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Short Communication

Molecular Characterization and Phylogeny of Chicken Anemia Virus Detected in Broiler Poultry Flocks in Punjab, Pakistan

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ABSTRACT

Precise diagnosis and precautionary measures for Chicken Anemia Virus (CAV) requires continuous surveillance of disease and understanding of local field CAV strains. The CAVs have been reported previously both in broiler chicken and layer birds in Pakistan; however, their molecular characterization is largely inadequate and requires continuous evaluation. In this study, sequencing and molecular analysis of specific regions overlapping VP1-VP2 and VP2-VP3 genes of CAV strain from outbreaks in broiler flock (n=45) were undertaken in the Punjab Province of Pakistan. The phylogenetic analysis clustered study strains of CAV in group II similar to those reported previously from China. These findings are helpful for an understanding of currently circulating strains of CAV; however, they require further analysis to intervene in necessary strategies to avoid future outbreaks in commercial poultry.

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SMN, MRK and AA Conceived and designed the work. AAS and MA analyzed the data, draft writing. SMN, AA and MRK drafted the manuscript.

Key words
CAV, Punjab, Sequencing, Phylogenetic analysis, Broilers

Chicken Anemia virus (CAV) has not only gained considerable economic importance in the poultry industry of Pakistan but also round the globe during past few years (Mohamed, 2010; Rehman *et al*., 2011). It is a DNA virus that belongs to the genus *Gyrovirus* of *the Circoviridae* family (Schat, 2009). It has 2.3 kb, negative sense single-stranded genome with three overlapping open reading frame which is encoded by three viral proteins i.e. VP1 with molecular weight of 51.6 kDa, which is the major protein of viral capsid, VP2 has molecular weight of 24kDa, a unique dual specificity protein phosphatase which acts as a scaffolding protein during virion replication and VP3 having molecular weight of 13.6 kDa also termed as apoptin, which has been recognized to induce apoptosis in the transformed cell lines (Peters *et al*., 2002). VP1 has the highest variability in nucleotide; therefore, it is used for molecular studies and genetic characterization of CAV (Noteborn *et al*., 1991).

The virus is transmitted both vertically and horizontally to the progeny (Bhatt *et al*., 2011). The disease is characterized by destruction of erythroblastoid, cortical thymocyte cells, muscular and subcutaneous hemorrhages, aplastic anemia, thymus atrophy leading to immunosuppression, which may be associated with secondary bacterial, viral, fungal or parasitic infections (Dhama *et al*., 2008). The infections may be either clinical or sub-clinical (Schat, 2003). The clinical disease appears in young chicks that acquire a vertical infection during 1-2 weeks of age. Older birds more than two to three weeks of age are also prone to disease but infection remains subclinical (Adair, 2000). Morbidities and mortalities due to the infection may be up to 55% and 80%, respectively. CIA infection may be relatively easy to diagnose on the bases of pathognomic signs and lesions showed by the affected birds. In this regard, it can be diagnosed based on the history of the flock, clinical signs, hematological changes and gross pathological findings during postmortem. A decrease in the hematocrit values is the sensitive indicator to diagnose clinically affected birds with CIAV (Rehman *et al*., 2011). For confirmatory diagnosis; however, isolation and identification of the CAV is required.

In Pakistan poultry industry, CAV is an emerging issue and unfortunately, little attention has been paid until now to investigate the problem of CAV. Only a few studies have proclaimed the presence of CAV in Pakistan (Rehman *et al*., 2011). However, all the previous studies were conducted solely based on the clinical picture and, to some extent, described serological titration. However, the genetic diversity of circulating virus is yet unknown that could predict molecular epidemiology of circulating strains in the country. This study is therefore designed to characterize the circulating virus and its phylogenetic relationship with a closely related virus that is reported worldwide from the poultry-rearing settings.

Materials and methods

Samples collection: Different organs (liver, spleen,

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bursa, thymus and bone marrow) were collected from forty-five broiler flocks located in different poultry rearing areas in the Punjab province of Pakistan. Each of the flock had clinical symptoms suggestive of CAV infection including anorexia, depression, and anemia. The age of the birds ranged from 10 - 25 days. Necropsy was performed and above-said samples were collected. The samples were stored at −80 degree centigrade until further processing for confirmation of CAV by genome-based in-vitro amplification (PCR reaction).

The extraction of the DNA was conducted from the supernatant of tissue homogenates by using BioinGentech veterinary PCR kit according to the manufacturer instructions. The oligonucleotide primers CAV-1 (5-CTAAGATCTGCAACTGCGGA-3) and CAV-2 (5-CCTTGGAAGCGGATAGTCAT-3) were used to amplify a 419 bp DNA fragment (Taylor, 1989). DNA of CAV was detected by modifying the method of Herman et al. (2012). The final volume of 20 ul PCR reaction mixture was used to perform Polymerase Chain Reaction which was comprised of 10 ul of Dream Taq Green PCR Master Mix 2x (Thermo Scientific, USA), 2 µl of template DNA, 1 ul of primers both forward and reverse (50 pm/µl each) and 6 µl of nuclease-free water. The amplification was optimized on BioRad T100 held down the following condition: initial denaturation of one cycle at 95 C for five minutes, next was thirty-five cycles at 95 C for thirty seconds, then 55.5 C for 45 seconds and 72°C for sixty seconds following denaturation, annealing and extension, respectively. Lastly, final extension was carried out at 72 C for five minutes. The gel electrophoresis unit was used to analyzed the amplified products. The gel electrophoresis unit was loaded to 1.5% agarose with stained by ethidium bromide and viewed under UV Transilluminator (Wajid et al., 2018).

Tissues were homogenized by using tissue homogenizer with sterile PBS i.e phosphate buffer saline. Centrifugation method was used to remove cellular debris and supernatants were collected and stored at −80°C.

Gene JET PCR Purification Kit (Thermo Fisher Scientific, USA) was used to purify the amplicons. By using the same primers as used previously and ABI PRISM genetic analyzer with Big Dye Terminator version 3.1 cycle sequencing kit by Applied Biosystems, USA. The processing of the purified DNA was sequenced in 3100 DNA Analyzer by Thermo Fisher Scientific. A reliable consensus sequence was assured by the sequencing of each genomic fragment which was carried out in both forward and reverse directions. The sequence of a specific region overlapping VP2-VP3 and VP1-VP2 gene of the study isolate (CAV/Maaz-UVAS/Pak/2017) has been submitted to GenBank with accession number MG981210. BioEdit version 7.2.6 (Hall, 1999) was used to align the obtained sequence and sequences reported earlier (GenBank) by using ClustalW, multiple alignments and cut into parallel lengths. Phylogenetic relationships of a partial region of overlapping VP2-VP3 and VP1-VP2 gene of the study isolate were clarified to the corresponding region of other already characterized viral isolates around the world at the genotype level and sub genotype. For subject matter, a tree was constructed by a neighbor-joining method employing Kimura-two parameter model with general time reversible, gamma distribution and invariants nucleotide sites calculated on 1000 bootstraps replication through the MEGA software V7.0.26 (Kumar *et al*., 2016). The evolutionary gap was deduced and manifested based on a number of nucleotide submissions per site. All those sites having missing data and gaps were removed from the data set by using the "complete deletion" option. Amino acid analysis of representative strains of each known genotype including the vaccinal ones was compared by Bio Edit.

Results and discussion

All the bird's flock suspected for CAV infection were of the age of 10 to 25 days. Birds in each of the affected flock showed growth retardation, overall weakness, anemia, pale comb and wattles, depression, with an increase in mortality. The postmortem findings were pale, enlarged liver and spleen, muscular hemorrhages, bursal atrophy and mild to the moderate atrophied thymus. The clinical signs, symptoms and postmortem lesions observed in our study were in concordance with the clinical signs and postmortems lesions evidenced earlier (Mariya and Konstantin, 2018)

The amplified DNA extracted from organ samples by agarose gel electrophoresis showed DNA bands of the same length as expected i.e. 419 bp. Out of forty-five samples, only four samples representing four distinct flocks were positive. However, owing to a similar sequence pattern, only one of them was processed further and included in subsequent phylogeny and residue substitution analysis.

The phylogenetic tree clearly showed the viral proteins gene sequence of CAV clustered with different viral strains (Fig. 1). The sequences of the amino acid obtained from the study virus were correlated with the other sequences already submitted in the GenBank. Our study isolate of CAV was categorized in group II. The topography of our phylogenetic tree showed the existence of two groups that were described in the previous study of Zhang *et al*. (2013). The phylogenetic tree also revealed that an identified sequence was related to different sequences such as AF311900/China, KF224935GD-K-12/China, DQ124936-AH4/China, DQ217400-SMSC-1P9WT/Malaysia, AF285882-SMSC-1 Malaysia, U65414/Australia and AB027470-TR20/Japan which are falling in

group II according to Zhang *et al*. (2013). However, the vaccinal strain used in Pakistan i.e Nobilis P4 matched with other nucleotide sequences in groups I as shown in (Fig. 1). Sequence analysis of the region overlapping VP2-VP3 and VP1-VP2 genes revealed maximum percentage identity (99.5%) with the same gene identified in China (AF311900) and 97.3% with Vaccinal stain Nobilis P4 (AJ980284) (Table I). Our studied isolate is marked by the black triangle and vaccinal isolate is marked by black square as shown in Figure 1.

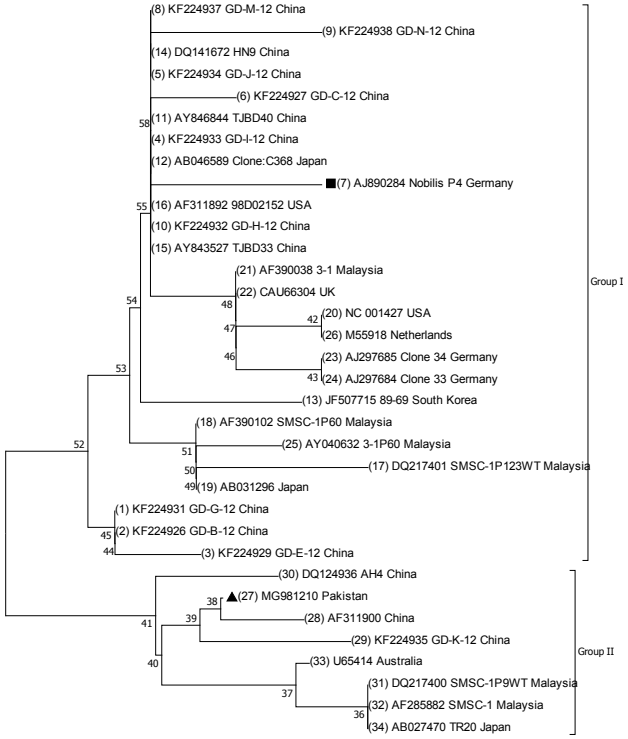

Fig. 1. The phylogenetic tree was inferred by the Neighbor Joining method as described by Saitou and Nei, 1987. The optimal length of the tree with sum of branch length = 0.11748525 is shown. The tree is sketch to scale having branch lengths in the same units which are used in evolutionary distances to infer the phylogenetic tree. Evolutionary distances were calculated by using the Poisson correction method as mentioned by Zuckerkandl and Pauling (1965) and are in the units of the number of amino acids swipe per site. The analysis is made from thirty-four amino acids sequences. All sites having gaps and missing data were eliminated. There were total 222 positions in final dataset. Evolutionary assessment was carried out in MEGA7 (Kumar *et al*., 2016).

Though CAV is endemic to poultry in Pakistan and, in this regard, several disease outbreaks have been reported on the basis of clinical symptoms during the year 2011 to 2012, especially in poultry-dense areas of the Punjab province (Islam et al., 2013). However, there is absolute paucity of laboratory-based disease evidence so far. Hence, the study provides the first-ever evidence of CIAV in Pakistan. The study employed polymerase chain reaction for confirmation of CAV infection in commercial broilers in the Punjab province. A similar approach has previously been employed to confirm the presence of genomic DNA corresponding to CAV in both broiler and layer flocks (Hegazy *et al*., 2010). The used primers and assay is considered to be more specific and sensitive for identification and isolation of virus considering the fact that DNA can be extracted from the same organs as used for virus isolation (Soine *et al*., 1993).

Table I. Estimates of evolutionary percentage identity between study sequences.

Sr. No	Accession number	Country	Percentage identity
1.	AF311900	China	99.5 %
2.	DQ124936	China	98.6 %
3.	U65414	Australia	98.6 %
4.	AF395114	Bangladesh	98.5 %
5.	AF311892	USA	98.2 %
6.	KX377129	India	98.2 %
7.	AB031296	Japan	98.2 %
8.	AB027470	Japan	98.2 %
9.	DQ217400	Malaysia	98.2 %
10.	AF390038	Malaysia	97.7 %
11.	KJ621020	India	97.3 %
12.	AJ890284	Germany	97.3 %
13.	KF224938	China	97.3 %
14.	M55918	Netherlands	97.3 %
15.	JF507715	South Korea	97.3 %

The amino acid number substitutions between sequences are described. Analysis were done by using the Poisson correction model (Zuckerkandl and Pauling, 1965). The analysis involved 14 sequences. Positions containing gaps and missing data were eliminated. Final dataset consists of 222 positions. MEGA7 was used for the evolutionary analysis (Kumar *et al*., 2016).

Despite frequent occurrence, the molecular characterization of CAV from Pakistan has not been reported so far. Indeed, it is very meaningful to characterize CAVs strains that are circulating in our environment to devise appropriate interventions, in terms of vaccine strain to be used, and improve methods of virus control. We used partial sequence analysis of the overlapping region of the VP1-VP2 gene to study the genetic characterization of the CAV sequence of Pakistan. Later, using a large sequence database reported from different countries such as Germany, China, USA, Bangladesh, Japan, Malaysia, Netherlands, South Korea, Brazil, Slovenia, Argentina and Australia, the study sequence were compared and annotated as per classification suggested previously by Zhang *et al*.

(2013) Sequence of VP1 gene is mostly used to evaluate the affiliation of other CAV isolates, simply because of the fact that most of the amino acid tradeoff between isolates occur in the VP1 gene, especially in the N-terminal half of the VP1 gene (Criag *et al*., 2009). A hyper-variable region extends from amino acids positions 139 to 151 in the VP1 gene (Renshaw *et al*., 1996). Islam et al. (2013) confirmed that five of sixteen commonly variable amino acid positions of the whole VP1 gene fall within the said specific region. The phylogenetic analysis showed that the studied CAV isolate sequence belongs to group II. The group II members represent much diversified geographic origins where most of the strains were isolated from China. Other isolates in group-II representing different countries included U65414 from Australia, DQ217400 and AF285882 from Malaysia and AB027470 TR20 from Japan. Our strain clustered very close to the isolates from China which indicates potential ancestor and origin of this virus. This is may be due to the involvement of migratory birds from China to Pakistan. While giving a detailed nucleotide sequence analysis, the study CAV strain closely resembled the isolates reported from China such as AF311900 (99.5 %), DQ124936 (98.6 %) and KF224938 (97.3%).

Pakistan poultry industry is now using live attenuated CAV vaccines that represent Nobilis P4. However, from the sequence analysis, it was evident that the amplified product was not a vaccine but a field variant. This is because a distant relationship was found between the vaccine strain and study strain where study strain clustered with viruses representing a group –II while vaccine strain clustered with viruses representing Group-I.

Conclusion

This is the first study that reports the genetic characterization of circulating Chicken Anemia Virus in the poultry farms of Punjab Province, Pakistan. Despite the fact, that the number of samples were limited, the phylogenetic analysis showed a close relationship with strains reported previously from China. Future studies covering a large geographical setting needs to be conducted.

Statement of conflict of interest

Authors declared no conflict of interest.

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