2009); 13(3): e121-e123.
21. Ahmed S, Ijaz T, Masood S, Ijaz S, Younus M, et al. Last Decade of Dengue–and the Next One. Advancements in Life Sciences, (2013); 1(1).
22. Deyde VM, Khristova ML, Rollin PE, Ksiazek TG, Nichol ST. Crimean-Congo hemorrhagic fever virus genomics and global diversity. Journal of virology, (2006); 80(17): 8834-8842.
23. Blakqori G, Delhaye S, Habjan M, Blair CD, Sánchez-Vargas I, et al. La Crosse bunyavirus nonstructural protein NSs serves to suppress the type I interferon system of mammalian hosts. Journal of virology, (2007); 81(10): 4991-4999.
24. Bergeron É, Vincent MJ, Nichol ST. Crimean-Congo hemorrhagic fever virus glycoprotein processing by the endoprotease SKI-1/S1P is critical for virus infectivity. Journal of virology, (2007); 81(23): 13271-13276.
25. Whitehouse CA. Crimean–Congo hemorrhagic fever. Antiviral research, (2004); 64(3): 145-160.
26. Swanepoel R, Gill D, Shepherd A, Leman P, Mynhardt J, et al. The clinical pathology of Crimean-Congo hemorrhagic fever. Review of Infectious Diseases, (1989); 11(Supplement 4): S794-S800.
27. Swanepoel R, Shepherd A, Leman P, Shepherd S, McGillivray G, et al. Epidemiologic and clinical features of Crimean-Congo hemorrhagic fever in southern Africa. The American journal of tropical medicine and hygiene, (1987); 36(1): 120-132.
28. Hussain T, Jamal M, ur Rehman T, Andleeb S. Dengue: pathogenesis, prevention and treatment–A mini review. Advancements in Life Sciences, (2015); 2(3): 110-114.
29. Papa A, Bino S, Velo E, Harxhi A, Kota M, et al. Cytokine levels in Crimean-Congo hemorrhagic fever. Journal of clinical virology, (2006); 36(4): 272-276.
30. Yaqoob A, Shehzad U, Ahmad Z, Naseer N, Bashir S. Effective treatment strategies against Ebola virus. Advancements in Life Sciences, (2015); 2(4): 176-182.
31. Papa A, Papadimitriou E, Christova I. The Bulgarian vaccine Crimean-Congo haemorrhagic fever virus strain. Scand J Infect Dis, (2011); 43(3): 225-229.
32. Daud S, Shahzad S, Shafique M, Bhinder MA, Niaz M, et al. Optimization and Validation of PCR protocol for three Hypervariable Regions (HVI, HVII and HVIII) in Human Mitochondrial DNA. Advancements in Life Sciences, (2014); 1(3): 165-170.
33. Ahmeti S, Raka L. Crimean-Congo haemorrhagic fever in Kosova: a fatal case report. Virology journal, (2006); 3(1): 1.
34. Vesenjak-Hirjan J, Punda-Polić V, Dobe M. Geographical distribution of arboviruses in Yugoslavia. Journal of hygiene, epidemiology, microbiology, and immunology, (1990); 35(2): 129-140.
35. Chinikar S, Goya M, Shirzadi M, Ghiasi SM, Mirahmadi R, et al. Surveillance and Laboratory Detection System of Crimean-Congo Haemorrhagic Fever in Iran. Transboundary and emerging diseases, (2008); 55(5-6): 200-204.
36. Papa A, Ma B, Kouidou S, Tang Q, Hang C, et al. Genetic characterization of the M RNA segment of Crimean Congo hemorrhagic fever virus strains, China. Emerging infectious diseases, (2002); 8(1): 50-53.
37. Mustafa ML, Ayazi E, Mohareb E, Yingst S, Zayed A, et al. Crimean-Congo hemorrhagic fever, Afghanistan, 2009. Emerging infectious diseases, (2011); 17(10): 1940-1941.
38. Nabeth P. Crimean–Congo Hemorrhagic Fever Virus. Perspectives in Medical Virology, (2006); 16299-324.
39. Papa A, Christova I, Papadimitriou E, Antoniadis A. Crimean-Congo hemorrhagic fever in Bulgaria. Emerg Infect Dis, (2004); 10(8): 1465-1467.
40. Yashina L, Petrova I, Seregin S, Vyshemirskii O, Lvov D, et al. Genetic variability of Crimean-Congo haemorrhagic fever virus in Russia and Central Asia. Journal of General Virology, (2003); 84(5): 1199-1206.
41. Afzal S. Endemic Crimean Congo Hemorrhagic Fever in Pakistan. Annals of King Edward Medical University, (2016); 22(3).
42. Karti SS, Odabasi Z, Korten V, Yilmaz M, Sonmez M, et al. Crimean-Congo Hemorrhagic Fever, Turkey. Emerging infectious diseases, (2004); 101379-1384.
43. Yilmaz G, Buzgan T, Torunoglu M, Safran A, Irmak H, et al. A preliminary report on Crimean-Congo haemorrhagic fever in Turkey, March-June 2008. Euro surveillance: bulletin Europeen sur les maladies transmissibles= European communicable disease bulletin, (2008); 13(33): 717-727.
44. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res, (2004); 32(5): 1792-1797.
45. Steele RG. Florida licensure statistics. J Fla Med Assoc, (1979); 66(9): 940-941.
46. Dunteman GH Principal components analysis. Chapter: Book Name. 1989 of publication; Sage.
47. Doytchinova IA, Flower DR. VaxiJen: a server for prediction of protective antigens, tumour antigens and subunit vaccines. BMC Bioinformatics, (2007); 84.
48. Ergönül Ö. Crimean-Congo haemorrhagic fever. The Lancet infectious diseases, (2006); 6(4): 203-214.
49. Alam MM, Khurshid A, Shaukat S, Suleman RM, Sharif S, et al. Epidemiology and genetic diversity of rotavirus strains in children with acute gastroenteritis in Lahore, Pakistan. PLoS One, (2013); 8(6): e67998.
50. Rasheed A, Ullah S, Naeem S, Zubair M, Ahmad W, et al. Occurrence of HCV genotypes in different age groups of patients from Lahore, Pakistan. Advancements in Life Sciences, (2014); 1(2): 89-95.

51. Raziq A, de Verdier K, Younas M. Ethnoveterinary treatments by dromedary camel herders in the Suleiman Mountainous Region in Pakistan: an observation and questionnaire study. J Ethnobiol Ethnomed, (2010); 616.
52. Olschlager S, Gabriel M, Schmidt-Chanasit J, Meyer M, Osborn E, et al. Complete sequence and phylogenetic characterisation of Crimean-Congo hemorrhagic fever virus from Afghanistan. J Clin Virol, (2011); 50(1): 90-92.
53. Tahmasebi F, Ghiasi SM, Mostafavi E, Moradi M, Piazak N, et al. Molecular epidemiology of Crimean- Congo hemorrhagic fever virus genome isolated from ticks of Hamadan province of Iran. J Vector Borne Dis, (2010); 47(4): 211-216.
54. Chinikar S, Ghiasi SM, Hewson R, Moradi M, Haeri A. Crimean-Congo hemorrhagic fever in Iran and neighboring countries. J Clin Virol, (2010); 47(2): 110-114.
55. Burt FJ, Swanepoel R. Molecular epidemiology of African and Asian Crimean-Congo haemorrhagic fever isolates. Epidemiol Infect, (2005); 133(4): 659-666.
56. Yashina L, Petrova I, Seregin S, Vyshemirskii O, Lvov D, et al. Genetic variability of Crimean-Congo haemorrhagic fever virus in Russia and Central Asia. J Gen Virol, (2003); 84(Pt 5): 1199-1206.
57. Ahmed AA, McFalls JM, Hoffmann C, Filone CM, Stewart SM, et al. Presence of broadly reactive and group-specific neutralizing epitopes on newly described isolates of Crimean-Congo hemorrhagic fever virus. J Gen Virol, (2005); 86(Pt 12): 3327-3336.