Original Article

Sequence and Phylogenetic analysis of the VP1 segment of chicken infectious anemia virus (CIAV) during an outbreak in Palestine

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Abstract

In this study, chicken infectious anemia virus (CIAV) was detected in two unvaccinated commercial broiler flocks, showing clinical signs relevant to Chicken infectious anemia (CIA) disease. The clinical signs included subcutaneous hemorrhage, depression and death. Necropsy findings were pale liver, severe atrophy of bursa of the Fabricius and thymus and discoloration of the bone marrow. DNA was extracted from the livers of the infected birds and the VP1 gene was detected by polymerase chain reaction. The virus strains were detected and genetically characterized in almost the whole VP 1 gene (1338 base pairs). The nucleic acid analysis showed that the two Palestinian strains had 99.9% similarity and 98.7% similarity to a strain from chickens in Bangladesh (Accession no. AF395114) and 98.5% to a strain from a human fecal sample (Accession no. JQ690762) from China in the same cluster. The lowest similarity (94.7%) was with a strain isolated from chickens in China (Accession no. KU645525). Alignment of the deduced VP1 amino acids showed that the two Palestinian strains shared 100% homology (with one silent mutation encoded Leucine at residue no 97(^{97}L)), classified as high pathogenicity based on glutamine at residue in position 394 (^{394}Q). The highest similarity was with the Bangladesh 99.8% (AAM20899), Brazil 99.5% (APQ44719) and China (AAZ40209) 99.5% strains, while the lowest was with the China strain (AFR46599) 96.6%. The study provided valuable information on the molecular characterization of CIAV strains in Palestine for the first time.

Keywords: Chicken Infectious anemia virus, viral protein 1, phylogenetic analysis

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Introduction

Chicken infectious anemia (CIA) is an economically important disease of the poultry industry worldwide. The disease is manifested in either subclinical or clinical signs; the subclinical state in broiler flocks is due to the presence of maternal antibodies (Yuasa *et al*. 1980). As maternal antibody levels decline, chickens can become susceptible to the infection. In addition, the risk of infection is also increased through the immunosuppressive effects of Marek's disease and infectious bursal disease (Rosenberger and Cloud, 1989). The clinical signs of CIA are characterized by severe anemia, thymus atrophy, bone marrow aplasia, low hematocrit values and reduced body weight (Rosenberger and Cloud 1998). The economic importance of the disease results from increased mortality and the poor performance of infected flocks (McNulty *et al*. 1991). The lymphoid depletion causes an immunosuppression state that leads to poor response to vaccination and is accompanied by secondary bacterial infections (Hoerr 2010).

Chicken infectious anemia virus (CIAV) is the causative agent of CIA disease. The virus belongs to the genus *Gyrovirus* of the family *Circoviridae* (Rosario *et al*., 2017) The viral genome is 2.3kDa single-stranded DNA, consisting of three overlapping open reading frames, encoding three proteins; viral protein (VP) 1, VP2, and VP3 (Noteborn *et al*., 1991). VP1 is the viral structural protein that forms the capsid of the virus particle and induces neutralizing antibodies in chickens. VP2 is a scaffolding protein that regulates the function of the non-structural VP3 protein named Apoptin (Lai *et al*., 2017). The VP1 gene has been the target for exploring the genetic diversity of CIAV strains because of its high variability (Renshaw *et al*., 1996). Phylogenetic analysis of the VP1 gene has been used to distinguish three genetically distinct genotypes (I, II, and III) groups (Islam *et al*., 2002). The geographical distribution of these groups varies worldwide (Brown *et al*., 2000; Oluwayelu, 2010). Phylogenetic analysis according to the sequencing of CIAV-VP1 is used to determine the pathogenicity of the virus and also helps epidemiologists in tracing the origin and following the spread of the virus (Yamaguchi *et al*., 2001). Also, homologous recombinant plays a role in generating genetic diversity in natural populations of CIAV(He *et al.* 2007).

The past few years have seen an increasing number of commercial breeder and broiler flocks in Palestine. Studies on the outbreaks and molecular characterization of CIAV in Palestine have not been reported before this study. Therefore, we performed the first genetic analysis of the VP1 gene from Palestinian CIAV genomes detected in commercial broiler flocks. The nucleotide and amino acid sequences of the local CIAV field strains were compared with the reference CIAV strains to determine the extent of genetic diversity. This study will be the baseline for tracking the CIAV based on genetic sequences.

Materials and Methods

Samples: Samples of livers were collected from two broilers flocks housed in private farms in Tulkarm city (Northern Palestine). Twelve samples originated from the broiler flocks (the population size was 7,000 and 10,000 birds) aged 22 and 20 days-old, with mortalities of 12% and 15%. The flocks were not vaccinated against CIAV. The two flocks share the same source of chicks, feed, as well as the veterinarian supervision.

The affected birds demonstrated clinical signs consistent with CIA, including roughened feathers and stunted growth. Most affected birds had died suddenly and two birds displaying the symptoms described above were chosen and necropsied.

DNA extraction, PCR amplification, and sequencing: The DNA extraction was carried out from 25 mg of the livers from birds showing clinical signs using the ISOLATE II Genomic DNA Kit (Bioline, USA) and according to the manufacturer's instructions. To analyze the VP1 gene of CIAV-positive viruses, we amplified three DNA fragments cover 1,425 bp using three sets of overlapping primers as previously described by (Kye *et al*., 2013).

The primers were amplified at position 88 bp downstream from the start codon of VP1 and one primer positioned 13 nucleotides upstream from the stop codon of VP1. The first primer pair (Forward 5`-GAGTACAGGGTAAGCGAGCTA-3'; Reverse, 5'-AGGGCACGTTATTATCTAGCG-3'), and gave DNA fragment of 563 bp. The second pair (Forward 5`-GCGAGAGTCGCCAAGATC-3'; Reverse, 5'-TACTGTTGCTCGTCGGGTCT-3'), with an amplicon size of527 bp. The third primer pair was (Forward 5`-CTGTTCCGACACATTGAAACC-3'; Reverse, 5'-CCCCAGTACATGGTGCTGTT-3'), with a product of 734 bp. The polymerase chain reaction was performed in the Veriti® Thermal Cycler (Applied Biosystem, USA), with the following reaction conditions: an initial denaturation step at 94 °C for 5 mins, followed by 30 cycles of 94 °C for 1 min, 52 °C for 45 seconds and 72 °C for 1 min with a final extension at 72 °C for 10 mins. The amplified PCR products were analyzed by electrophoresis on a 1% agarose gel with ethidium bromide. Unpurified PCR products of the VP1 gene with a specific size were submitted to Sanger sequencing (Syntezza Bioscience, IDT, Jerusalem) with the forward primers.

The nucleotide sequences results were retrieved by Finch TV 1.4 (https://finchtv.software.informer .com/1.4/) software, aligned and edited with Vector NTI 9.1.0® (Invitrogen, Carlsbad, CA), compared with 30 reference CIAV strains representing all species, serotypes and geographical locations. The phylogenetic tree was generated by the neighbor-joining method using the MEGA 10.0.5 ® software (Kumar *et al*. 2008) with 1,000 bootstrap replication.

The predicted amino acid sequences of almost the entire VP1 genes (from n=1 to 1,338 bp) retrieved from different reference CIAV strains were determined and compared in the current study. To assess the phylogenetic clustering and relationship among CIAV, the VP1 sequences were aligned by the ClustalW method (Kumar *et al*., 2008; Thompson *et al*., 2002) and

the phylogenetic tree was constructed using the neighbor-joining method with bootstrap values being determined by 1,000 replicates to assess the confidence level of each branch pattern using MEGA 10.0.5 ® software (Kumar *et al.*, 2008).

Results

Clinical signs and lesions: Examined birds showed signs of anemia, generalized weakness, depression, pale combs and wattles, stunting, growth retardation and a high mortality rate. The necropsy findings were watery blood, yellow fatty bone marrow, markedly atrophied thymus glands, atrophied bursa of the Fabricius and enlarged liver and spleen. Subcutaneous hemorrhages were also noticed.

PCR and sequencing: PCR was performed on the extracted DNA from the liver of diseased chickens from two broiler flocks and the products were analyzed by agarose gel electrophoresis. The results showed positive PCR reactions of correct size for the VP1-specific fragments. The expected size of the VP1 amplicons was 563 bp, 527 bp and 734 bp (including primers) agreeing with the published nucleotide sequences (Kye *et al.*, 2013) (Fig.1). The nucleotide sequence utilized in this study was deposited in GenBank with the accession number (MT583480 and MT583481).

The sequencing of the PCR products was performed as previously described (Syntezza, Applied Biosystems, Jerusalem). The C terminus of the VP1 coding region (9 nucleotides) was not reliably sequenced; therefore a shorter fragment (1338 nucleotides) was used in the phylogenetic analyses. On the nucleotide level, only one nucleotide substitution (nt positions 291) was found between both Palestine-Chicken strains. However, no predicted amino acid substitutions were detected between them (TT**A** and TT**G** both encode L), indicating a silent mutation. The sequences showed a 98.7% nucleotide sequence identity with CIAV strain isolated from chickens in Bangladesh (Accession no AF395114) (Islam *et al.*, 2002) and 98.5% CIAV detected in a human fecal sample in China (Accession no JQ690762)(Yu and Duan 2012), the lowest similarity was 96.6% with strain form chickens in China (Accession no KU645525).

The phylogenetic branching pattern of VP1 genes of the CIAV strains showed that closely relation to the CIAV strain isolated from chickens in Bangladesh (Islam *et al.*, 2002) and to a strain detected from a human fecal sample in China (Yu and Duan 2012) (Fig 2).

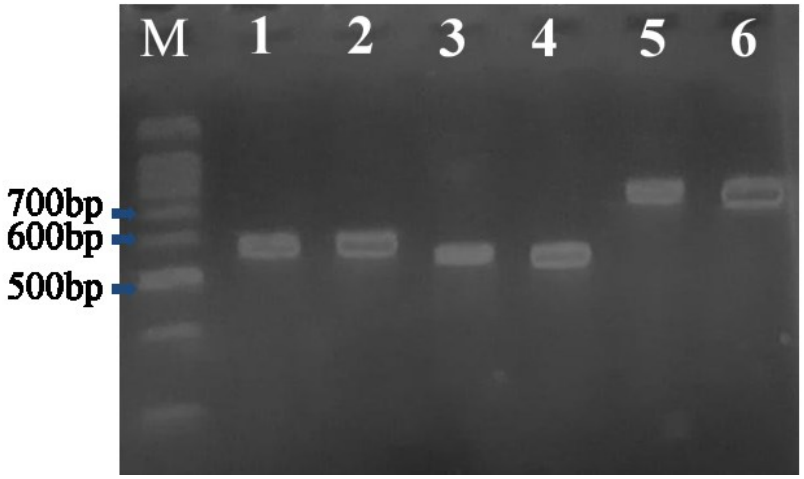

Figure 1 Agarose gel electrophoresis showing the three PCR products of the CIAV-VP1 gene from two broiler flocks. Lane 1 (M): DNA Molecular Weight Marker (GeneDireX, Inc., Taiwan), lane 1, and 2: 563bp from flock 1 and 2, lane 3, and 4: 527bp from flock 1 and 2, 5 and 6: 734bp from flock 1 and 2 respectively.

Amino Acid Alignment and Comparison with Other CIAV Strains: The predicted amino acid sequences of the VP1 gene of the Palestinian strains were aligned and compared with most universal CIAV isolates. The Palestinian CIAV viruses clustered together with a strain from chickens in Bangladesh (AAM20899) and from chickens in China (AAZ73172) (Fig. 3). The deduced 445-amino-acid sequence of VP1 protein revealed one substitution amino acid Asparagine (N) at position 22 (^{22}N), compared to Histidine (^{22} H) in the Bangladesh-chicken-AAM20899, and three residues ^{141}A, ^{370}T and ^{427}N compared to ^{141}S, ^{370}G and ^{427}D of the China isolate (AAZ73172) (Table 1). The amino acid at VP1 residue 394 was Glutamine (^{394}Q), which is crucial for the high pathogenicity of CIAV; it was also conserved in all listed isolates (Table 1)(Yamaguchi *et al.*, 2001).

Discussion

Chicken Infectious anemia (CIA) has been occurring as an economically important and re-emerging disease of broiler breeders and broiler chickens in several countries (Davidson *et al.*, 2004; Zhang *et al.*, 2013). For the first time we used molecular methods for the genetic characterization of CIAV to identify the CIAV strain in Palestine. The investigated broiler flocks showed clinical signs of subcutaneous hemorrhage and high mortality. The necropsy findings with severe atrophy of bursa of theFabricius and thymus and discoloration of the bone marrow, as well as hemorrhages subcutaneously, were in coordination with those caused by high virulence CIAV strain. (Hegazy *et al.*, 2010; Davidson *et al.*, 2004). The occurrence of disease at three weeks of age mostly

followed transovarial transmission from infected breeders (Rosenberger and Cloud, 1998). Beyond the scope of this study; other immunosuppressive diseases might be a potential for exacerbation of the clinical signs and mortality (Hagood *et al.*, 2000). No vaccine was used in Palestine against CIAV; this means that the detected virus is a field strain and should not be confused with a vaccine strain. The absence of control measures promoted the virus circulating in Palestinian breeder farms as well as being vertically transmitted.

At the molecular level; we used three PCR products containing almost the entire VP1 coding region (nucleotides 1 to 1338) of the positive samples. The two Palestinian strains were almost identical except for one nucleotide being different at position 291, suggesting the evolution of the virus and the occurrence of mutation. Although the number of samples used in this study was limited, our phylogenetic analysis of the nucleotide sequences revealed that CIAV strains detected in Palestine were genetically clustered with the Bangladesh CIAV strain and the strain from human fecal samples in China. Alignment of the deduced amino acid sequences of the two Palestinian strains showed 100 % sequence identity. The only nucleotide difference encoded a silent mutation which explains the same clinical signs and pathogenicity in the two broiler flocks. Comparison of the amino acid sequences of the Palestinian and 36 reference CIAV strains revealed that the VP1 gene appeared to be highly conserved. The greatest similarity was with the Bangladesh 99.8% (AAM20899), Brazil (APQ44719) 99.5%, China (AAZ40209) 99.5%, while the lowest was with China (AFR46599) 96.6%. The amino acid residue at positions 22 and 370 were the most variable location among all investigated strains; which seems to be non-relevant for the change in pathogenicity of CIAV. Previous studies have demonstrated that amino acid at position 394 in VP1 is crucial for CIAV virulence. When the amino acid was glutamine (Q), the virus showed high pathogenicity and when it was histidine, the virus had low pathogenicity (Eltahir *et al.*, 2011; Yamaguchi *et al.*, 2001). The Palestinian strains have a conserved ^{394}Q which is in agreement with the clinical signs and the necropsy findings of high virulence strains of CIAV.

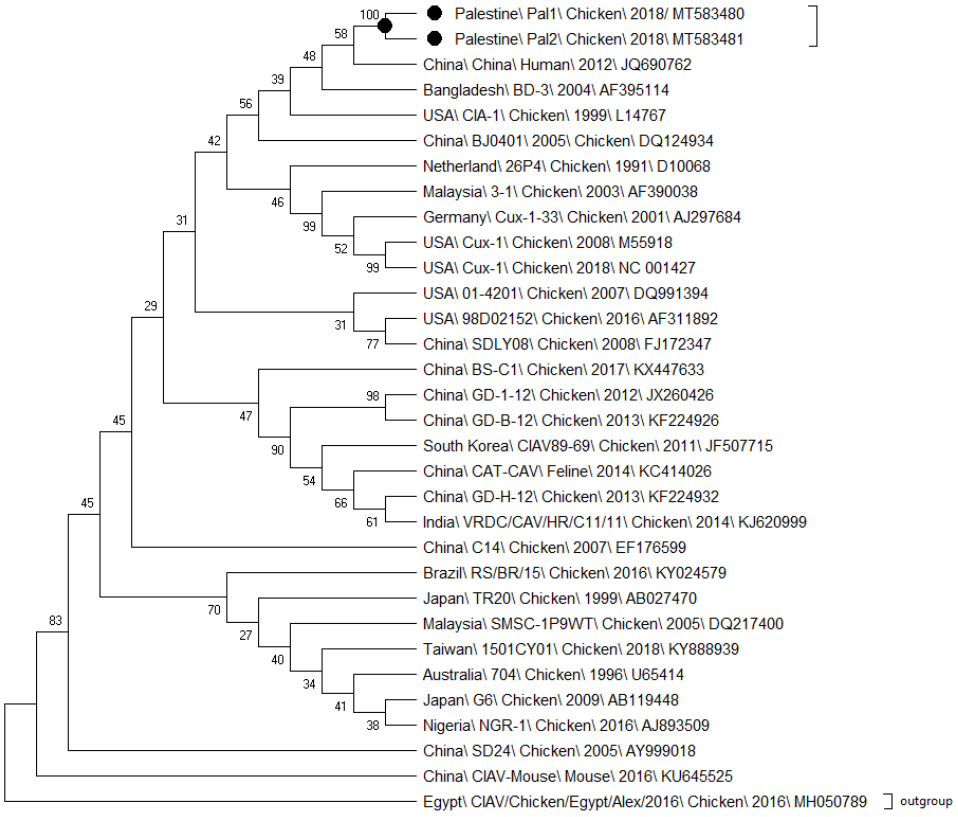

Figure 2 Phylogenetic relationship among Palestinian and reference CIAV strains based on the nucleotide sequence of a fragment of the VP1 gene.
Sequences from the present study are named as Palestine\Pal1\Chicken\2018\ 1 and 2 with accession numbers (MT583480 and MT583481), and indicated by the black circle. Names are given by Country\ Strain or Isolate\ Host\ Isolation year\ GenBank accession number. The numbers below the branches indicate neighbor-joining distances with 1,000 bootstrap replicates.

Taken together, these findings indicate that these Palestinian CIAV strain viruses have motifs commonly found in virulent viruses. The phylogenetic analysis of the VP1 nucleotide and amino acid sequences showed that the Palestinian strains formed a cluster with the Bangladesh CIAV strain isolated from chickens, which may indicate the origin of the Palestinian strains. Still, however, we cannot exclude the presence of subclinical low pathogenic strains in Palestine. The origin of the CIAV outbreak in Northern Palestine is a major open

question in the field. Even though there is no trade between Palestine and Bangladesh in poultry products, the close similarity to the CIAV reported in China could explain the ancestor of the virus. Since vaccination against CIAV is not done in Palestine at present, these two CIAV strains were likely introduced into the Palestine poultry population through the importation of infected poultry, poultry vaccines or other biological sources from another common origin.

A vaccine should be applied to breeder flocks for control of the continuous economic losses caused by CIAV. Our findings should be used as the baseline to follow up virus evolution in the region. More studies are required for exploring the epidemiological aspects of CIAV infection in Palestine; this will be of help in Middle Eastern countries in light of the absence of studies on the epidemiology and characterization of CIAV infection on commercial chickens in this region.

Conflict of interest: None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

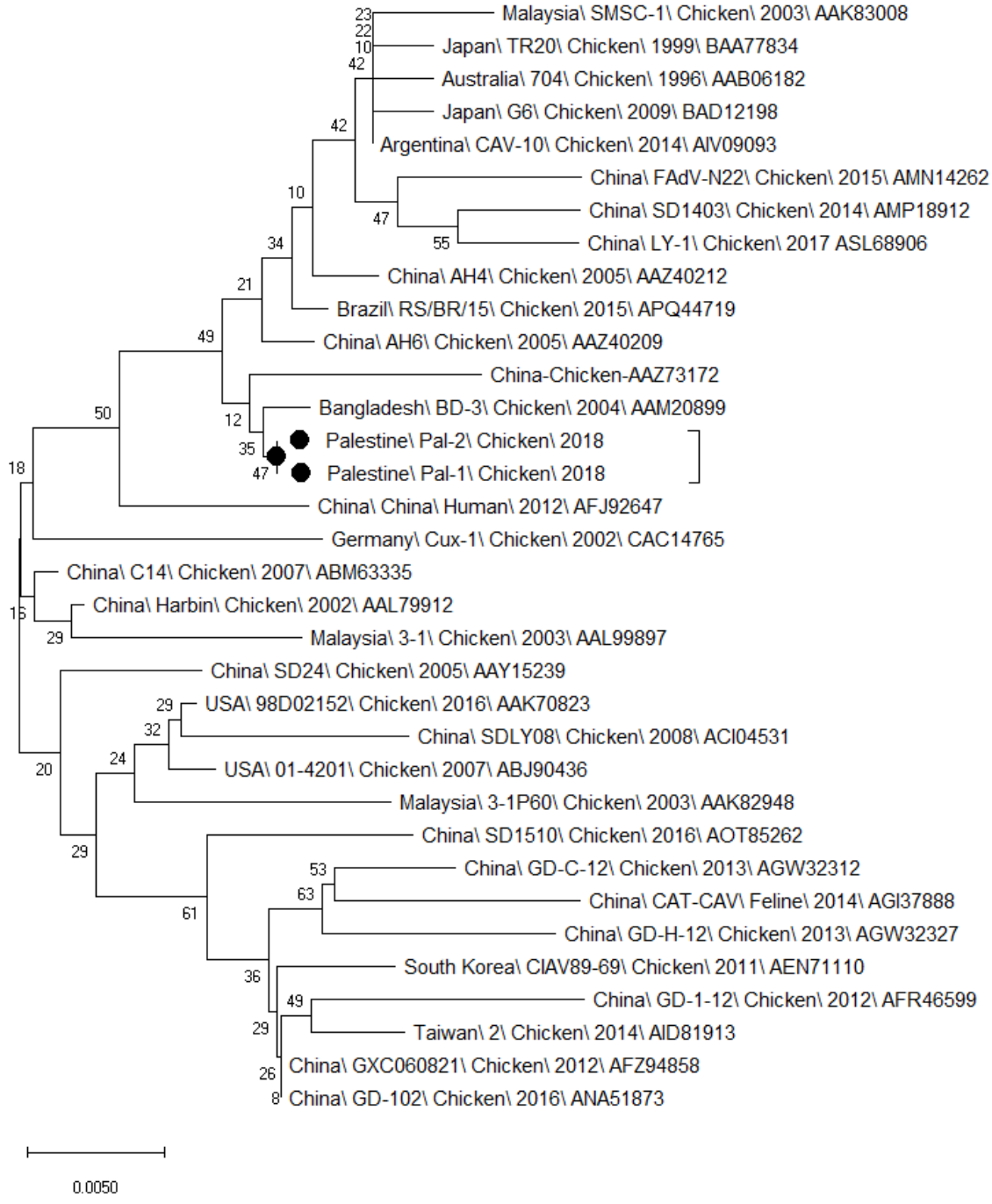

Figure 3 The phylogenetic analysis of 445 amino acids of CIAV strains based on the VP1 amino acid sequence detected in broiler in Palestine. Sequences from the present study are indicated with a black circle. Names are given by Country\ Strain or Isolate\ Host\ Isolation year \ GenBank accession number. The numbers below the branches indicate neighbor-joining distances with 1,000 bootstrap replicates.

Table 1 Amino acid differences between Palestinian and reference CAV strains

Strain	Amino Acid position														
	11	**22**	**41**	**51**	**63**	**75†**	**76**	**82**	**92**	**97**	**113**	**125†**	**139**	**141†**	**144**
Palestine-Chicken-1	R	N	R	N	N	I	I	L	G	L	L	I	Q	Q	Q
Palestine-Chicken-2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Bangladesh-chicken-AAM20899	.	H	.	.	.	.	.	.	.	.	.	.	.	.	.
China-Chicken-AAZ73172	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
China-Chicken-AAZ40209		Q													
Brazil-Chicken-APQ44719	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	151	**157**	**183**	**188**	**207**	**221**	**240**	**245**	**248**	**251**	**254**	**257**	**267**	**287**	**288**
Palestine-Chicken-1	L	V	N	M	E	F	V	D	N	R	E	M	K	A	T
Palestine-Chicken-2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Bangladesh-chicken-AAM20899	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
China-Chicken-AAZ73172	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.
China-Chicken-AAZ40209	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Brazil-Chicken-APQ44719	.	.	.	.	.	.	.	.	.	.	.	.	.	.T	.
	289	**290**	**293**	**294**	**310**	**315**	**320**	**327**	**341**	**357**	**370**	**371**	**375**	**376**	**394†**
Palestine-Chicken-1	T	A	T	Q	Y	T	G	P	K	V	T	A	E	L	Q
Palestine-Chicken-2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Bangladesh-chicken-AAM20899	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
China-Chicken-AAZ73172	.	.	.	.	.	.	.	.	.	.	G	.	.	.	
China-Chicken-AAZ40209	.	.	.	.	.	.	.	.	.	.	S	.	.	.	
Brazil-Chicken-APQ44719	.	.	.	.	.	.	.		.	.	S	.	.	.	
	413	**416**	**426**	**427**	**433**	**442**	**444**								
Palestine-Chicken-1	A	V	G	N	P	T	Y								
Palestine-Chicken-2	.		.	.	.	.	.								
Bangladesh-chicken-AAM20899	.	.	.	.	.	.	.								
China-Chicken-AAZ73172	S	.	.	D	.	.	.								
China-Chicken-AAZ40209	.	.	.	.	.	.	.								
Brazil-Chicken-APQ44719	.	.	.	.	.	.	.								

†Virulent motifs include amino acid residue on the VP1 protein known to be associated with high pathogenicity of CIAV for chickens as described by Yamaguchi et al., 2001(Yamaguchi *et al.,* 2001).

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Authors' contributions: I. Alzuheir carried out the design and drafting of the manuscript. N. Jalboush was responsible for reporting the clinical signs and necropsy, A. Fayyad carried out data analysis and M. Manasra was responsible for sequencing. The authors all read and approved the final manuscript.

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