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Molecular characterization of chicken anemia virus in Guangxi Province, southern China, from 2018 to 2020

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ABSTRACT

Background: Chicken anemia virus (CAV) causes chicken infectious anemia, which results in immunosuppression; the virus has spread widely in chicken flocks in China.
Objectives: The aim of this study was to understand recent CAV genetic evolution in chicken flocks in Guangxi Province, southern China.
Methods: In total, 350 liver samples were collected from eight commercial broiler chicken farms in Guangxi Province in southern China from 2018 to 2020. CAV was detected by conventional PCR, and twenty CAV complete genomes were amplified and used for the phylogenetic analysis and recombination analysis.
Results: The overall CAV-positive rate was 17.1%. The genetic analysis revealed that 84 CAVs were distributed in groups A, B, C (subgroups C1-C3) and D. In total, 30 of 47 Chinese CAV sequences from 2005-2020 belong to subgroup C3, including 15 CAVs from this study. There were some specific mutation sites among the intergenotypes in the VP1 protein. The amino acids at position 394Q in the VP1 protein of 20 CAV strains were consistent with the characteristics of a highly pathogenic strain. GX1904B was a putative recombinant.
Conclusions: Subgroup C3 was the dominant genotype in Guangxi Province from 2018–2020. The 20 CAV strains in this study might be virulent according to the amino acid residue analysis. These data help improve our understanding of the epidemiological trends of CAV in southern China.

Keywords: chicken anemia virus; genetics; recombination; genome; China

INTRODUCTION

Chicken infectious anemia (CIA) is caused by the chicken anemia virus (CAV). CIA is an important immunosuppressive disease characterized by aplastic anemia,bone marrow and lymphoid organ atrophy in young chicks aged less than 2-3 weeks [1]. CAV mainly damages the immune system of chickens and is likely to cause concurrent or secondary infection with other pathogens [2]. For example, CAV coinfection with other immunosuppressive viruses, such as Marek's disease virus (MDV), infectious bursal disease virus (IBDV) and avian leukosis virus (ALV), can not only mutually enhance pathogenicity in chickens, but also has a significant synergistic effect on immunosuppression [3-5]. Therefore, CAV infection can directly or indirectly cause substantial economic losses to the chicken industry.

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Conflict of Interest

The authors declare no conflicts of interest.

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CAV belongs to the genus *Gyrovirus*, family *Anelloviridae* [6]. CAV is a nonenveloped, circular, single-stranded DNA virus consisting of a circular genome of 2298 to 2319 nucleotides, with three parts or completely overlapping open reading frames (ORFs) [6]. ORF1 encodes the only capsid protein associated with virulence and replication, designated viral protein 1 (VP1) [7-9]. ORF2 encodes viral protein 2 (VP2), a scaffold protein and an auxiliary protein of VP1, which helps VP1 achieve the correct conformation [10]. ORF3 encodes viral protein 3 (VP3), a nonstructural protein that is thought to play a role in the apoptosis of thymic lymphoblasts and primitive haematopoietic cells [11].

CAV has spread widely in chickens worldwide since it was first reported in Japan in 1979 [12]. In 1996, CAV was first isolated and identified in chicken flocks in China, and then CAV was subsequently detected in chicken flocks in Guangdong Province, Jiangsu Province, Shandong Province and other places [13-17]. The genetic characterization of CAV in the above provinces of China has been reported [13-17], but the molecular evolution of CAV in Guangxi Province in southern China has not yet been reported. Guangxi Province is located on the border in southern China. There are currently thousands of chicken farms in Guangxi Province, and frequent introduction and transportation pose a risk for the spread and genetic recombination of CAV. Therefore, we report the genetic and recombination characterization of CAVs from Guangxi Province of southern China from 2018 to 2020 to better understand the genetic evolution of CAV in recent years in Guangxi Province, southern China.

MATERIALS AND METHODS

Sample collection, DNA/RNA extraction and CAV detection

A total of 350 liver samples were collected from deceased, diseased chickens that were not vaccinated with CAV from chicken farms in four cities in Guangxi Province in southern China (**Table 1**). Among the 350 liver samples, 51 were collected from 5–21-day-old chicken flocks, 232 were collected from 22–100-day-old flocks, and 67 were collected from 260–290-day-old flocks. All samples were stored at −80°C until processing. A commercial TransGen Biotech EasyPure® Genomic DNA/RNA Kit (TransGen, China) was used to extract DNA/RNA from the liver samples. CAV and Fowl adenovirus 4 (FAdV-4) DNA from the samples were detected directly by PCR. The primers and PCR procedure of CAV and FAdV-4 were performed according to previously described reports [18,19]. An isolate CAV GXC060821 (GenBank accession no. JX964755) was preserved in our laboratory and used as a positive control in the PCR. Avian influenza virus (AIV) (H5 and H9 subtypes) and avian leukosis virus subgroup J (ALV-J) RNA from the samples were used for cDNA synthesis, and then PCR for the detection of the viruses was performed according to a previously described procedure [20,21].

Full-length PCR amplification and sequencing

Twenty CAV-positive samples were randomly selected from CAV-positive samples for complete genome amplification. The primers for CAV complete genome amplification were designed based on the complete genome of the Cux-1 strain (GenBank accession no. M55918). The primers CAV1F 5′-CCGCGCAGGGGCAAGTA-3′ and CAV1R TCGCGGAGGGCAYGTTATTATCTA were applied to amplify an 872-bp fragment. CAV2F 5′-GCCCCATCGCCGGTGAGTTGA-3′ and CAV2R 5′-TGCCGGTTACCCAGTTGCCAVAC-3′ were applied to amplify a 907-bp fragment. CAV3F 5′-ATGAGACCCGACGAGCAAC-3′ and CAV3R 5′-CCACACAGCGATAGAGTGATTG-3′ were applied to amplify a 1254-bp fragment. The three primer sets covered the entire nucleotide sequence. CAV GXC060821 was used as a

Table 1. Information on samples collected from commercial chicken farms and 20 CAV genome sequences

Farm name	Breed	No. of chickens	Age of chickens in the farm	City	Positive rate of CAV (positive samples/ total samples)	Age of chickens with positive samples	Accession No.	Strain name	Year of collection	Age (days)
Farm A	Ma chicken	8,488	1–300 days	Nanning	13.5% (12/89)	48, 83, 63 and 75 days	MK484614	GX1801	2018	83
							MK484615	GX1804	2018	63
							MN103405	GX1805	2018	75
Farm B	Xiang chicken	11,230	20–110 days	Nanning	11.7% (2/17)	45 and 60 days	MK484616	GX1810	2018	45
Farm C	Xiang chicken	8,825	20–110 days	Baise	26.4% (14/53)	50, 96, 86, 89, 27 and 98 days	MN103402	GX1904A	2019	50
							MN103406	GX1904B	2019	50
Farm D	Three yellow chicken	7,800	20–110 days	Yulin	20.5% (8/39)	40, 60 and 90 days	MN103403	GX1904P	2019	90
							MW554706	GX2020-D1	2020	60
							MW579761	GX2020-D3	2020	60
							MW579762	GX2020-D6	2020	40
Farm E	Xiang chicken	10,002	20–85 days	Nanning	6.8% (4/59)	63 and 70 days	MN103404	GX1905	2019	70
Farm F	Xiang chicken	8,658	20–110 days	Nanning	16.1% (11/68)	40, 45,48 and 60 days	MN649258	GX1907A	2019	48
							MN649259	GX1907B	2019	48
							OK012319	GXWM201902	2019	40
							OK012320	GXWM201901	2019	40
Farm G	Wu chicken	9,965	20–120 days	Nanning	40.9% (9/22)	24, 46, 53 and 83 days	MN649254	GX1908W1	2019	53
							MN649255	GX1908W3	2019	53
							MN649256	GX1908L2	2019	24
							MN649257	GX1908L3	2019	24
Farm H	Hua chicken	6,590	20–110 days	Qinzhou	33.3%(1/3)	30 days	OK012318	GXQZ202001	2020	30

CAV, chicken anemia virus.

positive control in the PCR amplification. The PCR was developed with a 2× TransTaq-T PCR kit (TransGen) in a 50 μL volume including 25 μL of 2× TransTaq-T PCR SuperMix, 1 μmol/L each primer (20 μmol/L), 5 μL of DNA and 15 μL of distilled water. The PCR amplification reaction was performed as follows: 94°C for 5 min; 35 cycles of 94°C for 30 sec, 60°C for 1 min, and 72°C for 30 sec; and 72°C for 10 min. The purification and cloning of PCR products were performed according to the method used by Teng et al. [22], and the cloned vector containing the target gene was sent for sequencing (TaKaRa, China).

Phylogenetic analysis and molecular characterization of CAV

Sixty-four reference CAV sequences (information on CAV sequences is available in **Table 2**) were obtained from GenBank (https://www.ncbi.nlm.nih.gov). There were few CAV sequences after 2017 in China. Of the 64 reference CAV sequences, 27 were obtained between 2005–2016 in China and the remaining sequences (n = 37) were obtained between 1991–2019 in Japan, the USA, Britain, Australia, Italy, Malaysia, Tunisia and Argentina. The sequences in this study obtained by sequencing were assembled, and 20 complete genomes were generated using LaserGene version 7.1 software. A phylogenetic tree based on the complete CAV genomes was constructed using the neighbour-joining algorithm implemented in MEGA version 6.0 with 1,000 bootstrap replications. The nomenclature (Country/GenBank accession no/strain name/year) of CAV strains was used in the phylogenetic tree. Nucleic acid sequences and deduced amino acid sequence alignment of the VP1, VP2 and VP3 genes were performed by comparison with 64 reference CAV sequences using LaserGene version 7.1 software. Alignment reports were exported and analysed.

Recombination analysis

Twenty CAV genomes in this study and 64 reference CAV complete genomes of CAV were aligned using MEGA version 6.0 software. Then, the result was imported into recombination detection program 4 (RDP4) software. Seven independent methods (RDP, GENECONV, MaxChi, BootScan, Chimaera, SiScan and 3Seq) were implemented in RDP4 software using

Table 2. Reference strains retrieved from the GenBank database

Strain name	Country/Province	GenBank Accession No.	Reference	Year
Cux-1	Germany	M55918	[6]	1991
C369	Japan	AB046590	[8]	2000
C368	Japan	AB046589	[8]	2000
GD-J-12[a]	China/Guangdong	KF224934	[15]	2012
GD-K-12	China/Hainan	KF224935	[15]	2012
JN1503	China/Shandong	KU641014	[16]	2015
HB1404	China/Hubei	KU645514	[16]	2014
JS1501	China/jiangsu	KU645518	[16]	2015
SD1510	China/Shandong	KU598851	[16]	2015
CAU269/7	Australia	AF227982	[23]	2000
3711	Australia	EF683159	N.A.	2007
SD24[a]	China	AY999018	[24]	2005
6	China/Taiwan	KJ728817	N.A.	2012
Clone 34	Britain	AJ297685	[25]	2001
Clone 33	Britain	AJ297685	[25]	2001
c-CAV	USA	NC001427	[6]	1991
SMSC-1P60	Malaysia	AF390102	[26]	2001
3-1P60	Malaysia	AY040632	[26]	2001
HLJ15108	China/Heilongjiang	KY486137	[27]	2015
HB1517[a]	China/Hubei	KU645516	[27]	2015
HN1504[a]	China/Hunan	KU645512	[27]	2015
GXC060821	China/Guangxi	JX964755	N.A.	2006
GD-103	China/Guangdong	KU050678	N.A.	2014
GD-104	China/Guangdong	KU050679	N.A.	2014
GD-101	China/Guangdong	KU050680	N.A.	2014
N8	China/Guangdong	MK887164	N.A.	2016
LN15169	China/Liaoning	KY486154	[27]	2015
SD1505[a]	China/Shandong	KU645523	[27]	2015
HN1405	China/Hunan	KU645520	[27]	2014
SD1508[a]	China/Shandong	KU645519	[27]	2015
GD-1-12	China/Guangdong	JX260426	[28]	2012
TR20	Japan	AB027470	N.A.	1999
AH4	China/Anhui	DQ124936	N.A.	2005
CIAV-Shanxi7	China/Shanxi	MH186142	N.A.	2018
LY-2	China/Shandong	KX447637	N.A.	2016
SD1513	China/Shandong	KU645517	N.A.	2015
CAV-18	Argentina	KJ872514	[29]	2007
SMSC-1	Malaysian	AF285882	[30]	2000
SD1403	China/Shandong	KU221054	N.A.	2014
704	Australia	U65414	N.A.	1996
CAV-EG-14	Egypt	MH001565	[31]	2017
CAV-10	Argentina	KJ872513	[29]	2007
LY-1	China/Shandong	KX447636	N.A.	2016
CAV-EG-2	Egypt	MH001553	[31]	2017
CAV-EG-11	Egypt	MH001559	[31]	2017
CAV-EG-13[a]	Egypt	MH001560	[31]	2017
CAV-EG-26	Egypt	MH001564	[31]	2017
CIAV/IT/CK/909-06/18	Italy	MT813068	[32]	2018
CIAV/IT/CK/1196/19	Italy	MT813069	[32]	2019
CIAV/IT/CK/1155/19	Italy	MT813070	[32]	2019
CIAV/IT/CK/855/17	Italy	MT813071	[32]	2017
CIAV/IT/CK/1157/19	Italy	MT813072	[32]	2019
CIAV/IT/CK/986-2/18	Italy	MT813073	[32]	2018
CIAV/IT/CK/1099/19	Italy	MT813074	[32]	2019
CIAV/IT/CK/1153-2/19	Italy	MT813075	[32]	2019
CIAV/IT/CK/1188/19	Italy	MT813078	[32]	2019
CIAV/IT/CK/1180/19	Italy	MT813076	[32]	2019
CIAV/IT/CK/1186/19	Italy	MT813077	[32]	2019

(continued to the next page)

Table 2. (Continued) Reference strains retrieved from the GenBank database

Strain name	Country/Province	GenBank Accession No.	Reference	Year
CIAV/IT/CK/1188/19	Italy	MT813078	[32]	2019
CIAV_TN_7-1	Tunisia	MZ666088	[33]	2019
CIAV_TN_7-2	Tunisia	MZ666089	[33]	2019
CIAV_TN_7-3	Tunisia	MZ666090	[33]	2019
CIAV_TN_7-4	Tunisia	MZ666091	[33]	2019
CIAV_TN_7-5	Tunisia	MZ666092	[33]	2019
CIAV_TN_7-6	Tunisia	MZ666093	[33]	2019

N.A., not available.
[a]Recombination events were confirmed by previous reports.

default parameters to evaluate the potential recombination sequences [34]. The default settings were used for all methods, and the highest acceptable p value cut-off was set at 0.05. The parental sequences and localization of possible recombination breakpoints were assessed and identified. When a recombination event was supported by more than the above mentioned 6 methods with $p < 0.05$ and recombination consensus scores > 0.6, the recombination event was regarded as positive. The potential recombinant sequences, parental sequences and CAV sequences from the outgroup were analysed using simplot similarity in SimPlot version 3.5.1 [35].

RESULTS

Detection of CAV

The total CAV-positive rate was 17.1% (60/350), and all 60 CAV-positive samples were collected from 22–100-day-old chicken flocks (**Table 1**). CAVs were not detected in the samples collected from 5–21-day-old or 260–290-day-old chickens. However, 22 out of the 60 CAV-positive samples (36.7%) were coinfected with one each of FAdV-4 or H5 subtype AIV. CAV coinfection with FAdV-4 was detected in 16 samples, and 6 samples were coinfected with H5 subtype AIV. The detection results are shown in **Supplementary Table 1**.

Phylogenetic analysis of CAV

Twenty CAV genome sequences in this study were obtained by sequencing, with a total length of 2298 bp. Detailed information on the 20 CAV genome sequences is provided in **Table 1**. Phylogenetic analysis of complete CAV genome sequences (n = 84) by the neighbour-joining method separated the CAV strains into 4 distinct genotypes: groups A (n = 2), B (n = 3), C (n = 55) and D (n = 24) (**Fig. 1**). Group C comprised three distinct subgroups (subgroups C1-C3) based on the shape of tree branches (**Fig. 1**). Phylogenetic analysis suggested that genotyping of CAV in China showed obvious geographical characteristics and no significant correlation with sampling time (**Fig. 1**). The phylogenetic tree showed that 63.8% (30/47) of Chinese CAV sequences (n = 47) from 2005–2020 formed a special branch (subgroup C3) different from CAV sequences from other countries. Subgroup C3 (n = 32) was the dominant genotype in China from 2005–2020, and 93.8% (30/32) of subgroup C3 are Chinese strains. 15 complete CAV genome sequences from the present study were classified to subgroup C3 (GX1801, GX1804, GX1805, GX1810, GX1904P, GX1905, GX1907A, GX1907B, GX1908W1, GX1908W3, GX1908L2, GX1908L3, GXQZ202001, GX2020-D6 and GX2020-D3). Five out of 47 Chinese CAV sequences belonged to subgroup C1 (n = 12) (**Fig. 1**). None of the Chinese CAV sequences belonged to subgroup C2 (n = 11). Subgroup C2 mainly included British, American, Italian and Egyptian CAV strains. Group D comprised 9 Chinese CAV sequences (including GX2020-D1 and GX1904A) and 15 CAV sequences from Japan, Australia, Italy,

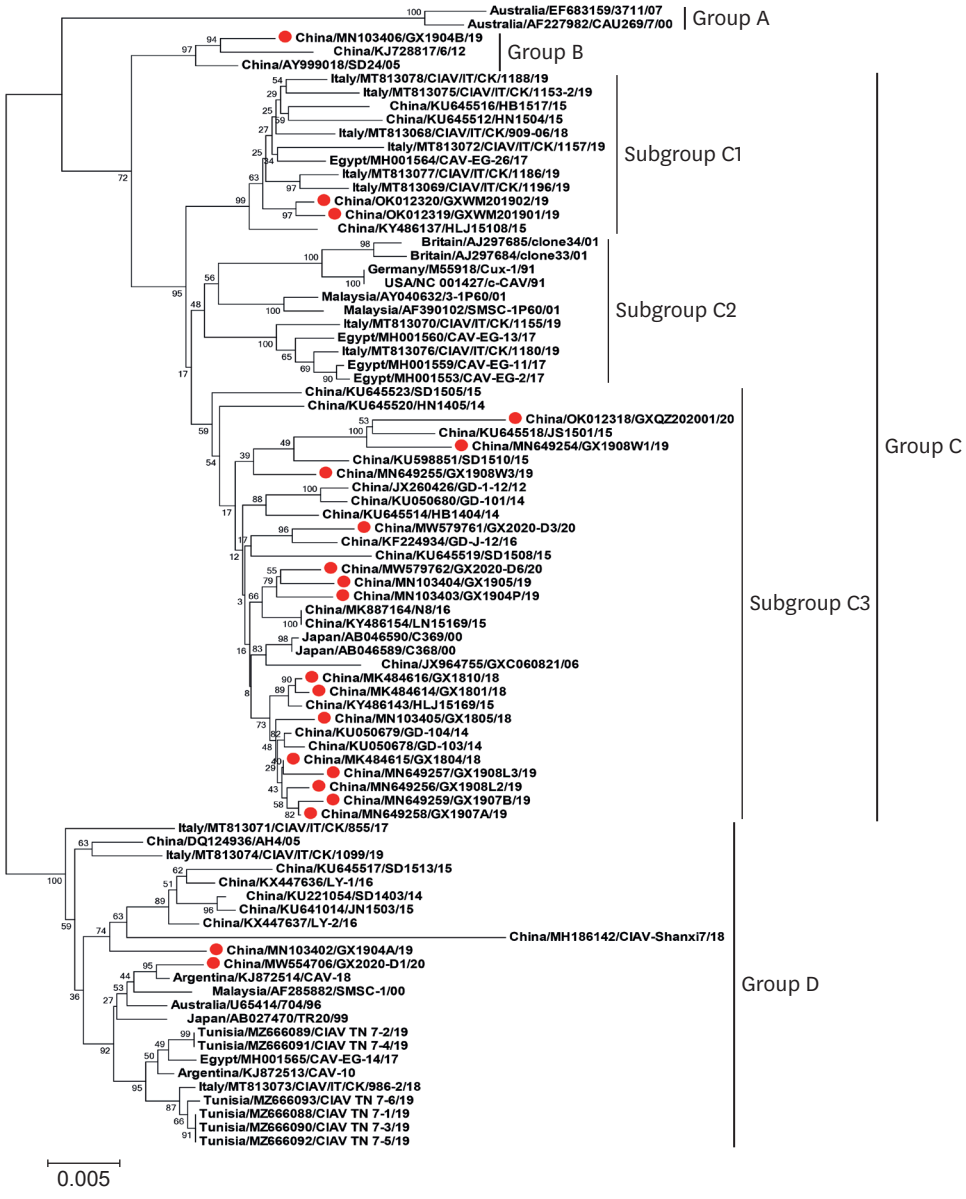

Fig. 1. Phylogenetic analysis of the 20 new complete CAV genome sequences and 64 reference complete CAV genome sequences available in GenBank. Values ≥ 60 are indicated on the branches (as percentages). The red circles represent the new complete CAV genome sequences.
CAV, chicken anemia virus.

Malaysia, Tunisia and Argentina. Group B included three Chinese CAV sequences (including GX1904B). Group A included two Australian CAV sequences. A distribution map showing the geographic distribution of Chinese CAV sequences from 2005–2016 and CAVs from 2018–2020 in Guangxi Province is shown in **Fig. 2**.

Molecular characterization

The nucleotide sequences of the 20 CAV genome sequences in this study exhibited 96%–99.8% homology. The nucleotide sequence identities of the complete genome were 94.8%–99.9% between the 20 CAV genome sequences and 64 reference CAV genome sequences from GenBank. GX1804 had the highest identity (99.9%) with strain GD-104 (accession

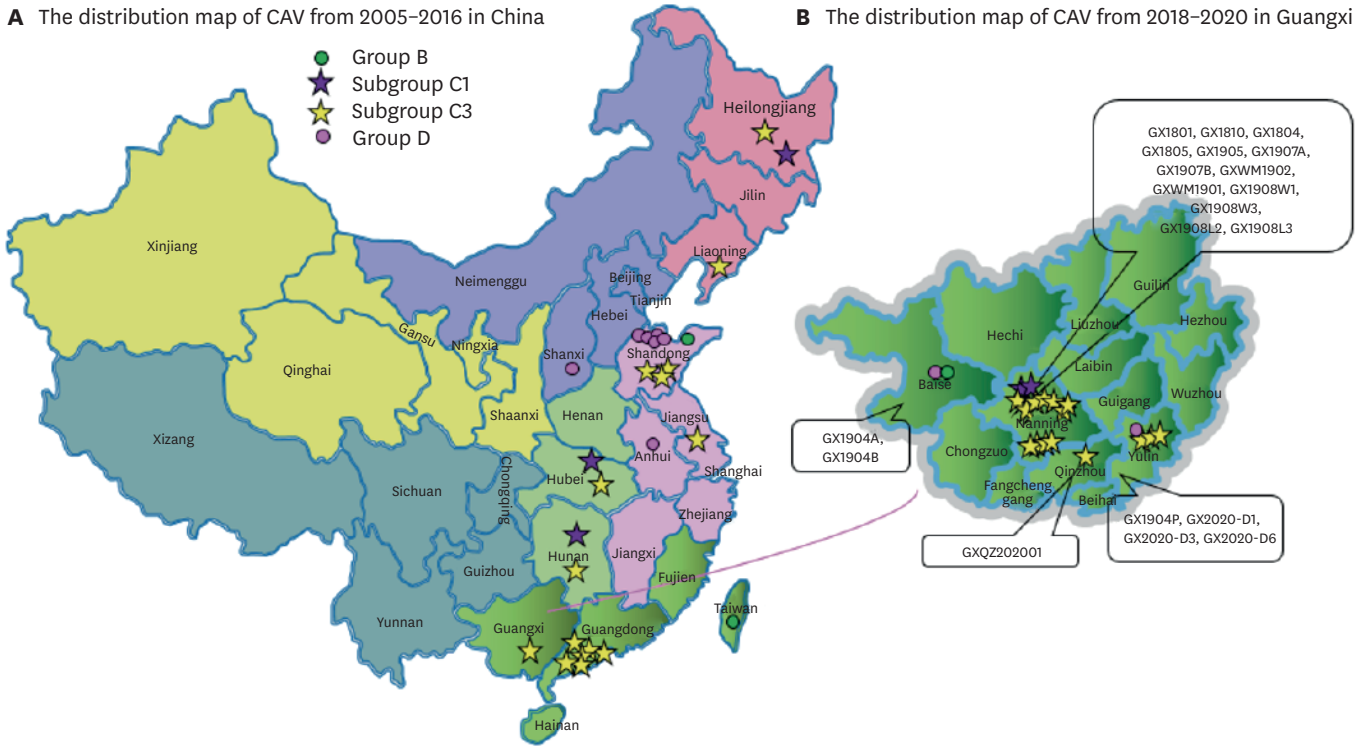

Fig. 2. The distribution map of CAV strains based on the available data of this study. (A) The distribution map of CAV strains according to geographical locations between 2005–2016 in China. (B) The distribution map of CAV based on the available sequences from 2018–2020 in Guangxi Province. The distribution of the CAV genotypes in each province is indicated by ★ (subgroup C1 and C3) and ● (group B and group D).
CAV, chicken anemia virus.

no. KU050679), a Chinese strain isolated in 2014. Strain GX1908W1 had the lowest identity (94.8%) with strain 3711 (accession no. EF683159), an Australian strain isolated in 2007.

All the VP1, VP2 and VP3 amino acid sequences from groups A, B, C (subgroups C1-C3) and D were aligned according to the genotyping from the phylogenetic analysis. The amino acid homology among the 20 CAV VP1 proteins obtained in this study was 97.1%–100%, with a similarity level of 100% among GX1908L2, GX1908L3, GX1805, GX1810 and GX1907A. Amino acid sequence identities among the 20 CAV VP1 and 64 CAV VP1 sequences retrieved from GenBank ranged from 96.4% to 100%. Comparison of the deduced VP2 and VP3 protein amino acid sequences of the 20 CAV strains in this study and 64 reference strains revealed overall predicted amino acid sequence identities ranging from 94.0% to 100%.

The main amino acid variations in VP1 were obtained by aligning the 20 CAV and 64 CAV VP1 amino acid sequences. The alignment results are shown in **Fig. 3**. The mutational positions were different among the genotypes. Because of the small number of sequences in group A and group B, the mutations of VP1 from group C and group D were analysed. The amino acid residues at position 370 of VP1 exhibited a relatively high mutation probability (**Fig. 3**). The mutation at position 370 accounted for approximately 61.9% (52/84) of the total number of VP1 amino acid sequences (**Supplementary Table 2**). The mutation at position 370 mainly manifested in Group B, Subgroup C1 and Group D (**Fig. 3**, **Supplementary Table 2**). Thirty-two out of 84 VP1 amino acid sequences had glycine (Gly) at position 370, and the remaining 52 VP1 amino acid sequences had alanine (Ala), threonine (Thr), serine (Ser) or arginine (Arg) (**Fig. 3**).

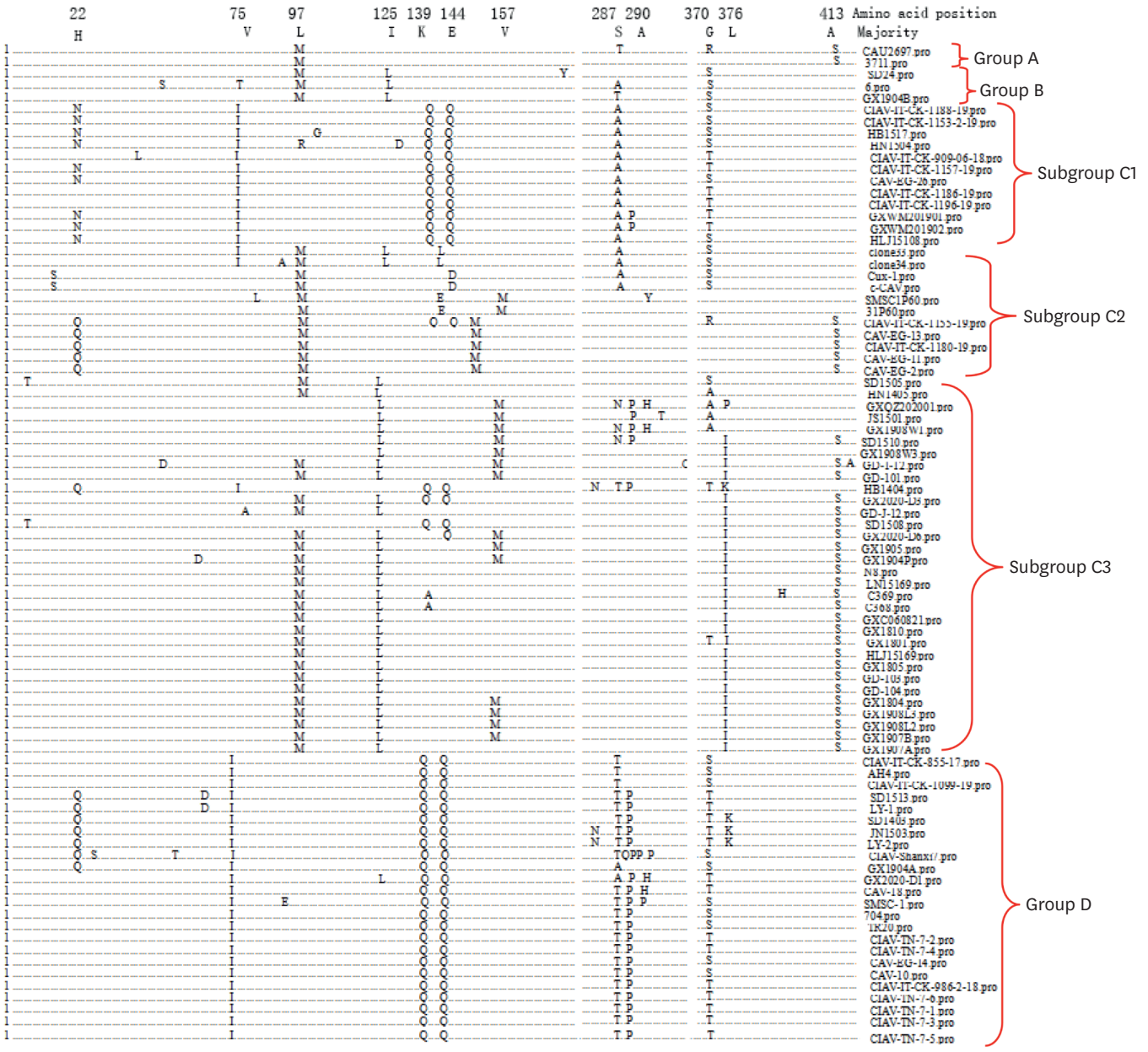

Fig. 3. Analysis of major mutations of amino acid residues derived from VP1 genes of CAVs.
CAV, chicken anemia virus.

The main amino acid variations in subgroup C3 were mainly concentrated at positions 97 (leucine [Leu] → methionine [Met]), 125 (isoleucine [Ile]→Leu), 157 (valine [Val]→Met), 376 (Leu→Ile) and 413 (Ala→Ser). The mutation probability in those positions was approximately 78.1%, 93.8%, 43.8%, 87.5% and 78.1%, respectively, based on the subgroup C3 CAV sequences (**Supplementary Table 2**). The amino acid substitutions in subgroup C2 were mainly concentrated at position 97 (Leu→Met), with a mutation probability of 100% (**Fig. 3**, **Supplementary Table 2**). The amino acid substitutions in group D were mainly concentrated at positions 75 (Val→Ile), 139 (Lysine [Lys]→glutamine [Gln]), 144 (glutamate [Glu]→Gln), 287 (Ser→Ala/Thr), 290 (Ala→Proline [Pro]) and 370 (Gly →Thr and Ser) (**Fig. 3**, **Supplementary Table 2**). The amino acid substitutions in subgroup C1 were mainly

concentrated at positions 75 (Val→Ile), 139 (Lys→Gln), 144 (Glu)→Gln), 287 (Ser→Ala) and 370 (Gly→Thr and Ser), all with a mutation probability of 100% (**Fig. 3**, **Supplementary Table 2**). The amino acid substitutions in group D and subgroup C1 had the same amino acid residue at positions 75, 139 and 144, but the residues differed between group D and subgroup C1 at positions 287 and 290 (**Fig. 3**). The above data reflect the uniqueness of the VP1 amino acid sequences of the group D, subgroup C1 and subgroup C3 genotypes at mutant positions; however, more sequences are needed for alignment. Analysis of the amino acid sequences of VP2 and VP3 showed that there were no special differences in amino acid sequences among the strains (data not shown).

The amino acids at positions 75, 89, 125, 141, 144 and 394 of VP1 related to CAV virulence were analysed by alignment with the virulent (Cux-1 and C368) and attenuated (C369) strains (**Table 3**). In this study, it was observed that the amino acids at positions 75, 89, 125, 141, 144 and 394 in the VP1 protein of 14 CAVs were Val, Thr, Leu, Gln, Glu and Gln, respectively, which is consistent with the virulent strain (C368) residues at the same positions. The amino acid at position 394Q in the VP1 protein of the remaining CAV strains from this study were consistent with the virulent strain (Cux-1).

Recombination analysis

RDP4 software and SimPlot 3.5.1 software were used to detect and confirm the potential recombination events using 20 new CAV genome sequences and 64 reference CAV complete genomes. The two potential recombination events (GX1904B and isolate 6) in CAV sequences are shown in **Supplementary Table 3**. The results showed that GX1904B of group B was a potential recombinant strain from the major parent isolate 704 (group D) and minor parent isolate HB1404 (group C). The simplot similarity implemented in SimPlot version 3.5.1 software confirmed that the beginning breakpoint and ending breakpoint mapped

Table 3. Key amino acid changes in VP1 among the 20 CAVs and Cux-1, C368, and C369

Strain	Amino acid position in VP1					
	75	89	125	141	144	394
Cux-1	V	T	I	Q	D	Q
C369	V	T	L	Q	E	H
C368	V	T	L	Q	E	Q
GX1801	V	T	L	Q	E	Q
GX1804	V	T	L	Q	E	Q
GX1805	V	T	L	Q	E	Q
GX1810	V	T	L	Q	E	Q
GX1904A	I	T	L	Q	Q	Q
GX1904B	V	T	L	Q	E	Q
GX1904P	V	T	L	Q	E	Q
GX1905	V	T	L	Q	E	Q
GX1907A	V	T	L	Q	E	Q
GX1907B	V	T	L	Q	E	Q
GX1908W1	V	T	L	Q	E	Q
GX1908W3	V	T	L	Q	E	Q
GX1908L2	V	T	L	Q	E	Q
GX1908L3	V	T	L	Q	E	Q
GXWM201901	I	T	I	Q	Q	Q
GXWM201902	I	T	I	Q	Q	Q
GX2020-D1	I	T	L	Q	Q	Q
GX2020-D3	V	T	L	Q	Q	Q
GX2020-D6	V	T	L	Q	Q	Q
GXQZ202001	V	T	L	Q	E	Q

CAV, chicken anemia virus.

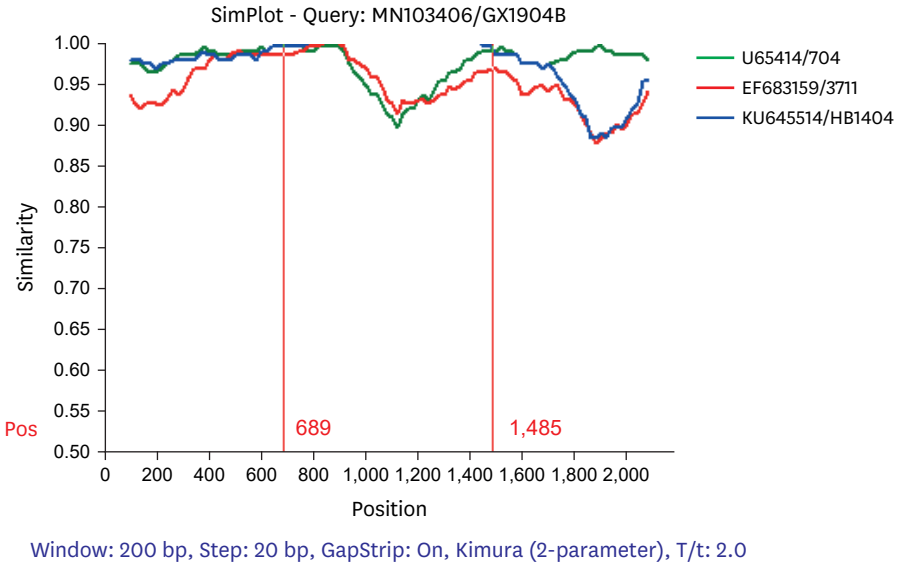

Fig. 4. A comparison of the sequences of three CAVs: 704, HB1404, and GX1904B. The GX1904B sequence was used as the query. The 3711 strain was included as an outgroup. The y-axis gives the percentage of identity with a sliding window of 200 bp and a step size of 20 bp.
CAV, chicken anemia virus.

to positions 689 and 1485, respectively (**Fig. 4**). The major parent isolate and minor parent isolate of isolate 6 were GX1908L3 (group C) and GX1904A (group D). The beginning breakpoint and ending breakpoint were mapped to positions 566 and 1584, respectively (**Supplementary Fig. 1**, **Supplementary Table 3**). SD24 was confirmed to be a recombination event and suspected to be a mosaicism originating from two genotypes by He et al [24]. The phylogenetic analysis classified SD24, isolate 6 and GX1904B to group B, suggesting that group B may be a branch of gene recombination strains. However, additional mosaicisms derived from group C strains and group D strains are needed to verify this conclusion. In this study, six recombination events (the CAV sequences are provided in **Table 2**), except for SD24, among 64 reference CAV sequences were used to construct the phylogenetic tree; however, previous reports have confirmed that the sequences were recombinants derived from different strains of the same genotype [15,27,31].

DISCUSSION

CAV was first reported in 1996 in China [13], and subsequently, the virus has spread widely in chicken flocks in China. In this study, none of the CAV samples collected from 5–21-day-old chickens were positive, which might have been due to the presence of maternal antibodies against CAV in chicks, allowing the chicks to avoid CAV infection. Sixty of the 350 samples were CAV positive, the positive samples were all from 22- to 100-day-old deceased or diseased chickens, and 36.7% of the CAV-positive samples exhibited coinfection of FAdV-4 and H5 subtype AIV. The detection results suggest that chickens aged over 3 weeks infected with CAV might be some cases of latent infection and secondary infections caused by FAdV-4 or other pathogens probably due to the decrease in maternal antibodies. However, because of the small number of samples, the correlation between age and CAV infection rate could not be determined, so it is necessary to collect more clinical samples from chickens of different ages for correlation analysis and to confirm the above conjecture.

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In this study, the CAVs were divided into four groups (groups A, B, C and D) according to the CAV classification method of Eltahir et al. [14]. Group C was separated into three subgroups (subgroups C1-C3) in this study. The 20 strains in this study were distributed in group B, subgroup C1, subgroup C3 and D, and 15 out of 20 strains belonged to subgroup C3, indicating that subgroup C3 was the dominant genotype in Guangxi Province, southern China. In addition, 63.8% (30/47) of Chinese CAV sequences from 2005-2020 were classified to subgroup C3 and 93.8% (30/32) of subgroup C3 are Chinese strains; A total of 19.1% (9/47) of the Chinese CAV sequences were classified to Group D. All eleven CAV sequences from Britain, the USA, Italy and Egypt were in subgroup C2. The CAV sequences from Japan, Australia, Italy, Malaysia, Tunisia and Argentina accounted for approximately 62.5% (15/24) of the total number of CAV sequences in Group D. The results showed that the genotype of CAV from China showed obvious geographic characteristics and no significant correlation with sampling time.

The classification of CAV has been previously described. Zhang et al. [15] reported that CAV was divided into five groups (A, B, C, D and E) based on 54 partial genomic sequences. CAV was separated into four major groups (A–D) by Eltahir using 55 complete VP1, VP2 and VP3 sequences [14]. Li et al. [16] analysed 121 complete CAV genomes and classified them into eight lineages. The reasons for the different CAV classification results obtained by different researchers are as follows: first, different reference strains were analysed by each researcher, and the CAV sequences might not have been complete sequences; second, the methods and software used to construct the phylogenetic tree were different (Zhang and Eltahir used the neighbour-joining statistical method in MEGA software to analyse CAV genetic evolution [14, 15], and, in contrast, maximum likelihood method statistical analysis based on the full-length genomes was implemented in RAxML software by Li et al. [16]); third, the genetic diversity of CAV and the number of CAV sequences used in phylogenetic analysis might explain the clustering of CAV. The fact that different classifications of CAV have been obtained indicates that additional CAV full-length genomes are needed to analyse genetic evolution.

The main amino acid variations in VP1 were obtained by aligning the 20 CAV and 64 CAV VP1 amino acid sequences. There were some special mutation sites among the intergenotypes in the VP1 amino acid sequences. Positions 125, 376 and 413 in the VP1 amino acid sequence of the subgroup C3 genotype with a high mutation probability above 78.1% occurred. The amino acid substitutions of subgroup C2 were mainly concentrated at position 97 with a mutation probability of 100%. The amino acid substitutions of group D and subgroup C1 had the same amino acid residues at positions 75, 139 and 144, but the amino acid residues at positions 287 and 290 differed between group D and subgroup C1. VP1 is the major structural protein of CAV and is believed to be involved in viral replication and virulence[7-9]. Therefore, the mutations at these positions among different genotypes might affect viral virulence and antigenic variation.

The pathogenicity of CAV in chickens is closely related to the amino acid residues of the VP1 protein. The amino acid at position 394 of VP1 has been demonstrated to be crucial for the pathogenicity of CAV, and all the highly virulent cloned strains had Gln at position 394, but all the low-virulence cloned strains had His [8]. Previous studies have shown that all changes at positions 75, 89, 125, 141 and 144 of the VP1 protein caused Cux-1 attenuation (the 394 position was still retained as Gln); however, if only one or four of them was changed, Cux-1 maintained high virulence [9]. The C368 strain was found to be highly virulent by Yamaguchi et al. [8]. The amino acids at positions 75, 89, 125, 141, 144 and 394 in VP1 of 14 strains

(GX1801, GX1804, GX1805, GX1810, GX1904P, GX1905, GX1907A, GX1907B, GX1908W1, GX1908W3, GX1908L2, GX1908L3, GX1904B and GXQZ202001) in this study were consistent with those of C368 at the same positions, which indicated that the 14 strains might be highly virulent viruses. The residue at position 394 of the remaining six strains was Gln, which was also consistent with the characteristics of virulent viruses. Therefore, these results indicated that the 20 strains all might be virulent viruses.

Recombination events in CAV occur frequently. Recombination events occur not only among different strains in the same group, but also across different genotype [24,36]. Previous studies have shown that the gene recombination of CAV can occur in both coding and noncoding regions [24,36]. The gene recombination event in this study involved the recombination of the coding region of CAV, which might impact the evolution of coding region genes of CAV.

In summary, 20 full-length CAV genomes originating from commercial broiler chicken flocks in Guangxi Province in southern China were characterized. CAV was separated into four major groups A, B, C (subgroup C1-C3) and D in this study, and subgroup C3 was the dominant genotype in Guangxi Province in southern China. VP1 protein analysis of 20 strains showed that these strains might be virulent, and one CAV recombination event among the 20 full-length CAV genomes was detected. The data in this study are helpful for understanding the recent genetic evolution of CAV in chicken flocks in southern China.

SUPPLEMENTARY MATERIALS

Supplementary Table 1

Detection results for CAV, ALV-J, AIV (H5 subtype and H9 subtype) and FAdV-4

Click here to view

Supplementary Table 2

Percentage of major mutations in deduced amino acids from VP1 genes based on 84 CAVs

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Supplementary Table 3

The potential recombination events in CAV sequences

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Supplementary Fig. 1

A comparison of the sequences of three CAVs: isolate 6, GX1908L3, and GX1904A. The isolate 6 sequence was used as the query. The 3711 strain was included as an outgroup. The y-axis gives the percentage of identity with a sliding window of 200 bp and a step size of 20 bp.

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