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ORIGINAL ARTICLE

Characterization and phylogenetic analysis of Brazilian chicken anaemia virus

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Abstract Chicken anaemia virus (CAV) was detected by a Nested-PCR assay in field samples from different regions of Brazil. The 539 bp amplified fragments of *vp1* gene from 44 field samples were sequenced and 10 new nucleotide sequences of CAV were observed. These sequences were phylogenetically analysed by Mega2 using neighbour joining distance methods with 1000 bootstrap replications. Phylogenetic analysis did not show correlation between CAV pathology pattern and genetic groups. The 10 nucleotide sequences of the Brazilian samples were also analysed together with 30 sequences of CAV strains previously described from other countries. The genetic variability observed was not related to the geographical distribution. Amino acid substitutions were detected at 9 positions of the Brazilian sequences and two of them had not been observed before, ^{65}R replacing the Q residue and ^{98}F replacing Y residue.

Keywords Chicken anemia virus (CAV) · Genotyping · Nested-PCR · Phylogeny · Genetics and virology

Introduction

Chicken anaemia virus (CAV) is a member of the *Circoviridae* family, *Gyrovirus* genus [1] and was first described in 1979 by Yuasa and colleagues in Japan [2]. CAV infections induce either clinical or sub-clinical signs [3] and both result in measurable economical. losses [4–6]. Signs and lesions include stunting, increased mortality, anaemia, bone marrow cell depletion, subcutaneous haemorrhage, and atrophy of secondary lymphatic organs [7]. This infection is often associated with secondary bacterial, viral infections or vaccination failures [8–11]. CAV can be vertically transmitted by either dam or sire to progeny [3, 12] and the transmission is greatly reduced but not entirely eliminated in immune breeders [13]. CAV has been detected worldwide by isolation, serology or DNA amplification in both laying and broiler chickens. The presence of CAV has been investigated in cell culture, sera and tissues by polymerase chain reaction (PCR) [13–17]. The CAV genome structure is composed by a circular ssDNA of about 2.3 kb with three open reading frames (ORFs) coding for proteins of 52 (VP1), 24 (VP2) and 14 (VP3) kDa [18].

CAV was known as a very conserved virus of one serotype [2] with several genetic groups [19], although

The nucleotide sequence data reported in this paper have been submitted to the GenBank nucleotide sequence database and have been assigned the accession numbers AY855079 to AY855088.

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an additional serotype has been recently reported [20, 21]. Renshaw et al. [22] identified an hypervariable region in VP1 and suggested that certain amino acid changes in this region could influence the rate of virus replication and spread of CAV strains in MDCC-MSB-1 cells. Recently, Chowdhury et al. [23] reported that low and high passage isolates were phylogenetically divergent and differed in their pathogenicities, but it would not accurately predict the phylogeny of the virus and rather it is more indicative of selection of a particular isolate in the cell culture.

Little is known about the phylogenetic analysis of CAV Brazilian samples. In the present work, we report the amplification of a *vp*1 fragment of the CAV from Brazilian field samples. The amplification products were sequenced and submitted to phylogenetic analysis by neighbour joining distance methods to evaluate genetic diversity and a possible correlation with their induced pathology.

Material and methods

Samples

One hundred and fifty field samples from commercial breeders, broilers and free-range chickens (originated by random crosses between different breeds introduced in Brazil following colonization, around 1500) [24] were analysed. They were selected from different states of Brazil and were collected from the year 2000 until 2004. Tissue samples (80), sera (55) and litter (15) were collected from flocks with or without clinical signs of chicken anaemia. Also, samples from flocks with diagnosis of infectious bursal disease (IBD), *Pasturella multocida* (PM) infectious laryngotracheitis virus (ILTV) and *Ornithobacterium rhinotracheale* (ORT) were tested (Table 1). A CAV commercial vaccine (Nobilis CAV P4, Intervet International, Netherlands) was also sequenced.

DNA amplification and sequencing

The DNA extraction methods, primers and cycling conditions for the Nested-PCR assay were performed as described previously [25]. The 539 bp amplification products were purified with GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Sweden). Both strands were sequenced using the Big Dye Terminator version 3.1 cycle sequencing RR-100 kit on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA).

Sequence analysis

The obtained nucleotide sequences and the deduced amino acid sequences were aligned using Clustal W [26]. GeneDoc software [27] was used to measure similarity degrees. Kimura 2-parameter pairwise distances [28], calculated for the different nucleotide sequences detected in Brazil were used to construct a phylogenetic unrooted tree by using the neighbour joining distance methods in the Molecular Evolutionary Genetics Analysis software MEGA 2 [29]. The statistical confidence of the tree topologies was performed by 1000 bootstrap replications on the same software.

In order to compare with the Brazilian samples in the phylogenetic studies (using the same analysis method described above), we detected seventy five

Table 1 CAV Brazilian sequences and its relation with clinical or serological status

GenBank accession No.	Number of samples[a]	Clinical/serological status[b]	State of origin[c]
AY855079	5	Clinically healthy	RS, SC, PR
AY855080	1	ORT antibody positive	RS, SC
AY855081	1	ORT antibody positive	RS, SC
AY855082	8	CAV, ILTV, PM clinical signs ORT antibody positive	RS, SC, PR
AY855083	1	Clinically healthy	RS
AY855084	1	Litter from clinically healthy flocks	RS
AY855085	1	IBD suspect	GO
AY855086	1	IBD suspect	PR
AY855087	1	IBD suspect	PR
AY855088	1	CAV clinical signs	RS

[a] Except AY855079 and AY855082 sequences occurred in only one sample. In these cases, two independent amplification and sequencing reactions were performed to assure the reliability of the results

[b] ORT (*Ornithobacterium rhinotracheale*); PM *(Pasteurella multocida);* ILTV (infectious laryngotracheitis virus); IBD (infectious bursal disease)

[c] Brazilian States: RS (Rio Grande do Sul); SC (Santa Catarina), PR (Paraná), and GO (Goiás).

CAV related sequences in GenBank, comprising the same genome region analysed in the present work and thirty of these sequences were selected to represent the diversity of CAV worldwide.

Results

From the 150 field samples analysed, 135 were Nested-PCR positive and from which 44 samples were selected for nucleotide sequencing representing flocks from different geographical regions. From the 539 bp amplification product sequenced, 481 nucleotides were further analysed. Twenty-three (52%) samples sequenced displayed more than one sequence in the same sample, including all CAV DNA sequences from São Paulo (8) and Minas Gerais (2) States. The remaining unambiguous 21 sequences (48%) were aligned and 10 different sequences (Table 1) with 92.0–99.0% similarity were found. Five samples detected in clinically healthy flocks had identical sequences and are represented here by GenBank accession number AY855079, which showed 100% identity with the amplification product of the commercial vaccine.

Phylogenetic analysis of Brazilian samples was performed to verify a possible relation of CAV induced pathology with a specific genetic group. Phylogenetic tree showed three groups (Fig. 1). The first (I) was formed by four sequences (GenBank accession numbers: AY855079, AY855084, AY855085 and AY855086) detected in clinically healthy flocks and litter. The second group (II) was formed by two sequences (GenBank accession numbers: AY855080 and AY855081) detected in flocks with ORT antibodies. The third group (III) was composed by four sequences (GenBank accession numbers: AY855082, AY855083, AY855087 and AY855088). The sequence AY855082 shared 99% similarity with AY855088 and both were detected in flocks displaying CAV clinical signs. Seven other samples had identical sequence to AY855082 and were detected in flocks with diagnosis attributed to CAV, PM, ILTV or with ORT antibodies. AY855003 was obtained from a clinically healthy flock and AY855087 was from a flock with IBD. These ten CAV Brazilian sequences did not show relation between genetic group and the state of origin.

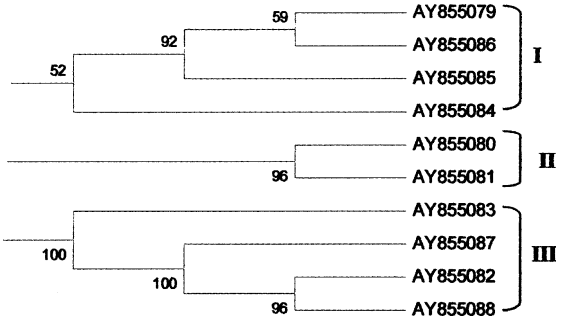

Fig. 1 Phylogenetic analysis among 10 Brazilian strains based on a fragment from the *vp1* nucleotide sequence of CAV using neighbour-joining distance method (1000 bootstrap replications). I, II and III: genetic groups

Brazilian CAV nucleotide sequences were compared with the 30 other CAV sequences deposited in the database to know if there was any geographical relation worldwide (Fig. 2). Phylogenetic trees constructed by using maximum parsimony or maximum likelihood methods also supported the same clustering as well as the bootstrap values (data not shown).

The alignment from the deduced VP1 amino acid sequences of the 10 Brazilian samples showed amino acid substitutions in nine different positions. Two of them presented amino acid substitution ^{65}R replacing the Q residue in AY855088 and ^{98}F replacing the Y residue in AV855085 (Fig. 3). AY855088 and AY855085 sequences were obtained from a free-range chicken with severe chicken anaemia symptoms and a commercial breeder flock with IBD, respectively.

Discussion

The 539 nucleotide sequences of the 2.3 kb CAV genome from commercial broiler and hens and free-range chickens from Brazil were obtained. The DNA samples were extracted from biological specimens without virus isolation in cell culture. This procedure enables us to characterize CAV that might not have replicated in vitro [19] and avoided the potential nucleotide changes that can occur during cell culture passage. The analysis of the nucleotide sequences obtained from the *vp1* amplification product of field samples showed that 52% of these had more than one sequence. Probably, these chickens were infected by more than one strain, or the different sequences constitute CAV *quasispecies.* This finding is in agreement with the observations of van Saten et al. [30] who reported ambiguous sequences in PCR products. Claessens et al. [31] also reported nucleotide differences in two clones obtained from the same sample.

Phylogenetic analysis of the 10 nucleotide sequences described in the present work displayed three groups. It was not observed any relationship between CAV pathology and the genetic group. Indeed, the severity of CAV disease is also influenced by the presence of other infectious pathogens. Interestingly, the

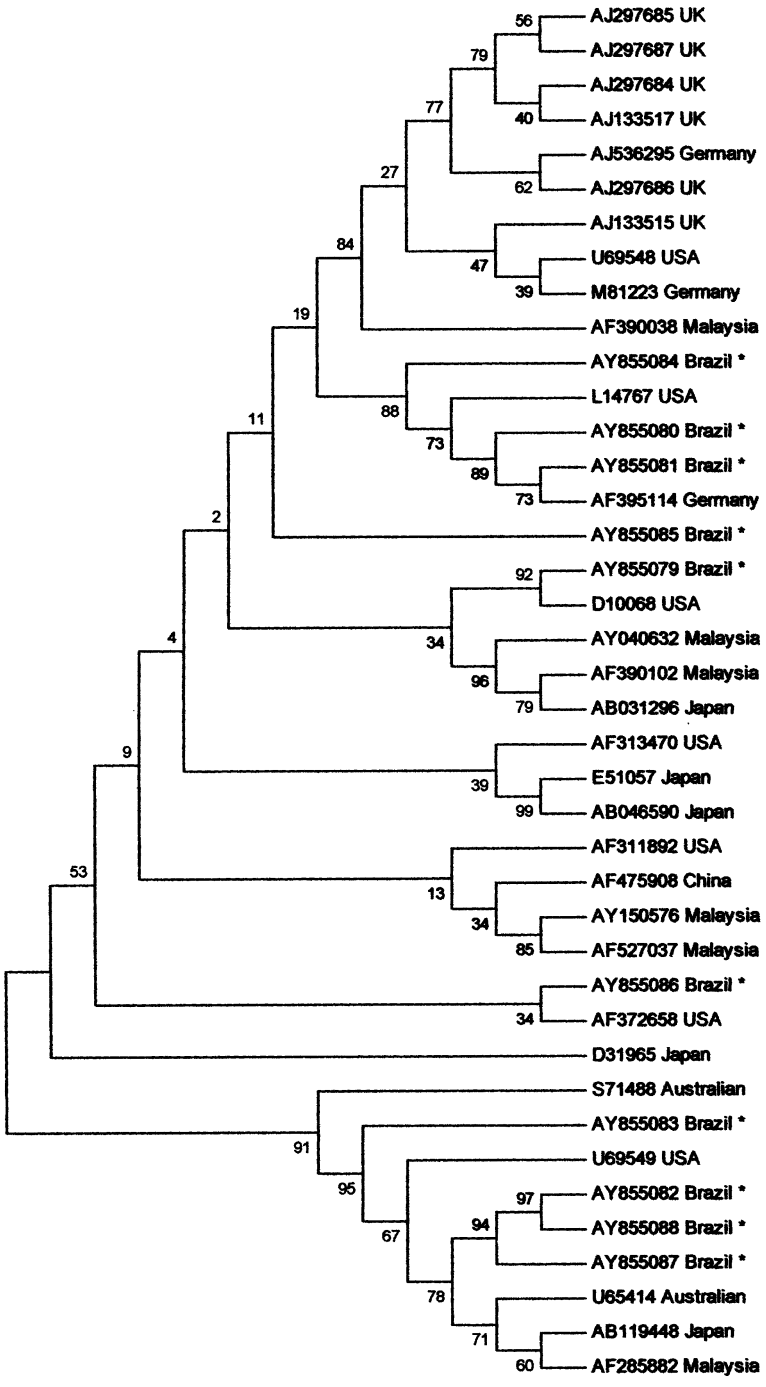

Fig. 2 Phylogenetic tree showing relation among 10 Brazilian and 30 *vp1* nucleotide sequences from different countries using neighbour joining distance method, with 1000 bootstrap replications. Asterisk(*) indicate Brazilian strains

AY855079 sequence, detected in clinically healthy flocks showed higher divergence with AY855088, detected in chicken with clinical signs of chicken anaemia in phylogenetic tree. These results were similar to an earlier study that indicated that isolates with different pathogenicities were also phylogenetically divergent [23].

The phylogenetic tree of Brazilian sequences and the 30 CAV described did not show a clear geographical correlation. Islam et al. [19] also reported that an Australian strain did not demonstrate any grouping with strains detected in five different countries. Brazilian sequences are not all found on a separate group. Therefore, these sequences are not more similar to each other than they are to CAV isolates of other countries. This result was similar to the observations of van Santen et al. analysing strains from USA [30]. However, some Brazilian nucleotide sequences displayed greater homology with USA strains what can be explained by the fact that some commercial chicken

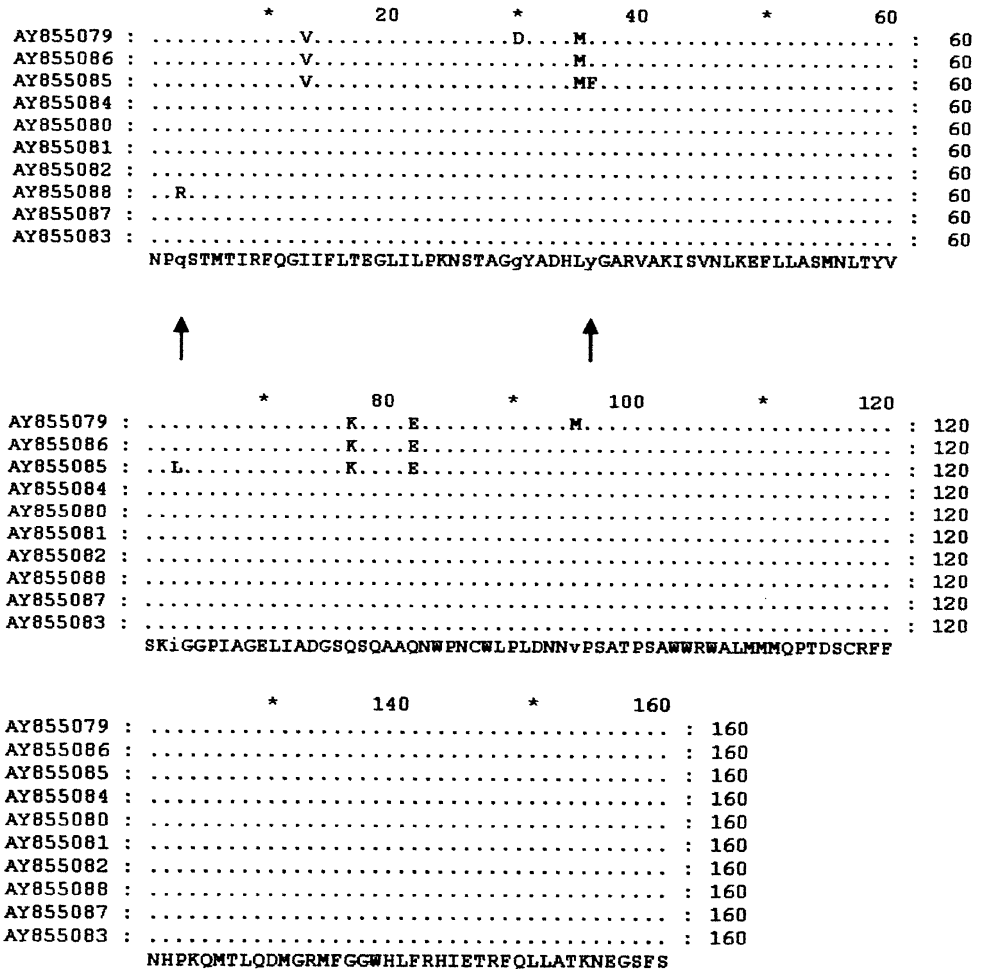

Fig. 3 Alignment of the deduced amino acid sequence of VP1 form Brazilian samples, starting in amino acid 63. The consensus amino acid sequence is shown below the aligned sequences. Arrows indicate the new amino acid changes (only observed in these samples)

lines and vaccines are usually imported from USA. Our results suggest that the diversity displayed was probably related to different origins of virus introduction more than its evolution inside Brazil.

Brazilian samples differed in 42 nucleotide positions that resulted in nine predicted amino acid substitutions. The VP1 alignment confirmed previous findings from Renshaw et al. [22] that described a hyper-variable region from amino acid positions 139 to 157. However, another variable region from amino acid positions 75 to 98 was also observed. Scott et al. [32] suggested that an amino acid substitution (T → A) at position 89 in the VP1 could be associated with the non-reactivity of one monoclonal antibody as well as the attenuation of some strains. We observed that the 10 Brazilian sequences had ^{89}T. Interestingly, only two of these were present in broilers with clinical signs of chicken anaemia, indicating that other factors are probably involved in the induction of the clinical disease. This discrepancy could be attributed to the use of clones sequenced from high passage viruses [32].

Seven out of the 10 Brazilian sequences had ^{75}I, ^{97}L, ^{139}Q and ^{144}Q and three had ^{75}V, ^{97}M, ^{139}K, and ^{144}E amino acid profile. These findings are in agreement with an earlier study of 14 CAV strains detected in 10 clinical samples obtained from commercial broiler chickens in Alabama State, USA [30]. The amino acids at positions 139 and 144 did not appear to be independent of each other, since a ^{139}Q is always followed by a ^{144}Q and a ^{139}K is always accompanied by a ^{144}E.

Renshaw et al. [22] suggested that ^{139}Q and/or ^{144}Q can influence the rate of replication or spread of infection in MDCC-MSB-1 cells. Seven out of the 10 Brazilian sequences were ^{139}Q and ^{144}Q, however, only two of them, were detected in flocks with CAV clinical signs. It was also verified that the three sequences that were ^{75}V, ^{97}M, ^{139}K and ^{144}E amino acid positions, were detected in flocks without CAV clinical signs and these residues could be associated with attenuated viruses. The AY855088 sequence, detected in broilers with severe signs of chicken anaemia presented amino acid change Q → R in position 65 not observed before,

which could be related to the pathogenicity of CAV strains. These questions will require more investigations to confirm correlations between pathogenicity and genetic determinants. The knowledge about these differences and their pathogenic potential may be useful in the future development of vaccines. However, sequence analysis and biological studies require careful interpretation concerning the mutation in the CAV genome.

In conclusion, when the Brazilian sequences were analysed alone, they formed three genetic groups that were not clustered when these sequences were analysed together with sequences from all over the world. Also, there was no clear relation between pathology and the genetic groups. The results obtained add new and important information about genetic variability of VP1 in Brazil and its putative correlation with CAV pathogenesis.

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