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ORIGINAL PAPER

Whole-genome characterization of avian picornaviruses from diarrheic broiler chickens co-infected with multiple picornaviruses in Iran

Niusha Haji Zamani[1] · Hossein Hosseini[2] · Zahra Ziafati Kafi[1] · Naser Sadri[1] · Ali Hojabr Rajeoni[1] · Reza Esmaeelzadeh Dizaji[1] · Aidin Molouki[3] · Mohammad Hossein FallahMehrabadi[3] · Mohammad Abdoshah[3] · Arash Ghalyanchilangeroudi[1]

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Abstract

Gastrointestinal symptoms in poultry are caused by several factors, such as infecting viruses. Several *avian picornaviruses* can cause diarrhea in these valuable animals. Poultry flocks in Iran suffer from gastrointestinal diseases, and information on picornaviruses is limited. In this study, two genera of avian picornaviruses were isolated from poultry and identified by the viral metagenomics. Fecal samples were collected from broiler chicken flocks affected with diarrhea from Gilan province Iran. The results showed that Eastern chicken flocks carried two genera of *picornaviridae* belonging to Sicinivirus A (SiV A) and Megrivirus C (MeV C). The Western chicken flocks carried SiV A based on whole-genome sequencing data. SiV A had type II ^{IRES} and MeV C contained a type IVB ^{IRES} 5′UTR. Phylogenetic results showed that all these three picornaviruses were similar to the Hungarian isolates. Interestingly, two different picornavirus genera were simultaneously co-infected with Eastern flocks. This phenomenon could increase and facilitate the recombination and evolution rate of picornaviruses and consequently cause this diversity of gastrointestinal diseases in poultry. This is the first report and complete genome sequencing of *Sicinivirus* and *Megrivirus* in Iran. Further studies are needed to evaluate the pathogenic potential of these picornaviruses.

Keywords Chicken picornavirus · Metagenomics · Phylogenetic analysis · Complete genome sequence · Iran

Introduction

Poultry provides food and fiber for humans through eggs and meat. The poultry industry suffers many losses, including various disease outbreaks such as diarrhea caused by bacteria, fungi, parasites, and viruses. Viral diarrhea is a common problem in chickens, caused by some viruses, such as the *Picornaviridae* family. Members of this family are small, non-enveloped, and icosahedral viruses that carry a plus-strand RNA molecule. The family is classified into 63 official genera such as, avian picornaviruses, *Sicinivirus*, *Megrivirus*, *Avihepatovirus*, *Tremovirus*, *Passerivirus*, *Sapelovirus*, and a continuously increasing number of novel, currently unassigned picornaviruses [1].

A picornaviral genome approximately 7–9 Kb in size, with a covalently bound viral protein (VPg) at its 5′ end and poly(A) at its 3′ end. The ORF is flanked by a long 5′ Untranslated Region (UTR) and a short and a long 3′ Untranslated Region (UTR). The 5′ and 3′ UTR contain five Internal Ribosome Entry Site (IRES) types structurally: conserved motifs and direct polyprotein translation. In addition to the highly structured 5′ UTR and 3′ UTR, other major RNA secondary structures have been identified in the open reading frame (ORF), which encodes a polyprotein of more than 2000 aa [2]. After translation, a single polyprotein is produced that can be functionally divided into three coding regions: P1, P2, and P3. The polyprotein contains the following organization: 5′UTR-(ORF: L-VP0(or cleaved intoVP4 and

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✉ Arash Ghalyanchilangeroudi
ghalyana@ut.ac.ir; arashghalyanchi@gmail.com

[1] Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

[2] Department of Clinical Sciences, Faculty of Veterinary Medicine, Karaj Branch, Islamic Azad University, Alborz, Iran

[3] Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran

VP2)-VP3-VP1-2A-2B-2C-3A-3B-3C-3D)-3′UTR-polyA-tail (Fig. 1). However, there is a potential second ORF in the 3′ part of the genome in *Megriviruses* [3, 4]. VP0 protein of some picornaviruses is proteolytically cleaved into VP4 and VP2 proteins [5]. In genera, the genomic organization of picornaviruses is conserved, and all picornavirus genes are orthologus within the family, with the exception of the L and 2A proteins [5, 6]. The L protein and viral protease 2A generally exhibit the highest rate of variation among picornavirus proteins [7].

The Picornaviridae family can cause some diseases in many vertebrate species, such as birds. Depending on the nature of each picornavirus, the problem can occur in severe to mild damage to the gastrointestinal tract, respiratory, nervous, hepatocellular, and cardio muscular systems [8, 9]. *Megrivirus*, isolated naturally from poultry, including turkeys and chickens [10], and sicinivirus, almost reported from chicken [11] are two commonly identified avian picornaviruses among avian species. The genera of Megriviruses include A–E species with IVB type from IRES in the 5′UTR (except type E), detected in chickens, turkeys, ducks, feral pigeons, harriers, and penguins. The genus of sicinivirus has a single species, sicinivirus A (SiV A), and its 5′UTR contains a II-type IRES.

Although chickens are considered the most important avian species, the diversity, and importance of picornaviruses in this group have been underestimated. Moreover, in previous studies, chickens have been considered one of the most important reservoirs for various picornaviruses that can overcome the species barrier by recombination [12]. Because of this issue's great importance, we have studied the metagenomics of some fecal samples of commercial broiler chickens, focusing on picornaviruses because of the lack of surveillance data on picornaviruses, which are considered a probable cause of diarrhea in chickens with clinical gastrointestinal symptoms. Therefore, the current study will contain the partial results of this metagenomics study.

Materials and methods

Sample collection

The fecal samples examined in the present study were collected from seven farms from December 2017 to June 2018. These seven farms were located in two different areas (Eastern and Western areas) of Gilan province, in the northen of Iran. Four and three farms were selected for each of the eastern and western areas, respectively. The farms were located in populated areas and were also the largest in terms of number of birds in Gilan province. Each farm contained 10,000–15,000 broilers. At the time of selection, we observed a certain proportion of broiler chickens showing clinical signs of enteric disease complex (EDC), 30–35% of the chickens per farm. We randomly selected ten chickens of the sick broilers for the study. The fecal samples were collected and taken transferred to the laboratory microbiology laboratory of the Veterinary Facultyof Tehran University, and immediately stored in 0.5 ml RNA later stabilization reagent (QIAGEN) at – 20 °C.

Sample preparation and RNA extraction

All samples from the same area (four samples from Eastern and three samples from Western areas) were homogenized and were pooled together and labeled with two different bar codes. genetic content of the sampleswas extracted using the RNXTM-Plus kit (CinnaGen, Tehran, Iran) according to the manufacturer's recommendations and stored at – 70 °C until use. Subsequently the pooled RNA samples were sent to Beijing Genomics Institute (BGI, China) and sequenced using the Illumina HiSeq 4000 platform in two separate runs, generating paired-end 150 bp reads.

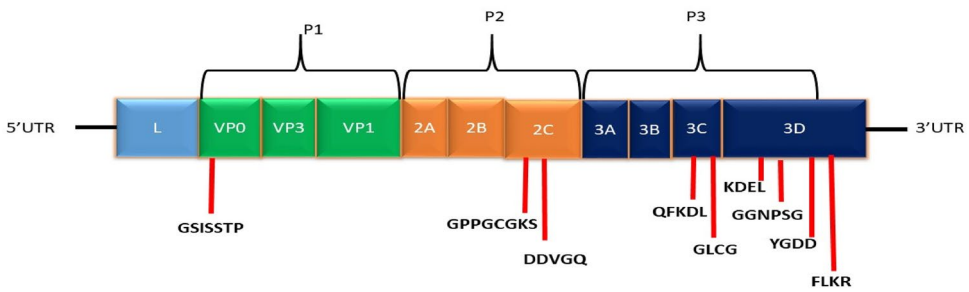

Fig. 1 Predicted genome organization of both Sicinivirus strains. The P1 region represents the structural proteins VP0, VP3, and VP1. The P2 region represents the nonstructural proteins, including 2A, 2B, and 2C. The P3 region carries the proteins 3A, 3B, 3C, and 3D. The predicted protein cleavage sites are indicated above each gene junction (one-letter amino acid code). The identified amino acid is sequenced in cleavage sites indicated under each protein

Quality control

Since the raw data obtained by sequencing always contain a certain amount of low-quality reads, the Trimmomatic [13] was used here to remove the paired-end reads as follows: (i) those with adapters, (ii) those containing more than 20% low-quality bases (SQ ≤ 20) (iii) those derived from PCR duplications, and (iv) those with a polyX sequence to improve the accuracy of the reads for follow-up analyses [13].

Contamination removal

Given the contamination of the ribosomal and host sequences, all clean reads that passed quality control were mapped to the ribosomal database (Silva.132) [14] and host database, respectively, using BWA (version 0.7.17, parameter: mem *–k* 30) [15]. Only the unmapped sequences remained for high-quality data.

Virus classification

BWA [15] (v0.7.17) was used to map the high-quality reads with the GenBanknon-fowl nucleotide database (NT), and the comparison results with a length less than 80% of the total length of the reads were filtered for high accuracy. Then, the remaining reads were classified into different virus families using the annotation information from the NCBI taxonomy database [16].

RNA sequencing and bioinformatics

Next-generation sequencing of RNA extracted samples was performed by Illumine Hiseq4000 (BGI, China). The next-generation sequencing data (reads) were analyzed with high accuracy using the web-based tool Genome Detective. The total known viral genomes were assembled by web-based Genome Detective [17]. Briefly, sequence results were evaluated in terms of read quality and bioinformatics analysis. The DIAMOND method identified viral reads, and non-viral reads were removed [18]. The viral reads were assembled de novo using metaSPAdes, and viral contigs were determined [17, 19].

Raw sequence data were analyzed and assembled using CLC genomic workbench version 21. Alignment of the complete genome sequences was performed with Clustal W neighbor-joining trees generated with the *P*-distance model from the program MEGA, version 7, using a 1000-fold bootstrap approach [20]. The obtained genome sequence was submitted to the NetPicoRNA 1.0 server [21] and also aligned with the available polyproteins of ChPV1 100C (KF979332) [12], Sicinivirus 1 strain UCC001 (NC_023861) [11], In addition some of these sequences are shown in Fig. 7 to predict possible polyprotein cleavage sites, and they are compared with different selection sequences available in GenBank, including picornavirus isolates from birds from other parts of the world.

Results

Samples and accession number

The sequencing results were 44,438,474 paired-end reads with an average read length of 150 bp. The trimmed read length was 50–134 bp. The 131,046 (1%) reads that did not pass quality control (QC) were removed. Non-viral reads, including host genome and other non-viral microorganisms, were excluded. Reads were mapped back to the viral contigs. After excluding bacterial genomes from this metagenomics analysis, several viral families were identified. However, only three complete genomes of picornaviride were reported in this study and other families will be published in another article. Therefore, we focused on these three picornaviruses and identified themusing BLASTx analysis of metagenomics reads.

The pooled fecal samples from Eastern areas of Gilan province contained one *sicinivirus* isolate and one *Megrivirus* isolate, whereas the pooled samples from Western areas contained only one avian picornavirus isolate, which was identified as a *Sicinivirus* strain. Two Sicinivirus isolates were named Iran_Sicinivirus (UT-G-Khajehnasiri) and Iran_Sicinivirus (UT-G-Masoumi), which was, our *Megrivirus* was named Chicken_megrivirus_strain_chicken_Karoon_2018_Iran (v).

The complete genome sequences of UT-G-Khajehnasiri, UT-G-Masoumi, and Karoon_2018_Iran were submitted to GenBank and assigned accession numbers MT215487, MT225405, and MH125198, respectively.

Genome analysis of the Siciniviruses

The first *Sicinivirus*-related reads covered ~ 99.2% of the 9884-nt-long genome of *Sicinivirus* (UT-G-Khajehnasiri), and the second *Sicinivirus*-related reads covered ~ 82% of the 9277-nt-long genome of *Sicinivirus* (UT-G-Masoumi). Based on the sequence data of the gene regions-encoding the capsid protein, both Sicinivirus isolates in the study were determined to be SiV A species.

Genome analysis of the Megrivirus

The Karoon_2018_Iran-related reads covered ~ 91.4% of the 9570-nt-long genome of *Megrivirus* (Karoon_2018). *Megrivirus* has a genome layout of VPg + 5′-UTR $^{IRES-IVB}$-[1AB-1C-1D/(2A1-2A2-2A3-)2A$^{4H-box/NC}$-2B-2C/3A-3C-3D]

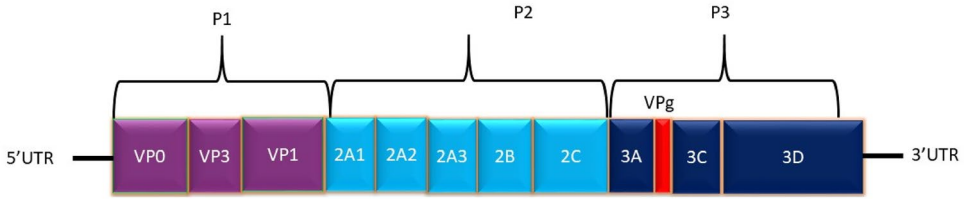

Fig. 2 Predicted genome organization of both Megrivirus strains. The isolate does not have L protein. The P1 region represents the structural proteins VP0, VP3, and VP1. The P2 region represents the non-structural proteins, including 2A1, 2A2, 2A3, 2B, and 2C. The P3 region carries the proteins 3A, 3C, and 3D

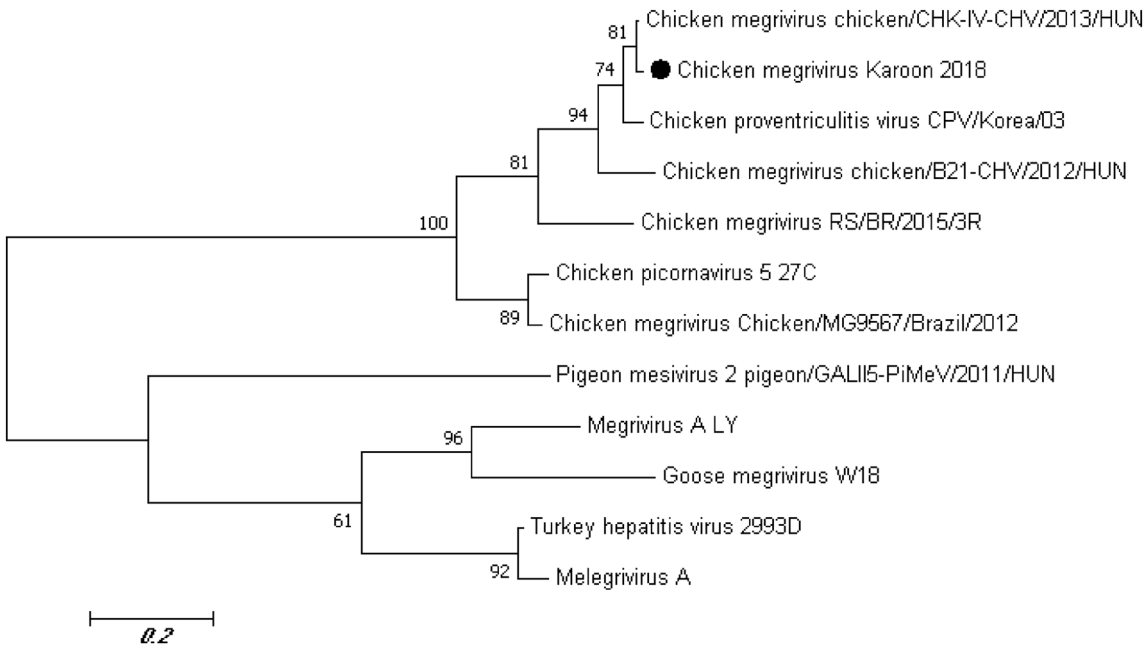

Fig. 3 Nucleotide acid–base phylogenetic relationships of the VP1 gene of Karoon-2018 detected from Iran and other Megriviruse strains. The phylogenetic tree was generated using Muscle Distance-based neighbor-joining model with MEGA (version 7.0.14). Numbers below branches indicate bootstrap values from 1000 replicates and bootstrap values. Horizontal distances are proportional to the minimum number of nucleic acid differences required to join nodes. The vertical lines are for spacing branches and labels. The scale bar represents the distance unit between sequence pairs. The virus genome characterized in this report is indicated as a black circle

Table 1 Estimates of evolutionary divergence for the VP1 gene of isolate Karoon-2018

		1	2	3	4	5	6	7
1	Chicken_megrivirus_Karoon							
2	Chicken_megrivirus_chicken/CHK-IV-CHV/2013/HUN	98.80						
3	Chicken_proventriculitis_virus_CPV/Korea/03	94.03	94.48					
4	Chicken_megrivirus_chicken/B21-CHV/2012/HUN	83.73	84.88	83.34				
5	Chicken_megrivirus_RS/BR/2015/3R	66.00	66.83	64.71	60.94			
6	Chicken_megrivirus_Chicken/MG9567/Brazil/2012	51.06	50.26	50.26	54.55	49.66		
7	Chicken_picornavirus_5_27C	47.29	46.47	47.39	51.52	53.22	92.97	

The number of base differences per site from between sequences is shown. The analysis involved seven nucleotide sequences as the closest results derived from BLAST. Codon positions included were 1st + 2nd + 3rd. All ambiguous positions were removed for each sequence pair. There were a total of 2002 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [20]

c-3′-UTR-poly(A) (Fig. 2). Our *Megrivirus* did not contain L protein in its genome (Fig. 2). Our Karoon_2018_Iran was classified as species C (MeV C) based on the sequence analysis of the capsid protein. The sequence analysis of the VP1 gene of this *Megrivirus* is shown in Fig. 3 and Table 1.

Analysis of the 5′UTR and 3′UTR of Sicinivirus

The 5′ UTR of UT-G-Khajehnasiri and UT-G-Masoumi are incomplete as the domains A–I of the potential the type II IRES are missing. Interestingly, UT-G-Masoumi has an unusually long spacer. The characteristic domains J, K, and L of type-II IRESes located at the partial 5′ UTR of the two *Sicinivirus*were also predicted by Bullman et al. [11]. Analysis of the RNA secondary structure of the 939 nt 5′ UTR sequence also revealed that the *Sicinivirus* strain JSY contains at least twelve stem-loop domains (A–L), and motifs are evident, including the pyrimidine-tract binding protein (PTB) binding sites, upper stem-loop I and the eIF4G binding domains J and K, as well as the conserved sequence motifs of GNRA, RAAA [22, 23] (Fig. 4). This is the first characterization of the complete 5′ UTR structure for the genus *Sicinivirus*. The predicted secondary RNA structure of the 3′ UTR of the Sicinivirus strain has a 48 nt 'barbell-like structure'. (Fig. 5).

Analysis of coding regions of Siciniviruses

The myristoylation site GSISSTP was recognized in the VP0 protein of both *Sicinivirus* strains UT-G-Khajehnasiri and UT-G-Masoumi, as reported by Bullman et al. [11]. VP1

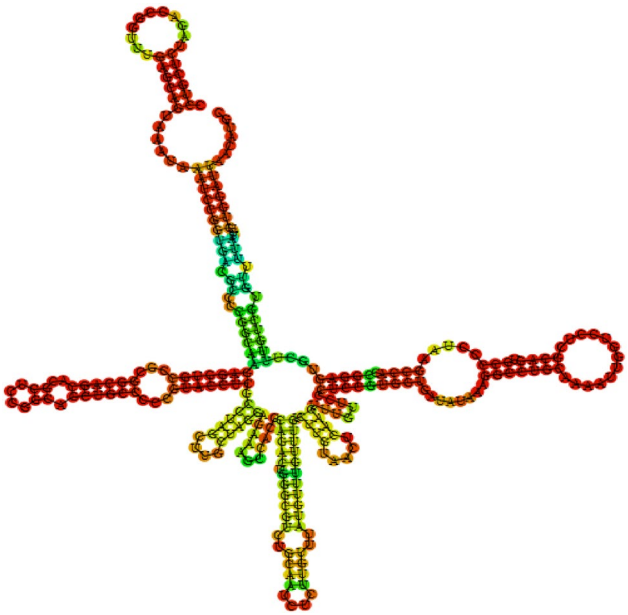

Fig. 5 The predicted secondary RNA structure of the 3′ UTR of the Sicinivirus strain possesses a 48 nt 'barbell-like' structure

sequence analysis showed that our *Siciniviruses* were similar to the Hungarian and Irish isolates (Fig. 6, Table 2). The 2C protein of both sicinivirus strains contains a conserved NTP-binding site GPPGCGKS motif and the DDVGQ associated with putative helicase activity [25]. The active-site cysteine in GLCG motif [26] and the RNA binding domain QFKDL are present in the 3C protein of our *Sicinivirus* strains (Fig. 7).

Fig. 4 Secondary RNA structures of 5′UTR of UT-G-Khajehnasiri. The main domains of the type-II IRES of UT-G-Khajehnasiri are annotated from A to L and from H to L, as previously proposed [22]. The three stem-loop structures (SL1–3) are identified using covariance analysis of the corresponding regions of UT-G-Khajehnasiri, where black arrows show either the covariant or structurally irrelevant (silent) nucleotides of UT-G-Khajehnasiri. *PTB* pyrimidine-tract binding protein, *pY* pyrimidine bases, The main domains (II, III) and hairpins (IIIa, IIIb, etc.) of UT-G-Khajehnasiri-IRES are labeled according to the corresponding regions of type-VIRES of hepatitis C virus Hellen and de Breyne [24]. Stem1and 2 are parts of the pseudoknot. Gray boxes indicate conserved sequence regions of type-IVIRESes Hellen and de Breyne [24]. Black arrow: conserved A–A missing pair of domain III. The conserved apical "8" structure is shown with a dashed box

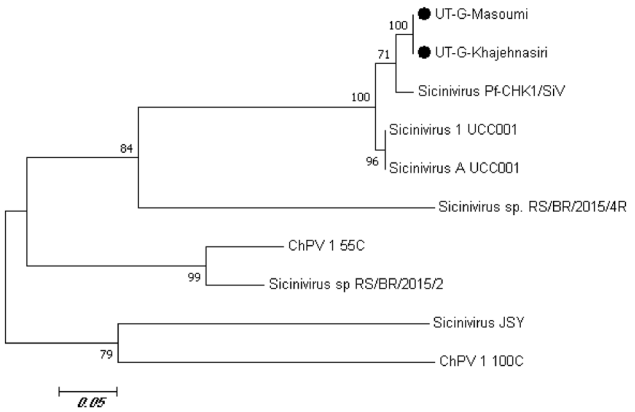

Fig. 6 Nucleotide acid–base phylogenetic relationships of the VP1 gene of both Sicinivirus strains detected from Iran and other Sicinivirus strains. The phylogenetic tree was generated using Muscle Distance-based neighbor-joining model with MEGA (version 7.0.14). Numbers below branches indicate bootstrap values from 1000 replicates and bootstrap values. Horizontal distances are proportional to the minimum number of nucleic acid differences required to join nodes. The vertical lines are for spacing branches and labels. The scale bar represents the distance unit between sequence pairs. The virus genome characterized in this report is indicated as a black circle ●

Analysis of the 5′UTR and 3′UTR of Megrivirus

The predicted secondary RNA structure of the 5′UTR and 3′ UTR of the *Megrivirus* strain was shown in Figs. 8, 9.

Phylogenetic and bioinformatics analysis

The UT-G-Masoumi and UT-G-Khajehnasiri isolates were 95.14% identical, and had the highest similarity to the Hungarian isolate (Sicinivirus_Pf-CHK1/SiV KT880665.1) and the Irish isolate (Sicinivirus_1_strain_UCC001_KF741227.1) based on the whole-genome sequencing data (Table 3, Fig. 10). Our *Megrivirus* had 91.51 and 89.33% identity with the Hungarian isolate (CHK-IV-CHV/2013/HUN_KF961187.1) and the Korean isolate (CPV/Korea/03_KJ690629.1), respectively, based on whole-genome sequencing analysis (Table 4, Fig. 10).

Discussion

Enteric viral infections are caused by various viruses, such as members of the Picornaviridae, and result in a broad range of consequences, from apparent, economically insignificant effects to severe and financially devastating. This study includes samples from two broiler chickens affected by diarrhoea and focuses on the investigatingoverall picornavirus diversity in poultry and the co-infecting picornaviruses in a single sample. The results showed that three picornaviruses from two broiler chickens suffering were identified from diarrhoea were identifiedusing a viral metagenomics approach. Based on the complete genome sequence data, 2/3 of these isolates were classified in the genus *Sicinivirus* (UT-G-Masoumi and UT-G-Khajehnasiri), and another belonged to the genus *Megivirus* (Karoon_2018_Iran). Previous studies have shown that, these picornaviruses were frequently detected in cloacal samplesfrom healthy and diseased chickens [11, 12, 27]. These infections are triggered by a variety of predisposing factors, not the least of which are the age and immune status of the affected birds and the virulence of the involved viruses. The information about the diversity of picornaviruses and their co-infections in poultry has not been available in Iran. This is the first detection and complete genome sequencing of two *Sicinivirus* and *Megrivirus* strains in Iran.

Bullman et al. first reported *Sicinivirus*., 2014, from Irish broiler chickens, and the whole genome was characterized by Boros et al., [11, 28]. This species has one species, known as SiV A, and contains five interspecies types. Complete genome analysis of the viruses studied revealed the presence of sequence motifs, previously found only in chicken picornaviruses, and the unusually long, highly structured spacer

Table 2 Estimates of evolutionary divergence for the VP1 gene of isolate both Sicinivirus strains

		1	2	3	4	5	6	7	8
1	Iran_Scinini_virus_(UT-G-Masoumi)(_MT225405)								
2	Iran_Sicinivirus_(UT-G-Khajehnasiri)(_MT215487)	95.14							
3	Sicinivirus_Pf-CHK1/SiV(KT880665.1)	86.25	84.35						
4	Sicinivirus_1_UCC001(KF741227.1)	84.90	86.44	83.98					
5	Sicinivirus_UCC1(KF366619.1)	79.96	80.92	79.26	87.73				
6	Sicinivirus_sp._RS/BR/2015/4R(MG846481.1)	74.82	74.26	74.58	74.71	73.52			
7	Chicken_picornavirus_1_55C(KF979331.1)	74.09	74.14	73.43	74.01	75.05	72.21		
8	Chicken_picornavirus_1_100C(KF979332.1)	73.51	73.50	72.97	73.33	73.52	70.52	82.06	

The number of base differences per site from between sequences is shown. The analysis involved seven nucleotide sequences as the closest results derived from BLAST. Codon positions included were 1st + 2nd + 3rd. All ambiguous positions were removed for each sequence pair. There were a total of 2002 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [20]

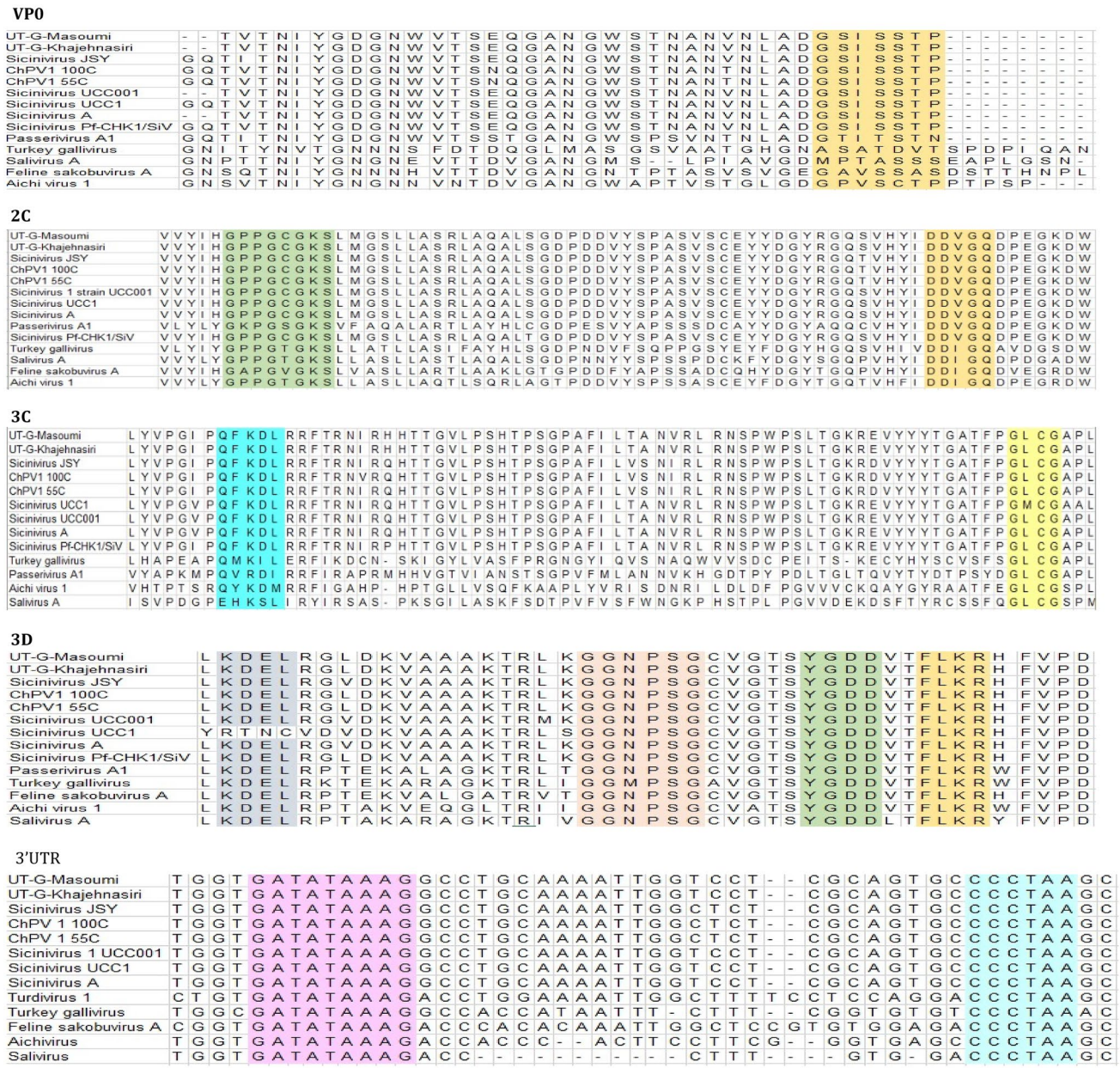

Fig. 7 Genome organization and the conserved picornaviral motifs. (A) The predicted genome organization possesses conserved picornaviral motifs of UT-G-Masoumi (MT225405), UT-G-Khajehnasiri (MT215487), Sicinivirus JSY (KP779642), ChPV1 100C (KF979332), ChPV1 55C (NC_024765), Sicinivirus 1 UCC001 (NC_023861) and Sicinivirus UCC1 (KF366619); Sicinivirus A (KF741227), Turdivirus 1 (NC_014411), Turkey gallivirus (NC_018400), Feline sakobuvirus A isolate FFUP1 (NC_022802), Aichi virus (NC_001918) and Salivirus A isolate 02394–01 (NC_012986)

in the 5′UTR of UT-G-Masoumi, suggesting an essential role for these motifs in the replication cycle of these viruses in chicken hosts. Viral proteins play a key role in viral life and the infection cycle; structural proteins assemble viral capsids, whereas nonstructural proteins replicate genomic RNA in conjunction with cell proteins [29–31]. VP0 is a capsid protein that is autocatalytically cleaved into VP2 and VP4. This cleavage stabilizes the mature virion and facilitates infectivity by cell entry [32]. VP1 is the most surface-exposed capsid protein and has antigenic significance and

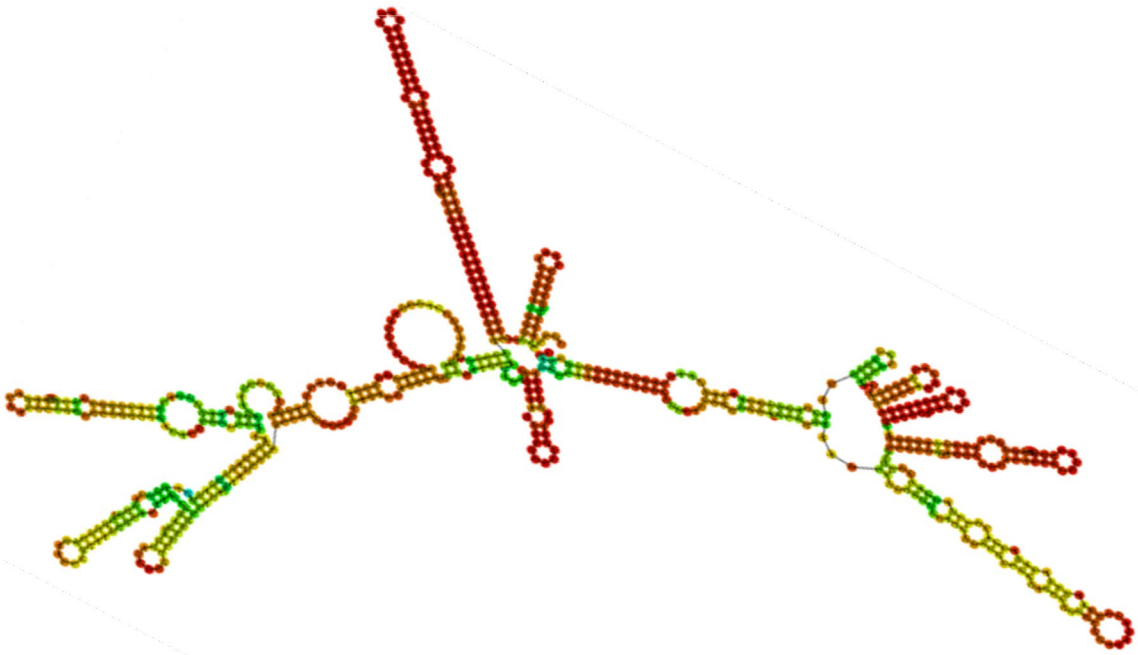

Fig. 8 Secondary RNA structures of 5′UTR IRES of Karoon-2018 with the positions of different nucleotides (in dark circles) indicated by black arrows and within frames of dashed. The main domains (II and III), helical segments (III 1–4), hairpins (III a; III c–f), and stems (Stem 1 and Stem 2) are similar to those of the similarly labeled structures of hepacivirus/pestivirus-like type IVB IRES are depicted. Gray boxes indicate conserved motifs of IVB IRESes [23]. White boxes indicate the conserved unpaired base pairs concerning DHAVs within the middle loop of domain II. The sequence and location of the apical "8" structure also identified in other avian picornaviruses are indicated with a black frame box [25]

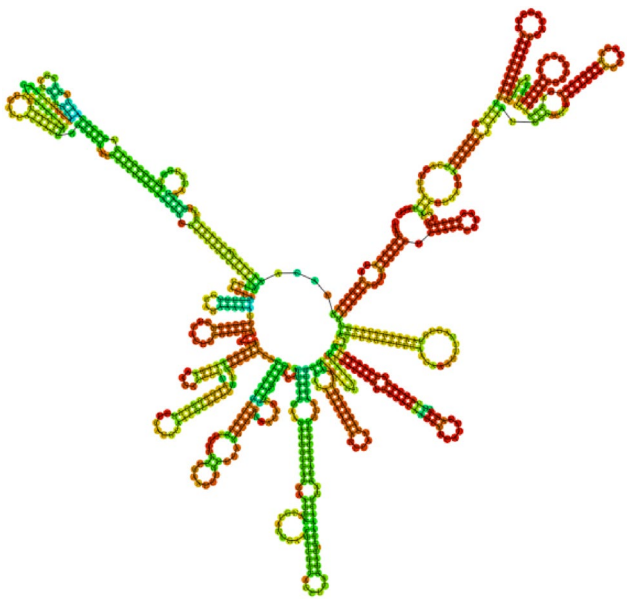

Fig. 9 The predicted secondary RNA structure of the 3′ UTR of the Megrivirus strain possesses a 48 nt 'barbell-like structure

plays a role in viral attachment [3]. One of the antigenic sites in VP1 is located in the G–H loop [18]. V P1 in UT-G-Masoumi and UT-G-Khajehnasiri is a 95–97% identical to Pf-CHK1/SiV (KT880665.1) which was our refrence gene sequence and the highest identical strain to our siciniviruses, so it can be concluded that these *Sicinivirus* share common antigenic sites. Protein 2C is an RNA helicase and an ATP-independent RNA chaperone critical for RNA replication and viability of enteroviruses [33, 34]. It inhibits the production of IFN and suppresses the activation of NF-κB and IFN by a specific mechanism [35]. Picornaviruses encode an unique protease 3C that plays an importantrole in the viral life cycle and virus–host interactions. Protein 3C is an important proteinase found in UT-G-Masoumi, UT-G-Khajehnasiri, and contained 3C protein sites in the initial analysis. Functions of 3C protein previously reported include mediating the cleavage of histone H3 in baby hamster kidney cells, the cleavage between P1 and P2, cleavage between glutamine-glycine (Q–G) pairs, and also activation of the transcription factors TFIIIC and TFIID and

Table 3 Estimates of evolutionary divergence for the complete genome of isolate both Sicinivirus strains

		1	2	3	4	5	6	7	8
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8	Chicken_picornavirus_1_100C(KF979332.1)	73.51	73.50	72.97	73.33	73.52	70.52	82.06	

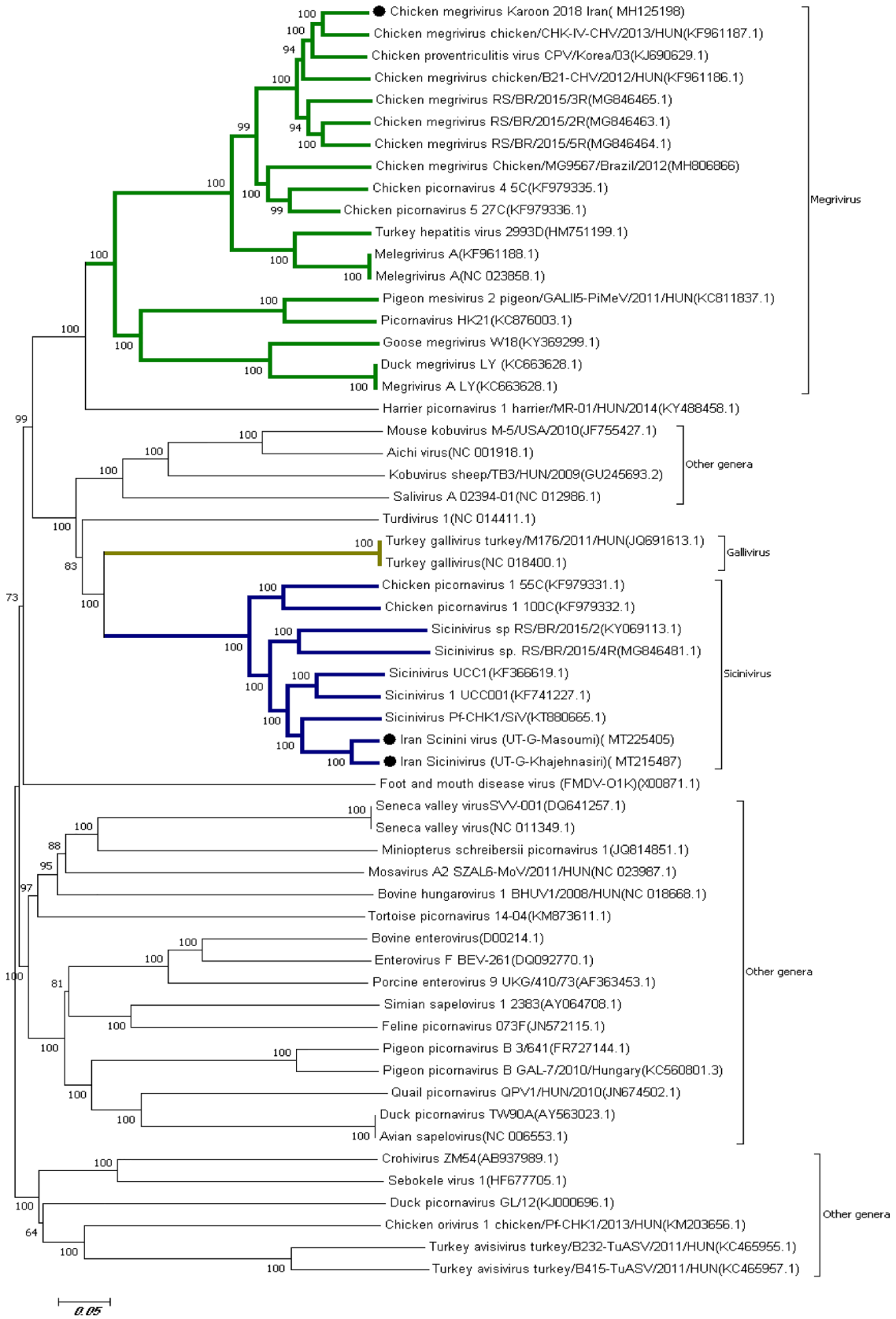
The number of base differences per site from between sequences is shown. The analysis involved eight nucleotide sequences as the closest results derived from BLAST. Codon positions included were 1st + 2nd + 3rd. All ambiguous positions were removed for each sequence pair. There were a total of 2002 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [20]

microtubule-associated protein 4 (MAP-4) in HeLa cells in some picornaviruses [36–41]. The 3D protein is a type of RNA-dependent RNA polymerase that does not affect IFN-γ receptor expression, but appears to be a viral factor for immune-editing [42, 43]. The shorter 3′-UTR is important for synthesis of the in (-) strand. Based on the whole-genome UT-G-Masoumi, UT-G-Khajehnasiri were similar to the Hungarian and Irish *Sicinivirus* strains.

The genus Megrivirus was first established in 2013 [44]. This genus has five official species, namely Megrivirus A–E (www.picornaviridae.com/megrivirus/megrivirus.htm). Megrivirus RNAs have been isolated from chickens, turkeys, ducks, feral pigeons, marsh harriers, and penguins. However, we identified *Megrivirus* in chickens, and this is probably due to transmission between different species, especially in turkeys (belongs to the same type 'MeV C' as our *Megrivirus*), raised close to each other. Our results showed that Karoon_2018_Iran was similar to Hungarian and Korean isolates according to whole-genome sequence studies; however, it shared higher identity with Hungarian *Megrivirus* isolates based on VP1 sequence data. As it is mentioned earlier VP1 has antigenic importance, therefore, it can be concluded that our *Megrivirus* is more antigenically similar to Hungarian isolate Megrivirus compared to Korean ones.

Our analysis showed that both novel chicken picornaviruses, including Karoon_2018_Iran and UT-G-Khajehnasiri, were excreted simultaneously from a single chicken. Inadequate cleaning of the concrete floor of the backyard where the sampled animal was kept could contribute to the presence of high picornavirus diversity in this bird, which was recently introduced into this environment. Co-infection with different picornaviruses could facilitate the evolution of picornaviruses and allow the emergence of recombinant picornaviruses, similar to the possible recombinant chicken megriviruses and chicken picornaviruses [3, 12]. Our avian picornaviruses in this study were interestingly similar to the Hungarian isolates. However, there was also high identity between UT-G-Khajehnasiri, UT-G-Masoumi, and Irish *Sicinivirus*, and the results also showed high similarity between the Karoon_2018_Iran and Korean *Megrivirus* isolate. This study was the first report on *Sicinivirus* and *Megrivirus* in Iran. Nevertheless, there were some limitations in the present study, such as the lack of published work and also surveillance data on these picornaviruses. As a result, the picornaviruses identified in the present study might be circulating in poultry farms in Iran or even in neighboring countries.

Although almost all previous studies in Iran have focused on an important picornaviruse named Avian Encephalomyelitis Virus (AEV), which causes an economically important disease in chicken, this study showed that unknown factors such as other picornavirus species circulating in poultry may be the cause of diarrhea in poultry flocks in Iran [45]. Apart from picornaviruses, several eukaryotic viruses including picobirna-, calici-, astro- and parvovirus-related viruses, have been isolated from the diarrheic chicken studied, so the assignment of a contributionof each related virus to the development of diarrhea must be linked tothe analysis of possible co-infecting bacteria, parasites, viruses, and the known genetic background of the host. Therefore, the discovery of additionalin poultry picornaviruses that might be involved in gastrointestinal infections could be an aid to in veterinary care; medical importance prevents economic losses. in addition, studying the pathogenicity of picornaviruses in poultry and conducting more rigorous surveillance of diarrheal disease agents such as bacterial and viral profiles in the poultry industry are highly recommended. As noted in this study, several genera of viruses such as picornaviruses may be involved in the development of diarrheal diseases. Therefore, it is necessary to monitor, further research, and take preventive measures for these pathogens that can cause serious problems in the industry.


Chicken megrivirus Karoon 2018 Iran(MH125198)
Chicken megrivirus chicken/CHK-IV-CHV/2013/HUN(KF961187.1)
Chicken proventriculitis virus CPV/Korea/03(KJ690629.1)
Chicken megrivirus chicken/B21-CHV/2012/HUN(KF961186.1)
Chicken megrivirus RS/BR/2015/3R(MG846465.1)
Chicken megrivirus RS/BR/2015/2R(MG846463.1)
Chicken megrivirus RS/BR/2015/5R(MG846464.1)
Chicken megrivirus Chicken/MG9567/Brazil/2012(MH806866)
Chicken picornavirus 4 5C(KF979335.1)
Chicken picornavirus 5 27C(KF979336.1)
Turkey hepatitis virus 2993D(HM751199.1)
Melegrivirus A(KF961188.1)
Melegrivirus A(NC 023858.1)
Pigeon mesivirus 2 pigeon/GALII5-PiMeV/2011/HUN(KC811837.1)
Picornavirus HK21(KC876003.1)
Goose megrivirus W18(KY369299.1)
Duck megrivirus LY (KC663628.1)
Megrivirus A LY(KC663628.1)
Megrivirus
Harrier picornavirus 1 harrier/MR-01/HUN/2014(KY488458.1)
Mouse kobuvirus M-5/USA/2010(JF755427.1)
Aichi virus(NC 001918.1)
Kobuvirus sheep/TB3/HUN/2009(GU245693.2)
Salivirus A 02394-01(NC 012986.1)
Other genera
Turdivirus 1(NC 014411.1)
Turkey gallivirus turkey/M176/2011/HUN(JQ691613.1)
Turkey gallivirus(NC 018400.1)
Gallivirus
Chicken picornavirus 1 55C(KF979331.1)
Chicken picornavirus 1 100C(KF979332.1)
Sicinivirus sp RS/BR/2015/2(KY069113.1)
Sicinivirus sp. RS/BR/2015/4R(MG846481.1)
Sicinivirus UCC1(KF366619.1)
Sicinivirus 1 UCC001(KF741227.1)
Sicinivirus Pf-CHK1/SiV(KT880665.1)
Iran Scinini virus (UT-G-Masoumi)(MT225405)
Iran Sicinivirus (UT-G-Khajehnasiri)(MT215487)
Sicinivirus
Foot and mouth disease virus (FMDV-O1K)(X00871.1)
Seneca valley virusSVV-001(DQ641257.1)
Seneca valley virus(NC 011349.1)
Miniopterus schreibersii picornavirus 1(JQ814851.1)
Mosavirus A2 SZAL6-MoV/2011/HUN(NC 023987.1)
Bovine hungarovirus 1 BHUV1/2008/HUN(NC 018668.1)
Tortoise picornavirus 14-04(KM873611.1)
Bovine enterovirus(D00214.1)
Enterovirus F BEV-261(DQ092770.1)
Porcine enterovirus 9 UKG/410/73(AF363453.1)
Simian sapelovirus 1 2383(AY064708.1)
Feline picornavirus 073F(JN572115.1)
Pigeon picornavirus B 3/641(FR727144.1)
Pigeon picornavirus B GAL-7/2010/Hungary(KC560801.3)
Quail picornavirus QPV1/HUN/2010(JN674502.1)
Duck picornavirus TW90A(AY563023.1)
Avian sapelovirus(NC 006553.1)
Other genera
Crohivirus ZM54(AB937989.1)
Sebokele virus 1(HF677705.1)
Duck picornavirus GL/12(KJ000696.1)
Chicken orivirus 1 chicken/Pf-CHK1/2013/HUN(KM203656.1)
Turkey avisivirus turkey/B232-TuASV/2011/HUN(KC465955.1)
Turkey avisivirus turkey/B415-TuASV/2011/HUN(KC465957.1)
Other genera
0.05

◂**Fig. 10** Nucleotide acid–base phylogenetic relationships of the complete genome of both Sicinivirus and Megriviruses strains detected from Iran and other picornavirus strains. The phylogenetic tree was generated using Muscle Distance-based neighbor-joining model with MEGA (version 7.0.14). Numbers below branches indicate bootstrap values from 1000 replicates and bootstrap values. Horizontal distances are proportional to the minimum number of nucleic acid differences required to join nodes. The vertical lines are for spacing branches and labels. The scale bar represents the distance unit between sequence pairs. The virus genome characterized in this report is indicated as a black circle ●

Table 4 Estimates of evolutionary divergence for the complete genome of isolate Megrivirus strain

		1	2	3	4	5	6	7	8	9	10	11
1	Chicken_megrivirus_Karoon_2018_Iran(_MH125198)											
2	Chicken_megrivirus_chicken/CHK-IV-CHV/2013/HUN(KF961187.1)	91.51										
3	Chicken_proventriculitis_virus_CPV/Korea/03(KJ690629.1)	89.33	89.96									
4	Chicken_megrivirus_chicken/B21-CHV/2012/HUN(KF961186.1)	87.37	87.96	87.60								
5	Chicken_megrivirus_RS/BR/2015/2R(MG846463.1)	86.99	87.13	87.26	88.24							
6	Chicken_megrivirus_RS/BR/2015/3R(MG846465.1)	84.94	84.97	85.15	85.11	88.45						
7	Chicken_megrivirus_Chicken/MG9567/Brazil/2012(MH806866)	79.53	78.22	80.24	78.79	79.01	78.96					
8	Melegrivirus_A(KF961188.1)	71.92	71.35	72.32	71.59	71.36	71.20	73.89				
9	Melegrivirus_A(NC_023858.1)	71.27	70.64	71.96	70.91	70.70	70.58	73.54	100.00			
10	Duck_megrivirus_LY_(KC663628.1)	50.63	50.23	50.98	49.97	49.98	50.18	50.47	53.98	54.85		
11	Goose_megrivirus_W18(KY369299.1)	49.69	49.69	50.41	49.50	49.21	49.76	49.45	52.83	53.63	79.59	

The number of base differences per site from between sequences is shown. The analysis involved eight nucleotide sequences as the closest results derived from BLAST. Codon positions included were 1st + 2nd + 3rd. All ambiguous positions were removed for each sequence pair. There were a total of 2002 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [20]

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Author contributions AG, AM, HH, MHFH conceptualization and design study, NHZ, ZZK, AHR performed the experiments, AG, HH, AM, MA analyzed data and sequence, AG, MA. AM, RED, and NS wrote the manuscript. All authors approved the article for publication.

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Declarations

Competing interests The authors declare no competing interests.

Ethical approval All institutional and national guidelines for the care and use of laboratory animals were followed.

Consent for publication All authors have read the manuscript and approved the final manuscript.

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