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Short Paper

Molecular characterization and the first full sequencing genome of chicken infectious anemia virus (CIAV) in Iran

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Abstract

Background: The chicken infectious anemia virus (CIAV) is an important pathogen that causes severe immunosuppression in young chickens. **Aims:** The study aims to characterize the genotype and full-length sequencing of CIAV strains in Iran. **Methods:** First, the collected thymus samples were investigated by conventional PCR for CIAV detection. Second, one of the CIAV positive samples (UT-Zahraee) was chosen for full genome sequencing. **Results:** Throughout 2017, we detected 13 CIAVs isolated from 40 broiler flocks of different provinces of Iran. A comparison of the complete sequences of the genome and homologies of the nucleotides revealed that UT-Zahraee had a high similarity with American and Egyptian CIAV isolates. Moreover, *VP1* sequence analysis showed that UT-Zahraee shared high homology with previously reported Iranian CIAV strains, Chinese, and Egyptian isolates. **Conclusion:** This study is the first report of full genome sequencing of CIAV strain from Iran. It will be beneficial to understand better the epidemiology and molecular characteristics of CIAV circulating in Iran.

Key words: Chicken infectious anemia virus, Full genome, Iran, Phylogenetic

Introduction

The chicken infectious anemia virus (CIAV) is a small, non-enveloped, icosahedral DNA virus with a circular, covalently linked, single negative-strand genome. It is the causative agent of chicken infectious anemia (CIA) (Pringle, 1999) and is classified in the genus Gyrovirus of the *Anelloviridae* family (Adams *et al*., 2017). The genome consists of approximately 2.3 kb nucleotides and three partially or completely overlapping open reading frames (ORF), which encode three viral proteins, designated as VP1, the major viral structural protein. The immunogenicity and virulence of the virus are determined by the presence of a few amino acids in this gene including VP2, scaffolding protein, and VP3, apoptin (Phenix *et al*., 1994; Oluwayelu *et al*., 2008; Hiremath *et al*., 2013). Different CIAV genotypes had been described based on phylogenetic analysis (Eltahir *et al*., 2011). CIAV is considered one of the most important poultry diseases and was identified in Japan for the first time in late 1979 (Yuasa *et al*., 1979). CIA is known as an economically important poultry pathogen worldwide with a highly immunosuppressive nature (Natesan *et al*., 2006). Simultaneous infection of chickens with other immunosuppressive viruses increases the severity of CIAV signs and decreases the resistance of older birds to the infection (Rosenberger and Cloud, 1989; De Boer *et al*., 1994). Recently, several CIAV outbreaks were reported from Iranian poultry flocks, and there is a serious concern about this economically important disease in the country. So, further studies should be conducted on the presence of CIAV in Iran (Farhoodi *et al*., 2007; Gholami-Ahangaran *et al*., 2011; Nayabian and Mardani, 2013; Gholami-Ahangaran, 2015; Kaffashi *et al*., 2017). Besides, to detect CIAV, the PCR technique is considered the choice assay of CIAV-DNA detection in chicken tissues, cell cultures, and vaccines (Hegazy *et al*., 2010). The study aims to characterize the genotype and full-length sequencing of CIAV strains in Iran and the comparison of the sequences of these strains with the strains of other countries.

Materials and Methods

Clinical samples and DNA isolation

Thymus samples (n=100) were collected from 40 commercial broiler flocks (aged 5-6 weeks which did not show any clinical signs) from different areas of Iran, including Ardabil, Isfahan, Golestan, Qom, and Yazd provinces during 2017. Thymus tissues from each flock were mixed and homogenized. Total DNA was isolated from the supernatant of 40 tissue pool homogenates

using a commercial DNA Extraction kit (Sinaclon DNA extraction kit DNP, Cat No. EX6071) according to the manufacturer's instructions. All samples were stored at -20°C. This article does not include any studies performed on humans or animals.

Detection of CIAV in samples

The forward primer 5´ AGC CGA CCC CGA ACC GCA AGA A 3´, and reverse primer 5´ ATC AGG GCT GCG TCC CCC AGT ACA 3´, were used to amplify *VP1* gene for CIAV detection with 1390 bp product size (Hiremath *et al*., 2013).

Full-length detection and phylogenetic analysis of UT-zahraee strain

The primers VP1 (VP1F 5´AGC CGA CCC CGA ACC GCA AGA A 3´, VP1R 5´ATC AGG GCT GCG TCC CCC AGT ACA 3´), VP2 (VP2F 5´AGC GCA CAT ACC GGT CGG CAG T 3´, VP2R 5´AGG GGT TCG GCA GCC TCA CAC TAT 3´), and VP3 (VP3F 5´ATG AAC GCT CTC CAA GAA G 3´, VP3R 5´ACT TAC AGT CTT ATA CAC CTT 3´) were used to amplify *VP1*, *VP2*, and *VP3* genes, respectively (Hiremath *et al*., 2013).

Genome sequences of VP1 coding region of CIAV and the full-length CIAV genome were retrieved from the GenBank. The alignment of the sequences was performed using the MEGA-7 software (Kumar *et al*., 2016). Sequence analysis was conducted using the CLC genome workbench version 3.5. Phylogenetic analysis was conducted using the Neighbor-Joining method, Kimura 2-parameter method (Kimura, 1980), and JTT matrix-based model, respectively, and bootstrapping up to 1000 replicates by MEGA-7 software (Kumar *et al*., 2016).

Results

Detection of CIAV by PCR

A total of 13/40 CIAVs were found to be positive in PCR (Fig. 1), and one isolate was used to complete genome sequencing. The CIAV isolate was named UT-Zahraee and was submitted to the GenBank with the accession number MT239353.

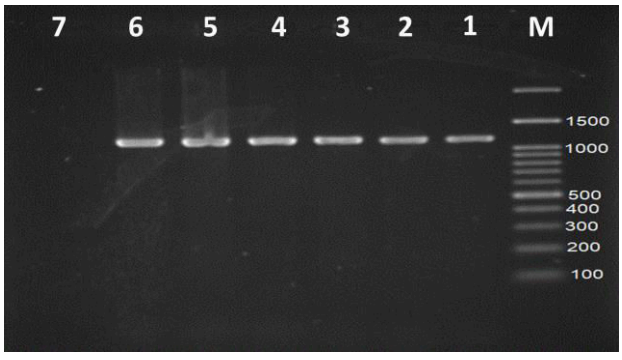

Fig. 1: PCR results of CIAV detection. M: 100 bp marker. 1: Positive control, 2-6: Positive CIAV isolates, 7: Negative control

Bioinformatics and phylogenetic analysis

The full-length genome of the UT-Zahraee was 2298 bps with a composition of 26% A, 18% T, 28% C, and 28% G, with a total G+C content of 55.91%. There were three overlapping open reading frames (ORFs) with nucleotide sequences of *VP1* (1350 bp), *VP2* (721 bp), and *VP3* (349 bp) (Fig. 2).

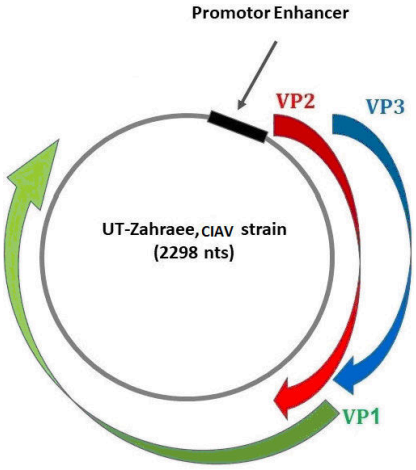

Fig. 2: Genome structure of UT-Zahraee, a CIAV strain

Phylogenetic analysis

Based on *VP1* gene sequences analysis, UT-Zahraee, and the previously reported Iranian CIAV strains were placed into the D genotype (Fig. 3). It shared high homology of 99.51%, 99.07%, 99.05%, 99.04%, and 98.84% with the previous Iranian isolate Shiraz1 (KU523251), a Chinese CIAV 17JL0310 (MK089241.1), two Iranian isolates IR1-CIAV (KU195689), IR5-CIAV (KU195693), and the Egyptian CIAV strain CIAV-EG-11 (MH001559.1), respectively (Table 1). However, based on full genome sequencing, UT-Zahraee isolate was classified as an A3 sub-cluster member with the high identity of 99.16%, 99.02%, and 98.57% with two American CIAV isolates, 01-4201 (DQ991394.1), Chicken_anemia_virus_3_(AF313470.1), and Egyptian CIAV strain CIAV-EG-11 (MH001559.1) (Fig. 4 and Table 2).

The critical amino acid substitution in VP1 protein of UT-Zahraee

At the amino acid level, the new CIAV strain showed virulent characteristics (Table 3). Some amino acid substitutions in the VP1 protein of UT-Zahraee included amino acid K at 139, Q at 141, Q at 144, and Q at 394 positions (Table 3). Some other variations in amino acids are I at 75, V at 157 (Table 3). Also, few reports have suggested that nucleotide at 141L and 144E (Table 3).

Discussion

In Iran, there is not enough information about CIAV's circulating in the poultry population (Nayabian and Mardani, 2013). However, recently, the widespread CIAV outbreaks in Iranian broiler farms were reported

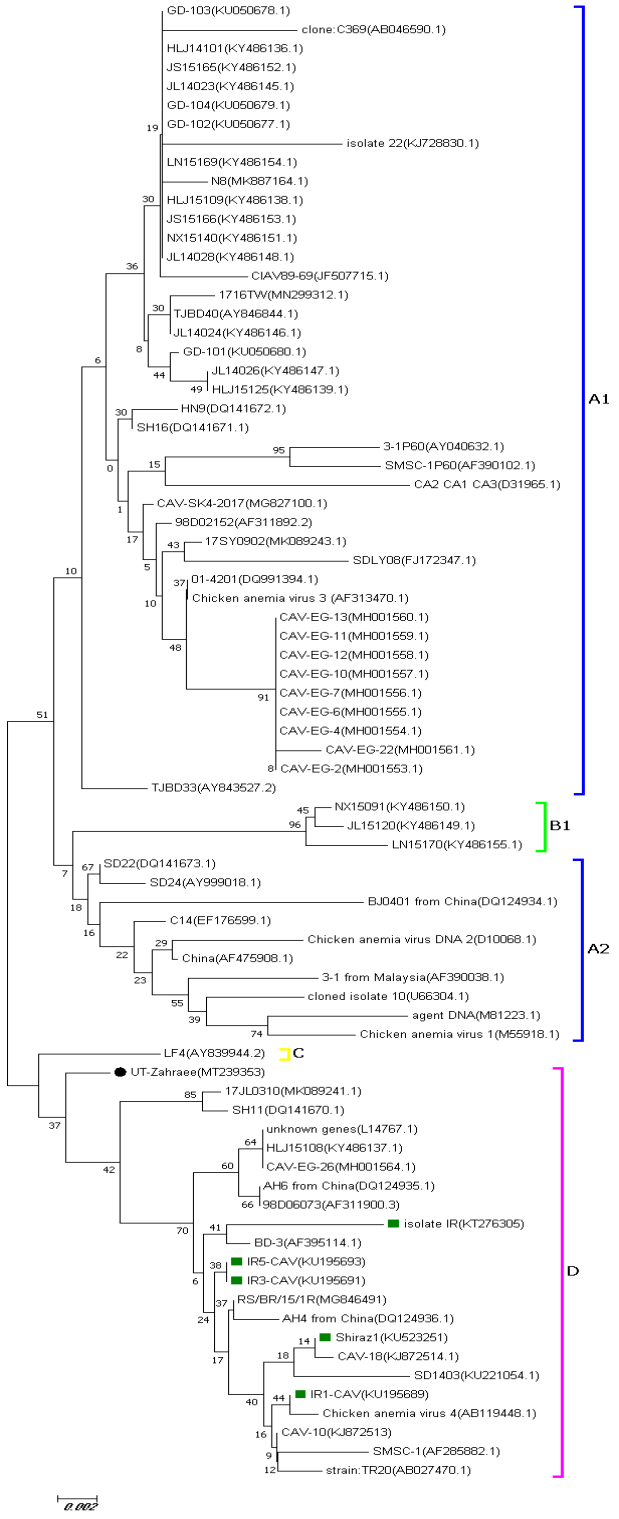

Fig. 3: Amino acid-base phylogenetic relationships of VP1 of UT-Zahraee and other CIAV isolates and other CIAV strains detected in Iran. The phylogenetic tree was generated using the JTT matrix-based model with MEGA [31]. The numbers below branches indicate bootstrap values from 1000 replicates. Horizontal 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 and previous Iranian isolates are indicated with a black circle (●) and green squares (■), respectively

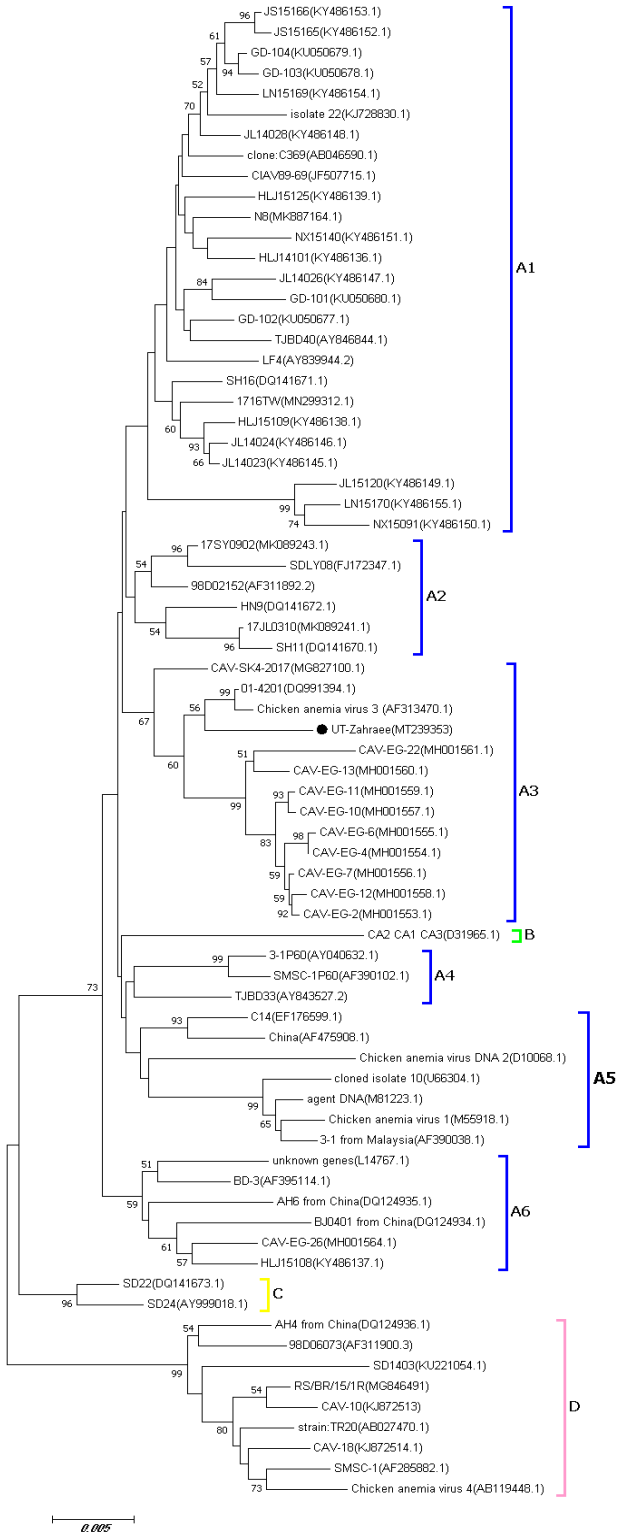

Fig. 4: Nucleotide acid-base phylogenetic relationships of the whole genome sequence UT-Zahraee and other CIAV strains detected in Iran. The phylogenetic tree was generated using the Clustal W-based Neighbor-Joining model with MEGA [31]. Numbers below branches indicate bootstrap values from 1000 replicates. 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 with a black circle (●)

Table 1: Estimates of evolutionary divergence for the *VP1* gene of UT-Zahraee isolate. The number of base differences per site is shown between sequences. The analysis involved 12 nucleotide sequences as the closest results derived from BLAST. Codon positions were 1st+2nd+3rd. All ambiguous positions were removed for each sequence pair. Evolutionary analyses were conducted in MEGA-7 (Kumar *et al*., 2016)

Number	CIAV strain	1	2	3	4	5	6	7	8	9	10	11	12
1	UT-Zahraee(MT239353)												
2	Shiraz1(KU523251)	99.51											
3	17JL0310(MK089241.1)	99.07	99.53										
4	IR5-CIAV(KU195693)	99.04	100.00	98.82									
5	IR3-CIAV(KU195691)	99.04	100.00	98.82	100.00								
6	IR1-CIAV(KU195689)	99.05	100.00	98.59	99.53	99.53							
7	CIAV-EG-11(MH001559.1)	98.84	97.10	98.16	97.86	97.86	97.64						
8	LF4(AY839944.2)	98.84	99.04	98.63	98.59	98.59	99.06	97.93					
9	BD-3(AF395114.1)	98.82	100.00	98.62	99.77	99.77	99.30	97.66	98.40				
10	Chicken_anemia_virus_4(AB119448.1)	98.60	100.00	98.40	99.53	99.53	100.00	97.23	98.86	98.86			
11	Isolate_IR(KT276305)	98.11	99.05	98.15	99.07	99.07	98.59	96.97	97.93	99.09	98.40		
12	NX15091(KY486150.1)	97.89	97.58	97.23	97.64	97.64	97.88	97.46	97.94	97.47	97.70	97.00	

Table 2: Estimates of evolutionary divergence for the complete genome of U-Zahraee isolate. The number of base differences per site is shown between sequences. The analysis involved 13 nucleotide sequences as the closest results derived from BLAST. Codon positions were 1st+2nd+3rd. All ambiguous positions were removed for each sequence pair. Evolutionary analyses were conducted in MEGA-7 (Kumar *et al*., 2016)

Number	CIAV strain	1	2	3	4	5	6	7	8	9	10	11	12	13
1	UT-Zahraee(MT239353)													
2	01-4201(DQ991394.1)	99.16												
3	Chicken_anemia_virus_3_(AF313470.1)	99.02	99.87											
4	CIAV-EG-11(MH001559.1)	98.57	98.85	98.76										
5	CIAV-EG-22(MH001561.1)	98.21	98.53	98.44	98.98									
6	SH11(DQ141670.1)	98.21	98.44	98.35	98.17	97.66								
7	SMSC-1P60(AF390102.1)	98.12	98.49	98.40	98.22	97.85	98.12							
8	GD-101(KU050680.1)	97.79	98.22	98.12	97.80	97.57	97.94	98.08						
9	AH6_from_China(DQ124935.1)	97.75	97.99	97.89	97.85	97.24	98.35	97.76	97.80					
10	3-1_from_Malaysia(AF390038.1)	97.70	98.03	97.94	97.76	97.52	97.85	98.12	97.61	97.76				
11	RS/BR/15/1R(MG846491)	97.59	96.87	96.77	96.57	96.02	96.82	96.77	96.67	96.97	96.57			
12	CA2_CA1_CA3(D31965.1)	97.62	97.74	97.62	97.25	97.13	97.63	97.74	97.46	97.14	97.52	96.33		
13	SD22(DQ141673.1)	97.23	97.85	97.75	97.38	97.15	97.52	97.80	98.08	97.52	97.47	97.41	97.14	

Table 3: The critical amino acid substitution in the VP1 protein of UT-Zahraee and other CIAVs

CIAV strain	Amino acid								
	75	89	125	139	141	144	157	287	394
JL14023(KY486145.1) Majority virulent	V	T	L	K	Q	E	V	S	Q
HLJ15125(KY486139.1) Low virulent strain	V	T	L	K	Q	E	M	S	Q
UT-Zahraee(MT239353)	I	?	I	K	Q	Q	V	S	Q
01-4201(DQ991394.1)	V	T	I	K	Q	E	V	S	Q
Chicken_anemia_virus_3_(AF313470.1)	V	T	I	K	Q	E	V	S	Q
IR6-CIAV(KU195694)	I	T	I	Q	Q	Q	V	A	Q
CIAV-EG-22(MH001561.1)	V	T	I	K	Q	E	V	S	Q
CIAV-EG-11(MH001559.1)	V	T	I	K	Q	E	V	S	Q
RS/BR/15/1R(MG846491)	I	T	I	Q	Q	Q	V	T	Q
GD-101(KU050680.1)	V	T	L	K	Q	E	M	S	Q
isolate_22(KJ728830.1)	V	T	L	K	Q	E	V	S	Q
SD22(DQ141673.1)	V	T	L	K	Q	E	V	S	Q
SH11(DQ141670.1)	I	T	I	Q	Q	Q	V	S	Q
AH6_from_China(DQ124935.1)	I	T	I	Q	Q	Q	V	A	Q
CA2_CA1_CA3(D31965.1)	V	T	L	K	E	E	V	S	Q
3-1P60(AY040632.1)	V	T	I	K	E	E	M	S	Q
SMSC-1P60(AF390102.1)	V	T	I	K	E	E	M	S	Q
3-1_from_Malaysia(AF390038.1)	V	T	I	K	Q	E	V	D	Q

several times (Toroghi *et al*., 2003, Farhoodi *et al*., 2007). Gholami *et al*., in 2011, found 73.33% CIAV-positive in broiler flocks by PCR. Nayabian and Mardani (2013) revealed a 46% prevalence of CIAV in Iranian broiler chickens, and their results also showed that the Iranian CIAV isolates carried a high genetic distance and were similar to the isolates from different countries. Similar to Nayabian and Mardanis' findings (2013), the current CIAV strain shared high homology to CIAVs isolated from different parts of the world. Therefore, it can be concluded that the circulating CIAVs in Iran's broiler farms contain high genetic variations (Nayabian and Mardani, 2013). At the first glance, the results might suggest that the equipment imported from some countries, especially from China, is responsible for the presence of this strain in Iran. On the other hand, according to VP1 sequence analysis, our CIAV isolate showed high homology with previously reported Iranian

isolates, which had shown to be genetically different from vaccine strains in Iran (Cuxhaven-1 and 26P4) (Nayabian and Mardani, 2013).

There are several CIAV outbreaks among broiler and layer flocks that have been reported from across the world such as the USA, Brazil, Australia, Asia, Spain, and Egypt from 2001 till now (Nogueira *et al*., 2005; Simionatto *et al*., 2006; Hegazy *et al*., 2010; Fang *et al*., 2017; Abdel-Mawgod *et al*., 2018; Aşkar, 2019). Also, CIAV has been detected in African and Cambodian backyard poultry (Oluwayelu *et al*., 2008; Kye *et al*., 2013), and these reports can raise concerns about the spread of the backyard chickens-CIAV strains to commercial poultry.

In this study, we detected 13 strains of CIAV among broiler chickens and reported a novel strain of CIAV named UT-Zahraee and was categorized in D and A3 Chinese cluster based on *VP1* gene and sequence analysis, respectively (Figs. 3 and 4).

Generally, chickens are the only natural and main host for CIAV, but this virus was also isolated in the other bird species, recently (Gholami-Ahangaran *et al*., 2013). In a study, chicken, turkey, and quail flocks were examined for CIAV infection and it was shown that whereas turkeys are not infected with CIAV, chickens and quails have a partially high infection rate to CIAV (Gholami-Ahangaran, 2015). Furthermore, the CIAV-antibody was not found in turkeys and ducks in the United Kingdom (McNulty *et al*., 1988), in crows, pigeons, and ducks in Japan (Farkas *et al*., 1998), and pigeons, ducks, and pheasants in Ireland (Campbell, 2001).

The genome sequence analysis of UT-Zahraee suggests that it is highly pathogenic, and it may have a direct/indirect role in increasing mortality or decreasing the flock performance (Sreekala *et al*., 2019). However, other immunosuppressive viruses and CIAV should be studied further to determine the main role of each virus in morbidity and mortality among poultry. In this regard, Yao *et al*. (2019) concluded that coinfection with Marek's disease virus (MDV), Reticuloendotheliosis virus (REV), avian leukosis virus (ALV), avian gyrovirus 2 (AGV2), and avian reovirus (ARV) were the main infection types of CIAV.

The immunogenicity and virulence of CIAV are usually determined by the presence of few amino acids in the *VP1* gene, the only structural protein in the virus encoding the viral capsid (Todd *et al*., 1990). In the current study, the phylogenetic analysis of VP1 embodied the diversity of CIAV, while the VP2 and VP3 embodied conservatism. According to previous investigations, amino acid Q (glutamine) at position 394 in VP1 may be a major determinant of pathogenicity, and amino acids at 139 and 144 are major concerns (Hailemariam *et al*., 2008). Researchers targeted *VP1* gene and genetically characterized CIAV, and they suggested that if both of the amino acids in positions 139 and 144 are glutamines, the virulence and replication ability of the virus are relatively weaker (Todd *et al*., 1990; Kye *et al*., 2013). Also, few reports suggest that nucleotides at 141L and 144E are associated with lower pathogenicity (Todd *et al*., 1990; Kye *et al*., 2013). UT-Zahraee had amino acid K at 139, Q at 141, Q at 144, and Q at 394 positions (Table 3).

This study can shed light on the genetic features of CIAV circulating among poultry flocks and provide information that can be used to develop diagnostics and preventive measures. Monitoring of CIAV circulating in the field at various intervals is recommended to determine the genotype and pathotype present in the field. Consequently, monitoring and improving the quality of vaccines may be a useful way to control the prevalence of CIAV.

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Conflict of interest

The authors declare that they have no conflict of interest.

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