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Characterization of a strain of quaranfil virus isolated from soft ticks in India. Is quaranfil virus an unrecognized cause of disease in human and animals?"

Devendra T. Mourya*, Pragya D. Yadav, Dimpal A. Nyayanit, Triparna D. Majumdar, Shilpi Jain, Prasad Sarkale, Anita Shete

ICMR-National Institute of Virology, Pune, India

* Corresponding author.

E-mail addresses: directorniv@gmail.com, dtmourya@gmail.com (D.T. Mourya).

Abstract

The soft ticks collected during a field survey in Karnataka state, India, in 1983, yielded a novel virus isolate, which caused mortality in an infant mouse upon inoculation. Attempts at characterizing the virus using the conventional methods were unsuccessful, which prompted us to study it by Next-Generation Sequencing (NGS). This virus isolate was obtained from the viral repository of National Institute of Virology, and an initial virus stock was prepared as a mouse brain homogenate. The virus stock showed cytopathic effects in different cell-lines and was used in NGS. Based on the complete genome sequence, obtained using *de novo* and reference mapping approach, the virus isolate was identified as a Quaranfil virus (QRFV) belonging to the family *Orthomyxoviridae*, genus Quaranjavirus. The genome size of the virus is 11,427 nucleotides which consist of 6 segments encoding six proteins. Homology analysis suggested this isolate as similar to QRFV of Afghanistan. *In silico* analysis showed the HA protein secondary structure to be a class III penetrance similar to Thogotovirus. QRFV

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was first isolated in 1953 from ticks [Cairo, Egypt] and subsequently reported from other geographical areas. This is the first report describing the presence of QRFV from India. This discovery emphasizes the need for investigating mild febrile illness cases with influenza-like symptoms, particularly in the area of high risk for tick bites.

Keywords: Bioinformatics, Virology

1. Introduction

Quaranfil virus (QRFV), an arbovirus, was first isolated from ticks (*Argas arboreus*) collected near Cairo, Egypt in 1953 [1]. Since then, it has been isolated from nestling cattle egrets, pigeon squabs and children suffering from mild febrile illnesses in Quaranfil, Egypt. Subsequently, different strains of QRFV have been isolated from ticks and seabirds in different geographical areas of Egypt, South Africa, Afghanistan, Nigeria, Kuwait, Iraq, Yemen and Iran [2, 3, 4]. Although serological studies in Egypt showed around 8% of the studied human population had neutralizing antibodies to QRFV, there is no known definitive evidence that QRFV causes clinical illness and diseases outcome in humans. The virus causes encephalitis-like symptoms and death in chickens, mice and guinea pigs [5, 6, 7]. Based on morphological and morphogenetic characteristics, this virus was previously assigned to family *Arenaviridae* [8]; however, recent genomic and serological data support its classification in the family *Orthomyxoviridae* [9].

The *Orthomyxoviridae* family consists of six genera: Influenza virus A, Influenza virus B, Influenza virus C, Isavirus, Thogotovirus, and Quaranjavirus. The QRFV belongs to a relatively new genus *Quaranjavirus* [9]. Orthomyxoviruses are negative-sense single-stranded RNA viruses having multi-segmented RNA genomes. The RNA segments in orthomyxoviruses code for polymerases essential for replication of the virus, polymerase PA (PA), polymerase PB1 (PB1), polymerase PB2 (PB2) and a surface protein, hemagglutinin (HA). Their high propensity for genetic reassortment often leads to the emergence of new viral strains threatening public health.

In this study, the unknown virus isolated from soft ticks, collected in 1983 in Karnataka State, India, was characterized using Next-Generation Sequencing (NGS) as QFRV. The QRFV has been reported to cause human infection; hence there is a need for surveillance for this virus in India. Complete genome sequencing of this virus would be the first step towards the development of diagnostic assays to detect QRFV infections and would help in understanding its geographical distribution in India.

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2. Materials and methods

2.1. Mice and ethical statement

CD1 suckling mice (0−2 days old) were used for preparing the initial virus stock. The present study had the approval of the Scientific Advisory Committee, Institutional Bio-safety Committee and Institutional Animal Ethics Committee (CPCSEA Registration No. 43/GO/ReBi/SL/99/CPCSEA). Institutional guidelines which adhere to National and International regulation were followed for the animal-related study.

CD1 mice used for the study were procured from the animal house of NIV, Pune. These mice were purchased from Charles River Laboratory, USA by NIV, Pune. The mice were in groups comprised of 1 mother and 8 pups. Mice were maintained under optimal housing conditions at a temperature of 24−26 °C with a 12/12 h light−dark cycle. The mother had free access to feed and water *ad libitum*.

2.2. In-vivo and in-vitro virus stock [strain 83939] preparation

The lyophilized virus isolate [NIV 83939] was received from the NIV Pune virus registry and revived by inoculating in CD1 suckling mice. The isolate was re-suspended in 0.5 mL bovine albumin phosphate saline (BAPS). Each infant was inoculated intracerebrally (ic) with 20 μL of the virus. The mice were observed on a daily basis for signs of illness. When showing clinical signs such as anorexia, inability to move followed by emaciation on the 5^{th}-day post inoculation (DPI), they were culled using >10% isoflurane anesthesia followed by cervical dislocation and their brains were harvested as 10% (w/v) suspension in 1.25% BAPS. The carcasses were disposed of as per the Institutional Bio-safety Committee's bio-waste disposal protocol.

To understand the susceptibility of this virus in various cell-lines, virus was diluted in 1 ml of sterile PBS and 100 μL inoculated in multiple cells-cultures (Vero CCL-81, Vero E6 cells (African green monkey kidney), Baby Hamster Kidney 21 (BHK-21), Rhabdomyosarcoma (RD), Pipistrellus ceylonicus bat cell culture, SW-13 (human adrenal cortex), A549 (human lung carcinoma) and porcine stable kidney (PS)) of different origin. *Pipistrellus* bat cell culture was developed in the NIV, Pune and the rest of the cells were procured from the National Centre of Cell Science (NCCS), Pune, India and maintained in NIV cell culture laboratory [10]. The media used for Vero CCL81, Vero E6, and RD was Minimum Essential Media (MEM); for BHK the media used was MEM+ 5% tryptose phosphate broth (TPB). The media used for SW-13 and A549 was Leibovitz's L-15 and F-12 Nutrient mixture respectively. Dulbecco's Modified Eagle's medium (DMEM) was used for both bat cells and PS cells. These media were supplemented with 10% Fetal Bovine Serum (FBS) and maintained at 37 °C. The inoculated cell cultures were observed daily for cytopathic effect (CPE).

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RNA was extracted from 1 mL of the supernatant of infected Vero-CCL81 using Tripure® (Roche, IN, USA) reagent and a QIAamp viral RNA mini kit (Qiagen, Germany) as per the manufacturer's protocol with a minor modification. The extracted RNA was eluted in 50 μL of nuclease-free water (NFW) and stored at −80 °C until further use. Similarly, 1 mL each of the cell culture supernatant and cell pellets of viral isolates were used for DNA extraction using a QIAmp DNA extraction kit (Qiagen) as per manufacturer's instructions. The DNA was eluted in 50μL nuclease-free water and stored at -20 °C until further use.

2.3. Identification of viral isolates using genus- and virus-specific qRT-PCR and RT-PCR

The RNA extracted from the infected cell culture fluid and the cell pellet was tested by a single step or nested RT-PCR assays for the detection of different viruses using specific primers and Superscript-III RT-PCR with platinum Taq DNA polymerase (Invitrogen).

The isolate being tick-borne, the cell culture fluid was predominantly tested for the presence of tick-borne viruses. The genus *Nairovirus* [family *Nairoviridae*]-specific PCR primers were used for RT-PCR as reported by Lambert and Lanciotti [11]. The samples were tested for the tick-borne Ganjam virus (GANJV) (genus *Nairovirus*) using a nucleocapsid gene-specific RT-PCR protocol reported by Yadav et al. [12] and for tick-borne encephalitis virus (TBEV) as described by Katargina et al. [13]. Genus-specific PCR targeting B, P, G and N genes of the tick-borne Thogoto virus (family *Orthomyxoviridae*) was also performed using the protocols reported by Olivia et al. [14]. For detecting flaviviruses, a nested RT-PCR designed for flavivirus genus, reported by Scaramozzino et al. [15] was used.

The isolate was also tested for the presence of Oya virus (Orthobunyavirus), as described by Yadav et al. [16]. RT-PCR targeting the S and L gene segments of Phlebovirus, Heartland and Severe Fever with Thrombocytopenia Syndrome virus (SFTS) as described by Mourya et al. [17] were also performed. Araguari and Dhori viruses (members of the family *Orthomyxoviridae*) and genus Thogotovirus were also tested by RT-PCR as reported by Da Silva et al. and Sang et al. [18, 19]. Screening for Bhanja virus, another tick-borne Phlebovirus, was done as per the protocol by Shah and workers [20]. A real-time RT-PCR for Crimean Congo hemorrhagic fever virus (CCHFV) a tick-borne virus of the *Nairoviridae* family, was also tested by a real-time RT-PCR [21] and also using primers and probe specific for the S-segment of the viral genome as described by Atkinson et al. [22]. RT-PCR for the detection of Kyasanur Forest disease virus (KFDV) was performed using NS5 gene-specific primers and probe as described by Mourya et al. [23]. Details of the primers used are also tabulated as Table 1.

https://doi.org/10.1016/j.heliyon.2019.e01368

Table 1. List of the different primers used in the identification of the novel virus.

Virus Target	Gene	Primers	Ref
Nairovirus	S segment	**Nairo forward:** 5′-TCTCAAAGAAACACGTGCCGC-3′ **Nairo reverse:** 5′-GTCCTTCCTCCACTTGWGRGCAGCC TGCTGGTA-3′	11
Ganjam virus	S segment	**GanjamVS-F1:** 5′-TCAGGATATGAGGTTAGCAT-3′ **GanjamVS-R1:** 5’ -TAATCATGTCGGATAGCATGT-3′	12
	L gene	**Ganjam VL-F1:** 5′-TCTGACTGGTAGAACAACTGGT-3′ **Ganjam VL-R1:** 5′- TGTGTTGTCTCCTGATATACAGATT-3′	
	M gene	**GanjamVM-F1:** 5′-GCACTAGCAGGTTAATCTCAA-3′ **GanjamVM-710R:** 5′-GAGCTACAATTGCTGTATCTT-3′	
Tick-borne encephalitis virus	NS3	**TBENS3 3005F:** 5′-TGTGACACIGGAGTSATGGG-3′ **TBENS3 3162R**: 5′-GTGTGRCTIGCIGGCCA-3′	13
Thogoto virus	N gene	**THONF:** 5′-CCTGCAGGGGCGGAAGTTATG-3′ **THONR:** 5′-AAAATCCTCGCAGTTGGCTATCA-3′	14
	B gene	**Thogoto B gene F:** 5′- GGCCCAACCCTCCTATCTTACTGC -3′ **THogotoB gene R:** 5′-ATCTCTTTGGCTGCATCCTCTACA -3′	
	P gene	**THogotoP geneF:** 5′-ATCTACCCCAGCATCAGTCATCACCT -3′ **THogotoP geneR:** 5′-CTTCCATCTGAGGGTTGTTG -3′	
	G gene	**THogotoG geneF:** 5′-ATTACCCGCCCAGCAGAGATGAGTTA -3′ **THogotoG geneR:** 5′-CCTCCAGCAGTTCGTCCTTAGAGA -3′	
	N gene	**THogotoN geneF:** 5′-CCTGCAGGGGCGGAAGTTATG -3′ **THogotoN geneR:** 5′-AAAATCCTCGCAGTTGGCTATCA -3′	
Flavivirus	NS5 gene	**FLV3cFD2:** 5′-GTGTCCCAGCCGGCGGTGTCATCAGC-3′ **FLV1MAMD:** 5′-AACATGATGGGAAAGAGAGAGAA-3′ **FLV2FS778:** 5′-AAGGGTAGCACGGCTATTTGGT-3′	15
Oya virus	S segment	**OYA RTP F1:** 5′-ACCGCCTCTCTGGATACCTT-3′ **OYA RTP R1:** 5′-CAAATCCAAGGTAAAGGGCA-3′ **OYA RTP F2:** 5′-TGCCCTTTACCTTGGATTTG-3′ **OYA RTP R2:** 5′-AATGCTGCCTTAATTGCCAC-3′	16
Phlebovirus	S gene	**SVFS-A Fwd:** 5′-ACACAAAGCTCCCTA G-3′ **SVFS-A Rev:** 5′-ACACAAAGACCCCCTAG-3′	17
	L gene	**SVFL-A Fwd:** 5′-ACACAAAGGCGCCCAATC-3′, **SVFL-A Rev:** 5′-ACA CAA AGA CCG CCC A-3′	
	M gene	**SVMF:** 5′-ACAAAGACGGTGC-3′ **SVFMR:** 5′-ACACAAAGACCGGTG C-3′	
Araguari	Nucleoprotein	**ARAGOS5F**: 5′-GATRMLATCTTGTGGCAG-3′ **ARAGOS5R:** 5′- ARAACTCCMAGRTCTTC -3′	18
Dhori viruses	P gene	**DHORINF2_384:** 5′-TGGTACCCTTTTCTTGCTTCACTCC-3′ **DHORINR2_1012:** 5′- TGCTCTTCCTCGGCTCAAACACCA-3′	19
Bhanja virus	S gene	**Bhanja S seg F1:** 5′-CACAAAGAAGCCGCTAACGA-3′ **Bhanja S seg R1:** 5′-TGACTCAAAGGATCGCAG AA-3′ **Bhanja S seg F2:** 5′-TGAATGTCTCCTGGGTCTCAA-3′ **Bhanja S seg R2:** 5′-ACACAGAGAAGCCGCAGAAT-3′	20
Crimean Congo	P gene	**CCHF-F2 :** 5′-TGGACACCTTCACAAACTC-3′ **CCHF-R3:** 5′-GACAAATTCCCTGCACCA-3′	21
KFDV	NS5 gene	**KFD NS5 3S:** 5′-GTCAGATGAACAAAATCGCTGG-3′ **KFD NS5 4R:** 5′-TCATCCCCACTGACCAGCAT-3′	23

https://doi.org/10.1016/j.heliyon.2019.e01368

2.4. Next-Generation Sequencing of virus from DNA and RNA samples of the virus isolate

Libraries were prepared using the RNA extracted from the infected cell pellet and cell culture supernatant. The concentration of the extracted RNA was quantified by Qubit® 2.0 Fluorometer (Invitrogen) using Qubit RNA High Sensitivity (HS) kit and a rRNA depletion protocol was used to remove host ribosomal RNA using the NEB NextrRNA depletion kit (New England Biolabs). Re-quantification of the purified RNA was performed again by using Qubit® 2.0 Fluorometer (Invitrogen) and used for RNA library preparation with TruSeq Stranded mRNA Library preparation kit (Illumina). Nextera-XT DNA library preparation kit (Illumina) was used as per the manufacturer's instructions for preparation of DNA sequencing libraries. DNA as well as RNA libraries were quantified using the KAPA Library Quantification Kit (Kapa Biosystems, Roche) as per the manufacturer's protocol and loaded on the Illumina Miniseq NGS platform. The FASTQ files generated after the completion of the run were analyzed using CLC Genomics Workbench software Version 10 (CLC, Qiagen).

A *de-novo* assembly program as implemented in CLC Genomics Workbench was used to assemble contiguous sequences (contig) for the paired-end reads of DNA as well as RNA. RNA and DNA contigs generated were searched using the BLAST program to identify the virus. After identification of the virus, reference mapping was performed using the reference sequence downloaded from the GenBank. For the functional annotation of the putative protein, a homologous protein comparison was done using BLASTx. These six genomic sequences of the quaranfil virus have been deposited in the GenBank database under accession nos. MG770331−MG770336. All the data in this study is already accessible in the paper.

2.5. Characterization and phylogenetic analysis of the virus isolate

Reference sequences for all six genes of all genera belonging to the *Orthomyxoviridae* family were retrieved from GenBank to perform the phylogenetic analysis. ClustalW algorithm (MEGA Version 7.0) was used as the method of sequence alignment [24]. The phylogenetic trees were created for PA, PB1, PB2, HA, the hypothetical and the unknown gene. General Time Reversible (GTR) + G + I nucleotide substitution model was used to generate a phylogenetic tree using maximum likelihood method along with 1000 bootstrap replications. Percent nucleotide (nt) divergence and amino acid (aa) divergence was also calculated using the P-distance method. The mass of the putative protein was calculated from the protein molecular weight prediction site (https://www.bioinformatics.org/sms/prot_mw.html). The secondary structure of hemagglutinin (HA) protein was predicted using the PSIpred software.

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3. Results and discussion

3.1. Virus isolation in mice and susceptibility of vertebrate and invertebrate cells

Signs of sickness appeared in one of the eight inoculated mice on the 3rd DPI. The mouse stopped feeding and showed reduced mobility. On the 4th DPI, three mice were anorexic, weak and emaciated while others were feeding adequately. On the 5th DPI, one mouse was dead and all others showed anorexia, emaciation and significantly reduced mobility. All the mice were culled and their brains were harvested.

Multiple cells-lines (Vero *CCL81*, Vero E6 cells, BHK-21, RD, PS, Pipistrellus ceylonicus bat cell culture) were found to be susceptible for this virus while SW-13 and A549 did not show any cytopathic effect (Table 2). The infected Vero-CCL and BHK-21 cells displayed CPE and the cells and supernatants were used as raw samples to obtain NGS data.

3.2. Identification of viral isolates using genus- and virus-specific qRT-PCR and RT-PCR

RT-PCR and real-time RT-PCR assays were found negative for GANJV, CCHFV, KFD, TBEV, Thogotovirus, Oya virus, Aragose virus, Bhanja, SFTS, and Dhori viruses. The initial screening of the isolate demonstrated it to be distinct from all the RT-PCT and qRT-PCR tested viruses.

Next-Generation Sequencing of virus from DNA and RNA samples of the virus isolate as Quaranfil virus.

Table 2. Susceptibility of vertebrate cell culture to QRFV with passage number and day on which the CPE observed.

Cell lines	Media used	Passage number of cells	CPE on PID* (passage 2)
VeroCCL-81	MEM	33	PID 4
Bat embryo (Pipistrellus ceylonicus)	DMEM	84	PID 4
PS (porcine stable kidney)	MEM	12	PID 3
BHK-21 (baby hamster kidney)	MEM +5% TPB	102	PID 3
SW-13 (human adrenal cortex)	DMEM	67	No CPE
RD (Rhabdomyosarcoma)	MEM	82	PID 4
Vero-E6	MEM	75	PID 4
A549 (human lung carcinoma)	Ham's F-12K (Kaighn's)	105	No CPE

* PID, post-infection days.

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The raw data generated using cell culture, the supernatant and the cell pellet were combined for each DNA (138 MB) and RNA sample (72.4 MB), carried out individually in independent runs. This was analyzed further using CLC genomic workbench (Qiagen). A total of 10,960 and 3,101 contigs were generated from DNA and RNA paired-end reads with an average length of 446 nucleotides (nt) and 432 nt, respectively. BLAST analysis of the DNA contigs generated from *de-novo* assembly led to the identification of an intracisternal particle from Syrian hamster. Reference mapping of the DNA reads led to the retrieval of complete clone sequence for Syrian hamster intracisternal A-particle gene. A nucleotide identity of 99% was observed when the retrieved sequence was compared with the reference genome (HAMIAP18C) downloaded from GenBank [25]. Analysis of RNA contigs generated by the *de-novo* assembly was performed using BLASTx. BLASTx identified the sequences to be similar to QRFV with more than 85% query coverage for PA, PB1, PB2, HA, NP and unknown gene (96%, 97%, 97%, 97%, 98% and 96%) from *Orthomyxoviridae* family respectively. Subsequent to the identification of this unidentified virus isolate, reference mapping was performed for all six segments of QRFV. Complete six gene segments of the virus were retrieved and reconfirmed using BLASTn. These retrieved sequences showed highest homology for nucleotide PA, PB1, PB2, HA, NP and unknown (90%, 91%, 91%, 92%, 91% and 92% respectively) to Quaranfil virus origin from Afghanistan. The final complete genome of 11,427 nucleotides was obtained from the Indian isolate of QRFV. QRFV has an average GC content of 49.6%, demonstrating that the genome has almost equal amounts of AT and GC content. Six genomic segments were obtained, that encoded for the structural as well as non-structural proteins required for its replication, transcription and other essential functions. The genomic characterization of the virus is tabulated in Table 3. Analysis of the 5′ and 3′ termini showed the presence of conserved residues, which were in agreement with the published sequence [9]; however, we were unable to obtain the entire 5′ and 3′ ends for all the segments.

Table 3. Genomic characterization of QRFV segments retrieved from next-generation sequencing of the *Quaranjavirus* isolate from India

Sr. No.	Segment	Accession number	Nucleotide/Amino Acids	Putative protein mass (kDa)	Putative Function	Accession number of homologous sequence	% GC content
1	PA	MG770331	2420nt/778 aa	88.92	Polymerase	ACY56279.1 (96%)	50.6
2	PB1	MG770333	2385nt/777aa	88.06	Polymerase PB1	ACY56282.1 (97%)	48.2
3	PB2	MG770335	2410nt/783 aa	90.79	Polymerase PB2	ACY56278.1 (97 %)	47.7
4	HA	MG770332	1617nt/511 aa	57.2	Hemagglutinin	ACY56281.1 (97%)	49.3
5	NP	MG770334	1708nt/527 aa	59.31	Hypothetical protein	AEW22798.1 (98%)	50.1
6	ML	MG770336	887nt/266 aa	29.55	Unknown	ACY56280.1 (95%)	52.1

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3.3. Characterization and phylogenetic analysis of quaranfil virus

Single Open Reading Frame (ORF) was identified for different segments of QRFV and translated into protein. Four of the six-genomic segments of QRFV encode protein starting with an initiation codon having a strong Kozak sequence (RNNAUGG) [26]. The segment encoding for polymerase protein (PA) has moderate Kozak sequence, whereas the HA protein has a weak Kozak sequence. The presence of a weak Kozak demonstrates that the HA protein would be expressed in low levels or it might have an efficient binding affinity towards polymerase with which it can maintain its concentration within the cell as compared to the sequence with strong Kozak sequences such as polymerase PB1, polymerase PB2, hypothetical (NP; Probable) and the unknown protein.

We could identify the presence of four conserved motifs based upon the sequence analysis of polymerase basic protein I (PB1) belonging to Thogoto and Quarjagroup viruses: motif I – ^{332}KxNECLxP^{340}; motif II – ^{331}GMxMGMxNx (2) STxLAL^{454}; motif III – ^{470}VxSSDD^{475}; motif IV – ^{499}LKX (2)GxNxSx (2)K^{510} also depicted in Fig. 1. The presence of these conserved motifs indicates the PB1 protein to be conserved for the tick-isolated viruses belonging to the orthomyxovirus family.

The nucleoprotein and HA proteins are the major structural determinants associated with the genomic RNA of orthomyxoviruses and used for typing. A comparison of nucleoprotein sequences revealed conserved regions in the Thogoto virus and Quaranjavirus. However, the conservation of amino acids was lesser towards the N-terminal, for the compared group, than for the second half (Fig. 2) towards the C-terminal which is also the proposed site for RNA interaction. A similar observation was reported for

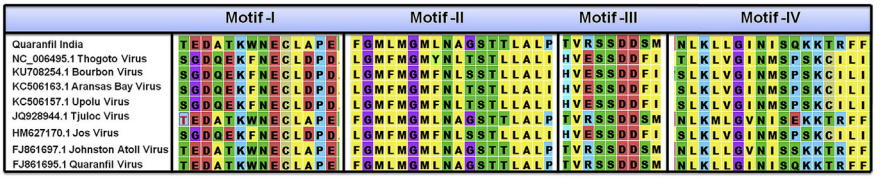

Fig. 1. Multiple alignments of closely associated viruses with QRFV PB1 (RNA polymerase) protein.

Fig. 2. Multiple alignments of closely associated viruses with QRFV nucleoprotein protein.

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Thogoto virus, Dhori virus and Influenza A virus by Briese et al., [26]. Classical bipartite nuclear localization signal has been observed in Thogoto viruses [27] and similar bipartite nuclear localization signal was observed for Jos virus [28]. However, a similar bipartite nuclear localization signal for nucleoprotein of QRFV could not be observed. The predictprotein (https://www.predictprotein.org/) software predicted nucleoprotein to be localized within the nucleus. Further, a bipartite nuclear localization signal (^{207}EKTVRKRKGGVITLEKKAVVVLETVHLKRNED^{238}) was predicted using the cNLS Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi).

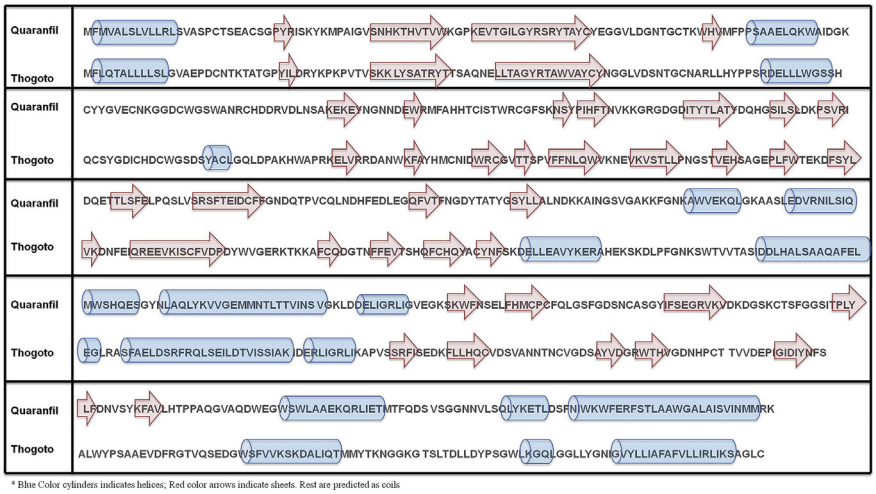

Fig. 3. Comparison of HA protein secondary structures between Thogoto and QRFV using the PSIPRED software.

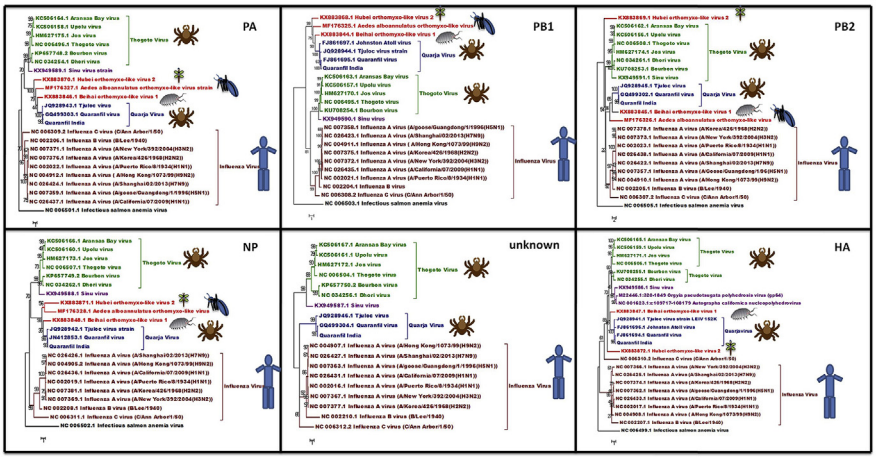

Fig. 4. A Phylogenetic tree for the different gene segments of QRFV: A discrete gamma distribution model along with (+G, parameter, and +I) was used to model the rate of evolutionary difference among the 20 sequences among orthomyxovirus family. Bootstrap method of 1000 re-sampling was used to determine the robustness of the generated tree.

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Table 4. Percent nucleotide and amino acid divergence for each segment of Indian Quaranfil virus with other reference orthomyxoviruses segments.

Compared Virus	PA%		PB1%		NP%		HA%		PB2%		Unknown%	
	Nt	AA	Nt	AA	Nt	AA	Nt	AA	Nt	AA	Nt	AA
Quaranfil virus	12.01	7.48	8.94	2.55	8.93	2.87	8.16	2.73	8.59	2.77	7.89	6.25
Johnston Atoll virus	**	**	24.96	16.4	**	**	30.33	26.92	**	**	**	**
Josvirus	67.76	86.91	62.58	77.73	69.22	88.79	61.76	76.1	68.02	87.72	72.24	92.49
Tjuloc virus	28.49	25.03	25.72	13.54	25.14	14.42	25.03	15.63	25.99	15.85	34.29	39.84
Upolu virus	69.49	88.23	62.2	78.06	70.81	89.79	63.85	77.67	67.84	87.86	73.9	92.03
Aransas Bay virus	67.81	87.74	62.38	77.78	70.47	89.79	62.23	77.14	68.38	87.45	71.39	92.89
Bourbon virus	68.24	87.74	62.84	78.22	68.63	87.55	61.07	76.78	68.83	89.19	71	89.92
Sinu virus	68.23	87.18	62.81	77.59	69.62	89.19	62.94	78.83	70.55	91.32	74.09	93.63
Influenza A virus (A/Puerto Rico/8/1934(H1N1))	67.92	88.07	63.27	76.05	70.71	91.06	71.69	92.05	66.98	84.84	69.18	84.71
Influenza B virus (B/Lee/1940)	68.87	88.42	61.44	74.37	69.37	90.41	71.43	91.45	66.09	85.87	71.57	94.14
Influenza A virus strain A/Hong Kong/1073/99(H9N2)	68.31	87.93	63.04	75.77	70.99	91.68	71.47	91.95	66.53	85.39	69.18	84.71
Influenza C virus (C/Ann Arbor/1/50)	71.55	90.55	63.08	77.42	70.05	91.28	71.23	91.19	68.15	87.72	72.16	93.75
Thogotovirus	68.79	88.73	62.31	78.11	72.34	92.34	63.13	78.54	68.47	88.13	72.59	91.97
Infectious salmon anemia virus	70.65	91.38	64.22	79.86	68.76	87.47	72.12	92.65	73.3	92.49	**	**
Influenza A virus (A/goose/Guangdong/1/1996(H5N1))	68.62	87.93	62.63	75.63	**	**	69.48	91.72	67.47	85.25	67.99	84.71
Influenza A virus (A/New York/392/2004(H3N2))	68.25	87.79	63.36	75.63	70.1	91.27	72.77	91.22	67.16	84.57	70.26	84.71
Influenza A virus A/Korea/426/1968(H2N2)	68.11	88.07	63.22	75.63	70.1	91.27	71.71	90.64	67.16	84.71	69.78	85.49
Influenza A virus A/Shanghai/02/2013(H7N9)	67.97	87.97	62.95	75.77	70.99	91.27	71.71	90.56	67.21	85.17	68.11	84.71
Influenza A virus A/California/07/2009(H1N1)	68.34	87.87	62.97	75.74	70.9	91.46	71.13	92.18	66.17	84.9	68.59	85.1
Dhori virus	69.34	87.92	**	**	67.92	88.6	64.88	77.76	69.91	89.57	71.36	90.32

Nt: Nucleotide divergence; AA amino acid divergence; ** data not available.

However, further experimental study needs to be performed in order to confirm this observation.

Cysteine residues play an important role in maintaining the stability and structural integrity of the protein structure. The HA gene has 5 conserved cysteine-residues between Thogotovirus and Quaranjavirus group, indicating its evolutionary significance to maintain the tertiary structure of the protein. PsiPred based secondary structure comparison of Thogotovirus and QRFV demonstrated a similar pattern of the helix and sheet prediction Fig. 3. The MEMSAT-SVM prediction also demonstrated a single transmembrane helix (483−509) towards the end of the protein sequence. This suggests that HA of QRFV has Class 3 type penetrenes [29].

Morse et al. demonstrated that the glycoprotein of Thogotovirus shared a common ancestor with a gp64 protein of Baculovirus demonstrating a distinct lineage for Thogoto-group viruses as compared to the influenza group viruses [30]. A similar observation from glycoprotein demonstrating a recent ancestry between Thogoto-group viruses and Baculoviruses gp64 was reconfirmed in our work. However, we also observed that Quarjagroup viruses shared a recent ancestry with Beihai Orthomyxo-like virus (currently unclassified virus) isolated from the woodlouse. Based on our analysis we conclude that Quaranjavirus and Thogotovirus have distinct evolutionary lineage based on the sequence homology (Fig. 4) or the other possibility is of the functional requirement that these membrane proteins had a convergent evolution.

The phylogenetic tree was generated for all six segments of sequenced QRFV. We observed that QRFV groups along with the Johnston Atoll virus, Tjuloc virus and QRFV (Fig. 4) as a separate group (Quaranjavirus). This group is separate from the other genera of the *Orthomyxoviruses*; a similar observation was previously published paper [9]. Table 4 provides the overall nucleotide and amino acid divergence between the orthomyxovirus reference sequences used in the study to compare with the Indian QRFV. The least diverged sequence to our isolate appeared to be the QRFV isolate from Afghanistan (about 12% divergent at the nucleotide level and 7.5 % at the amino acid level).

4. Conclusions

This virus has been isolated from ticks, birds as well as from the children suffering from mild febrile illness in Egypt. The mode of transmission has been associated with ticks since it has also been isolated from the ticks. The genome data presented here suggest that this virus has evolved from the *Orthomyxoviridae* cluster. Two important characteristics in the genomes of these viruses are their higher rate of antigenic shift and drift. Reassortment between the genomes is the main characteristics of this group of viruses. The available information suggests that the virus has a very

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low level of pathogenicity in humans; however, data from Egypt suggest that about 8% of the population had antibodies to this virus [9]. In the region were the QRFV virus occurs, it is not considered a pathogen of the potential importance. Public health diagnostic laboratories in the countries hence neglect the diagnosis of the virus. This leads to infection with this virus being undiagnosed and unreported in humans.

This virus requires detailed investigations, particularly in cases of febrile illness with influenza-like symptoms, especially from rural areas or villages near the fringes of forests or bird sanctuaries. The potential for transmission of the virus by tick bites and human-to-human transmission remains to be investigated. Re-assortment between homologous and non-homologous subtypes is known to occur in influenza viruses of different host species. Similarly, recombination has been found in Thogotovirus and Dhori virus [possessing 6 genome segments]. We observed homology between the QRFV unknown segment and the nucleocapsid region of Thogotovirus and influenza viruses. The remote possibility of recombination and re-assortment among Quaranfil virus infections with other *Orthomyxoviruses* particularly influenza viruses should be investigated. Living in close proximity to areas having close interaction with animals and ticks may carry a risk of infection with these *Orthomyxoviruses*.

Declarations

Author contribution statement

Devendra T Mourya: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Pragya D Yadav: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Dimpal A Nyayanit: Analyzed and interpreted the data; Wrote the paper.

Triparna D Majumdar, Shilpi Jain, Prasad Sarkale, Anita Shete: Performed the experiments.

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Competing interest statement

The authors declare no conflict of interest.

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Additional information

No additional information is available for this paper.

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