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Original Article

Full-Length Genomic Characterization of Chicken Anemia Virus in Turkey

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ABSTRACT

Chicken infectious anemia (CIA) is an immune-suppressive disease caused by chicken anemia virus (CAV). It is characterized by lymphoid atrophy, aplastic anemia, especially in chicks. In this study, full-length genomic characterization of CAV DNA from the broiler flocks in Turkey and phylogenetic analysis were aimed.

In the study, CAV DNA were found positive for 37 (53%) flocks with PCR studies from thymus tissues of each 70 broiler flocks. And 17 purified CAV DNA PCR products from these 37 CAV isolates were full length sequenced with the NGS method (Illumina MiSeq).

Also with the phylogenetic analyses, full length PCR products of 17 purified CAV isolates have been determined as 2298bp genome size and 99% similarity with each other. The highest similarity (99%) has been detected with the isolates from China and Taiwan. Furthermore, a 97-98% similarity has been detected with vaccine strains (Cux-1, 26P4 and Del Ros) and also 88-90 % similarity has been detected with GyV4 and GyV3 isolates.

As a result, in the study full length genomic characterization of CAV DNA from the 7 regions of Turkey were determined. And also all Turkish CAV isolates and vaccine strains were in group 2 according to the phylogenetic tree were obtained. But these isolates and vaccine strains were not found in the same group with GyV3 and GyV4 strains. Besides, these CAV isolates were showed more similarity to the isolates reported from Taiwan and China than the vaccine strains.

INTRODUCTION

Chicken infectious anemia (CIA) is an immune suppressive disease caused by CAV. Disease is characterized by aplastic anemia and generalized lymphoid atrophy in young chickens. CIA disease can cause significant economic losses in the poultry industry due to immunosuppression, subclinical, horizontal and vertical spread, resistance to disinfection, and lack of protective vaccination against CIA infection in commercial broilers (Schat, 2003).

Vaccination strategies are based on the preventing of vertical transmission of virus by immunization of breeder flocks and the passage of adequate maternal antibody to the young chicks (Schat, 2003). However, Aşkar & Yıldırım (2011) reported protective maternal antibody titer decreased from 2 weeks of age and CAV positivity was increased in commercial chicken flocks from 3 weeks of age. Three different commercial live vaccines (Cux-1, 26P4, Del Ros strains) have been used in Turkey.

Chicken anemia virus (CAV, Gifu -1 strain) was first isolated by Yuasa *et al.,* in 1979. CAV has been classified in the Gyrovirus genus of the Anelloviridae (Rosario *et al.,* 2017; ICTV 2018). CAV consists of 2298

or 2319 bp single stranded ambisense or negative sense DNA genome. The viral genome contains three complete major open reading frames (ORF) that encode for 3 viral proteins; VP1, VP2, and VP3 (Noteborn *et al.,* 1991). VP1 is the major viral structural protein which is associated with viral replication, cell infection ability and virulence. Although the amino acid (aa) composition of CAV is very conservative, VP1 displays significant variability in certain regions (e.g., amino acid positions 139-151). If both of the amino acids in position 139. and 144. are glutamines, virulence and replication ability of the virus is relatively weaker (Farkas, 1996; Renshaw *et al.,* 1996; Yamaguchi *et al.,* 2001; Schat, 2003).

Previous epidemiological studies on the molecular and serological determination of CAV infection in Turkey and the other countries (Hadimli *et al.,* 2008; Aşkar & Yıldırım, 2011). In recent years, sequence differences in the CAV genome and genome similarities with Gyrovirus of other organisms, especially human beings, have been examined (Krapez *et al.,* 2006; Hailemariam *et al.,* 2008; Kim *et al.,* 2010; Abo Elkhair *et al.,* 2014, Li *et al.,* 2017). In 2011, a new human virus called 'Human Gyrovirus' (HGyV) which shows homology with CAV was isolated from the human skin surface (Sauvage *et al.,* 2011). In China, two Gyrovirus species were identified in 2012, and they are named as GyV3 and GyV4. These viruses were found in fecal samples of human consuming CAV infected and/or vaccinated chickens (Phan *et al.,* 2012; Zhang *et al.,* 2012). In addition, it was reported that CAV genome showed many similarities with these viruses that caused infection in humans (Chu *et al.,* 2012; Maggi *et al.,* 2012). This data suggests that, after consumption of chicken infected with CAV or injected with CAV, CAV has the potential to threaten human health (Zhang *et al.,* 2013).

In this study, full-length genomic characterization of CAV from the broiler flocks of in Turkey and phylogenetic analysis were aimed. Furthermore, the sequences of representative 132 full length CAV isolates in CAV vaccine, human GyV3 and GyV4 including GenBank by phylogenetic methods were compared.

MATERIAL AND METHODS

Clinical Samples and DNA isolation

Thymus samples (n=700) were collected from 70 commercial broiler chicken flocks (aged 5–6 week and didn't show any clinical signs) after slaughter in the 7 different regions of Turkey during 2015-2016. Thymus tissues (n=10) from each flock were mixed and homogenized. Therefore, 70 tissue pools were obtained. Total DNA was isolated from the supernatant of the 70 tissue pool homogenates by using a commercial QIAamp MinElute Virus Spin Kit (Qiagen Science- Cat No.: 57704) according to the manufacturer's instructions. All samples were stored at -80 ºC.

Detection and Amplification of CAV Genome

PCR analyses were performed for the determination of CAV DNA and obtaining the full length genome from tissues. During PCR amplification, primer pairs of CQ1F 5'-CAATCACTCTATCGCTGTGT-3', CQ1R 5'-TTCGTCCATCTTGACTTTCT-3' and CQ2F 5'-GGCTACTATTCCATCACCATTCT-3', CQ2R 5'-GCTCGTCTTGCCATCTTACA-3' were used to amplify 1778-bp and 831-bp size fragments, respectively, covering the entire nucleotide sequence (Zhang *et al.,* 2013). PCR were performed in a 50 µL reaction volume containing 1 µL forward primer, 1 µL reverse primer, 1,5 µL DNA, 1,5 µL dNTPs (10 mM), 12.5 µL 10x Platinum Pfx Taq DNA polymerase buffer, 0.5 µL Platinum Pfx Taq DNA Polymerase (Thermo Fischer Scientific Cat. No. 11708039) and 32 µL ultra-pure distilled water. PCR cycle conditions were 94 ºC for 3 min for pre-denaturation, followed by 30 cycles of 94 ºC for 60s, 57 ºC for 60s, and 68 ºC for 180s; a final extension step was set at 72 ºC for 7 min prior to termination of the reaction at 4 ºC.

All PCR for each sample were performed in duplicate. The PCR products were analyzed in 1% agarose gels stained with ethidium bromide.

Sequencing, Alignment and Phylogenetic Analysis of Full-length CAV Genome

DNA libraries were obtained 17 CAV isolates PCR products of purified using Nextera XT DNA Library Preparation Kit (Illumina, Cat No. FC-131-1096). Then, adapters containing the index arrays (P5&P7 probes and Illumina-compliant sequence primers) were added to fragments tagged with amplification process. Samples were purified (AmpureXP bead, Beckman Coulter kit) and quantity determination was made (Qubit dsDNA HS Assay Kit). In 4 nM concentration obtained, concentration was reduced 20 pM by applying MiSeq denaturation process from DNA libraries. From the obtained library, 600 µl amount was taken and loaded to MiSeq cartridge, and cartridge was placed in device, new generation sequence (NGS) was performed.

Galaxy software was used to filter read errors of the device and remove erroneous readings from FASTQ data obtained by NGS (Illumina MiSeq). Genomic data was obtained from readings with quality control completed after pre-processing without using reference genome (Denova method). Thus, full length genome belonging to our sample was mapped by combining small reading pieces. These steps were repeated for all examples. Galaxy Web portal (https://usegalaxy.org/) was used during the mapping of array data.

The obtained 17 full length genome data were compared with 132 CAV full-length genome which are including vaccine strains, GyV3 and GyV4 reference genomes previously reported by deposited in GenBank. To compare multi-genome sequence alignment studies and VP1 protein amino acid sequences, Clustal Omega (Multiple Sequence Alignment) was used to apply multiple alignments among them. In phylogenic tree formation, Phylogeny and PHYLIP packaged software were used. Obtained phylogenetic analysis data were tested in "in-silico", and verification of study was performed.

RESULTS

Detection and Amplification of CAV Genome

According to PCR results conducted using two different primer pairs, CAV DNA presence was detected from 37 of 70 commercial broiler flocks. In tissue samples with non-determined CAV, it was confirmed whether a DNA inhibitor existed using internal control. Thus, the PCR results were verified. After PCR analyses, 17 PCR products were sequenced and the PCR gel images were given in Figure 1.

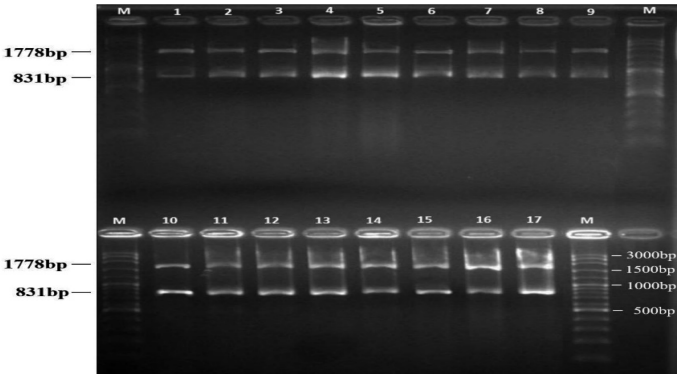

Figure 1 – Electrophoresis of PCR products of 17-purified CAV DNA isolates.

Sequencing, Alignment and Phylogenetic Analysis of Full-length CAV Genome

PCR products were sequenced after measurement of concentration values (in range of 10-81 ng/µl) by Qubit. After sequencing obtained FASTQ data were applied to array analysis, and thus the whole genome sequence was determined. In the study, it was determined that all isolates were in 2298bp genome size.

Phylogenetic distance matrices of 17 purified CAV isolates from Turkey, 132 CAV strains and 2 HGyV full-length genome sequences were analyzed. Obtained affinity relations are given by Phylogenetic tree in Figure 2.

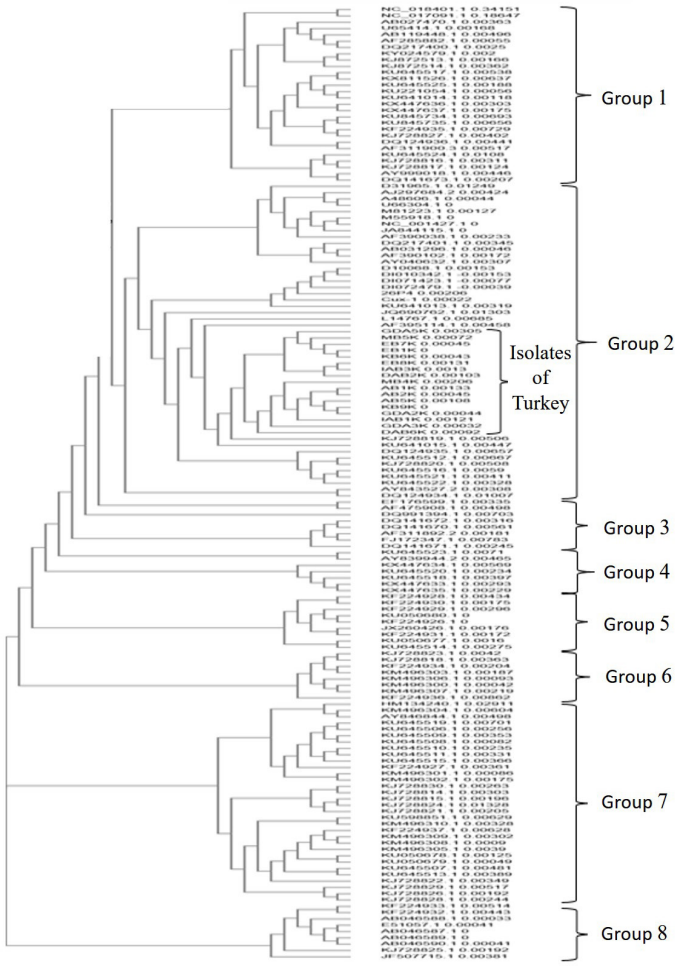

Figure 2 – Full-length genome sequences phylogenetic tree of 17 purified CAV genome in turkey, 132 CAV and 2 Human Gyrovirus genome in GenBank using Neighbour joining method that used clustal tree format, distance correction on, exclude gaps off parameters. Phylogenetic tree files were obtained from shuffle the sequence data matrix five times.

Eight groups were detected according to the phylogenetic tree obtained from the genome sequence of 151 isolates. All field isolates, reference genome and vaccine strains were found in the 2nd group of phylogeny. According to the data obtained from the phylogenetic tree, the highest similarity of Turkey CAV isolates were determined with China (Access no: KU641015, DQ124935, KU641016, KU645521, KU645522) and Taiwan isolates (KJ728819, KJ728820)

in 99% percentage. Also, high similarity with a 97% percentage was determined with vaccine strains (Cux-1, 26P4 and Del Ros).

When the Turkey's CAV genome data were compared with Human Gyrovirus genome data in GenBank, full length genome nucleotide similarities were found with GyV3 and GyV4 between 88-90% percentages.

VP1 protein amino acid sequences of reference genome, vaccine strains and 17 purified CAV isolates were compared with the Clustel Omega (Multiple Sequence Alignment). According to the obtained data (given in Table 1), CAV's (from field) VP1 proteins amino acid sequences showed similarities among each other, but there were differences with reference genome and vaccine strains.

Table 1 – Differences of VP1 protein aminoacid sequences among, vaccine and Turkey CAV isolates.

	CAV Isolates	Amino acid substitution in VP1 protein														
	Consensus Positions	14.	22.	75.	92.	97.	139.	144.	157.	251.	254.	287.	290.	324.	370.	446.
	Reference genome Cux-1 (M55918)	S	H	V	G	M	K	D	V	Q	G	A	A	S	S	G
1.	TR 1	.	N	I	.	L	Q	Q	.	R	E	.	P	.	T	.
2.	TR 2	A	N	I	.	L	Q	Q	.	R	E	.	P	.	T	.
3.	TR 3	A	N	I	.	L	Q	Q	.	R	E	.	.	.	T	.
4.	TR 4	A	N	I	.	L	Q	Q	.	R	E	.	.	.	.	.
5.	TR 5	A	N	I	.	L	Q	Q	.	R	E	.	.	.	T	.
6.	TR 6	A	N	I	.	L	Q	Q	.	R	E	.	.	.	.	.
7.	TR 7	A	N	I	.	L	Q	Q	.	R	E	.	.	.	.	.
8.	TR 8	A	N	I	.	L	Q	Q	.	R	E	.	P	.	.	.
9.	TR 9	A	N	I	.	L	Q	Q	.	R	E	.	.	.	T	.
10.	TR 10	A	N	I	.	L	Q	Q	.	R	E	.	.	.	T	.
11.	TR 11	A	N	I	.	L	Q	Q	.	R	E	.	.	.	T	.
12.	TR 12	A	N	I	.	L	Q	Q	.	R	E	.	.	.	T	.
13.	TR 13	A	N	I	.	L	Q	Q	.	R	E	.	P	P	.	.
14.	TR 14	A	N	I	.	L	Q	Q	.	R	E	.	.	.	.	.
15.	TR 15	A	N	I	.	L	Q	Q	.	R	E	.	.	.	.	.
16.	TR 16	A	N	I	.	L	Q	Q	.	R	E	.	.	.	T	.
17.	TR 17	A	N	I	.	L	Q	Q	.	R	E	.	.	.	.	.
19.	26P4 (AJ890284)	A	.	.	D	.	.	E	M	R	.	T	.	.	.	S
20.	Del Ros(AF313470)	A	.	.	.	.	.	E	.	R	E	S	.	.	G	.

A: Alanin, G: Glisin, L: Leucine, P: Prolin, T: Treonin, H: Histidine, I: Isoleucine, M: Methionine, S: Serin, V: Valin, R: Arginin, D: Aspartat, E: Glutamate, Q: Glutamine.

DISCUSSION AND CONCLUSIONS

In this study, presence of chicken anemia virus (CAV) DNA was investigated in flocks of commercial broilers from 7 different regions of Turkey. This study is the first to demonstrate the CAV genome sequence in Turkey by new generation sequencing (NGS-Illumina) method. According to the data obtained from the study, these genomes sequence similarity were analyzed with commercial vaccine strains (26P4, Cux-1 ve Del Ros) and GyV3, GyV4 viruses which are thought to be related with CAV and has been identified as infection agent in humans. So, important data were obtained about the spread of vaccine strains in field.

When the sequence and phylogenetic results were analyzed, obtained isolates from the field were determined in 2298 bp genome length and have more similarity with the China and Taiwan CAV isolates than the commercial vaccine isolates. This situation suggests that obtained isolates did not directly relate with the vaccine strains (Cuxhaven-1, 26P4 and Del-Ros) used in Turkey. Comparing amino acid sequences of VP1 protein, which is reported to exhibit variability, it was observed that field isolates have a high rate of similarity at 99%, and have different sequences from vaccine strains.

It was reported that the amino acid in the 394. position of VP1 protein has a major genetic determinant of virulence (Yamaguchi *et al.,* 2001). Ganar *et al. (*2017) determined glutamine aa. in the 394. position of VP1 protein of all 11 CAV field isolates obtained from India. Similar to results of Ganar *et al.,* glutamine aa. was determined in the 394. position of all 17 isolates isolated from Turkey in this study. This data suggests that all obtained isolates may be pathogenic. In a very variable region, it was reported that 139. and 144. positions had significant role in viral replication and spread in cell culture (Renshaw *et al.,* 1996). Li *et al. (*2017), were reported in their study that in 24 field isolates glutamine aa. were determined in

394 position whereas it were determined in the 139. and 144. positions in the 16 isolates. In this study, also the glutamine aa. in 139. and 144. positions in all 17 isolates were determined. This data showed that obtained isolates in this study may have a low viral replication and spread in cell culture.

When the similarities of our CAV isolates from Turkey were compared with human pathogenic GyV3 and GyV4 viruses which shows high similarity to CAV' strain in recent years (Chu *et al.,* 2012; Maggi *et al.,* 2012; Zhang *et al.,* 2012) and other CAV isolates in Gen Bank, low similarity (88-90 %) has been determined with other CAV isolates.

As a result of this study, CAV were determined from the 7 regions of Turkey and all determined CAV isolates have 2298bp genome size. According to the phylogenetic tree all Turkish CAV isolates and vaccine strains were in group 2 (Table 1) but these were not in the same group with Gyv3 and GyV4 (group 1) isolates. Besides, these CAV isolates were showed more similarity to isolates reported from Taiwan and China, than the vaccine strains. In addition, there is a need for further studies on the presence of Gyv3 and GyV4 in humans and phylogenetic analysis in Turkey.

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ETHICS STATEMENT

This study was approved by the Animal Welfare Committee of Kırıkkale University (R.N.:15/01).

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