Molecular Epidemiology of Chicken Anemia Virus (CAV) in Southeastern Chinese Live Birds Markets

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SUMMARY. Between January 2004 and December 2005, cloacal swabs from essentially healthy chickens and silky chickens from live birds markets in Guangdong and Hunan provinces in southeastern China were screened for chicken anemia virus (CAV) by polymerase chain reaction. Phylogenetic analysis of the major structural protein VP1 sequences showed no clear genotype cluster and no correlation with the geographic origin of CAV strains. Virus evolution at the amino acid level was very slow, which corresponds to a strong negative selection of the VP1 gene in China and worldwide. A high proportion (87%) of birds was CAV positive, suggesting that many farms in the region were infected. Further investigations are necessary to evaluate the economic losses caused by CAV and the cost–benefit of vaccination.

RESUMEN. Epidemiología molecular del virus de anemia infecciosa aviar en los mercados de aves vivas del sureste Chino.

Entre Enero del año 2004 y Diciembre del 2005, se tomaron hisopos cloacales principalmente de aves sanas y gallinas de plumas sedosas de los mercados de aves vivas de las provincias de Guangdong y Hunan en el sureste de China. Estos hisopos fueron evaluados para detectar la presencia de virus de anemia infecciosa aviar mediante la reacción en cadena por la polimerasa. El análisis filogenético de la secuencia de la proteína estructural viral VP1 no mostró un claro grupo genotípico ni una correlación con el sitio geográfico de origen de las cepas del virus. La evolución viral a nivel de los aminoácidos fue muy lenta, lo que corresponde a una fuerte selección negativa del gen de la proteína viral VP1 en China y alrededor del mundo. Una alta proporción de las aves (87%) fue positiva a anemia infecciosa aviar, sugiriendo que muchas granjas en la región estaban infectadas. Se requieren investigaciones posteriores para evaluar las pérdidas económicas causadas por esta enfermedad y la relación costo beneficio de la vacunación.

Key words: chicken anemia virus, China, live birds markets

Abbreviations: aa = amino acid(s); CAV = chicken anemia virus; IBDV = infectious bursal disease virus; nt = nucleotide(s); PCR = polymerase chain reaction; SPF = specific pathogen free; VP = virus protein

Chicken infectious anemia is caused by a virus that was first isolated in Japan in 1979 in specific-pathogen-free (SPF) chicks (27). Characteristic symptoms are aplastic anemia paired with hemorrhagic lesions. Immune suppression and generalized lymphoid atrophy are often associated with secondary viral, bacterial, or fungal infections (19). Direct mortality caused by chicken anemia virus (CAV) is usually relatively low but increased consumption of antibiotics and depressed weight gain represent an economic threat especially for the broiler industry and SPF egg producers.

CAV is the only member of the genus *Gyrovirus* (*Circoviridae*). It is a nonenveloped, icosahedral virus of about 25 nm in diameter with a negative-sense single-stranded circular DNA genome. The viral genome consists of 2.3 kilobases, with 3 partially overlapping open reading frames (13) for VP1, the major viral structural protein (51.6 kDa); VP2, a novel dual-specificity protein phosphatase (16) (24 kDa); and VP3, a nonstructural protein (13.6 kDa) named apoptin. VP1 and VP2 are the targets of neutralizing antibodies (19).

The VP1 gene is more variable than VP2 and VP3 (5), and the worldwide genetic diversity of CAV VP1 is about 4.4% and 5.8% at nucleotide (nt) and amino acid (aa) level, respectively (5).

CAV has been found worldwide (19). In China, CAV was first isolated in 1996 from 25- to 40-day-old broilers (29). A survey in domestic poultry in 5 Chinese provinces (Beijing, Guangdong, Zhejiang, Shanghai, and Tianjin Shi) farms showed a 42% overall seroprevalence (28). So far, all viruses seemed to belong to the same serotype (19), although Spackman *et al.* (20,21) reported a putative second serotype. The emergence of additional new serotypes cannot be excluded and would have important consequences for vaccine efficacy and serodiagnosis. Although a few VP1 sequences of CAV from China are available on GenBank, no systematic analysis of Chinese strains has been performed to the best of our knowledge. Despite the high seroprevalence in the country (28) and the immunosuppression associated with an early infection, CAV vaccines have not been introduced in China.

Chicken farming provides an important source of cheap animal proteins. In China, 10.1 million metric tons of chicken meat and 28.7 million metric tons of eggs were produced in 2005 (http://faostat.fao.org). As a result of severe acute respiratory syndrome (caused by a group II or IV coronavirus related to group III infectious bronchitis virus), and avian influenza virus outbreaks in southeastern China, the surveillance of avian viruses has been intensified, and sanitary conditions and control have been improved. Within the framework of the enhanced laboratory surveillance, we have characterized CAV on a molecular level in live bird markets in two provinces in southeastern China.

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Fig. 1. Phylogenic analysis of the nucleic acid sequence of 26 new complete CAV VP1 sequences from Guangdong and Huan provinces, China, and 47 relevant complete VP1 sequences currently available on GenBank. The Nobilis® P4 vaccine strain is also shown. Numbers at

MATERIALS AND METHODS

Field samples. Cloacal swabs were collected from apparently healthy chickens ($n = 13$) or silky chickens ($n = 94$) at live birds markets in Guangdong ($n = 99$) and Hunan ($n = 8$) provinces between January 2004 and December 2005. Sellers on live poultry markets buy the birds from a wholesale market where both birds from large industrial farms and from familial farms are mixed. Meat chickens from large industrial farms are between 50 and 60 days of age, whereas chickens from familial farms are usually older than 4 mo. Old layers and breeders are also sold on these markets. Samples were stored at -80 C in virus transport medium (phosphate-buffered saline + 6 antibiotics) and had either never been thawed or thawed just once (for isolation attempt) before being processed for the present study.

DNA isolation, polymerase chain reaction (PCR), and sequencing. DNA was extracted with the QIAmp viral RNA mini kit as recommended by the manufacturer (QIAGEN GmbH, Hilden, Germany; http://www1.qiagen.com/literature/), it was screened for CAV genome, and the full VP1 gene of positive samples was amplified in a nested format for sensitivity reasons as described previously (5), with Takara Ex Taq® DNA polymerase (Takara Biotechnology, Dalian, China). A commercial live attenuated vaccine (Nobilis® CAV P4; Intervet B.V., Boxmeer, The Netherlands) was used as positive PCR control and to optimize the different PCRs. Sequencing was carried out in both directions with the Big Dye Terminator v.3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) on a capillary sequencer (model 3130xl; Applied Biosystems) using the PCR primers as sequencing primers. Nonredundant sequences were submitted to GenBank under the accession numbers AM407817 to AM407881. Strains were designated using the following nomenclature: World Health Organization country code_host code/year.month/province code/sample number; with CK for chicken, SCK for silky chicken; GD for Guangdong, and HN for Hunan province.

Data analysis. Sequences were analyzed using the Bioedit program (7). Forward and reverse sequences were aligned with ClustalW (4). Phylogenetic and molecular evolutionary analyses were based on the entire VP1 gene sequences, using MEGA version 3.1 (9). Phylogenic analysis of nucleic acid and deduced amino acid sequences was done with the neighbor-joining method, Kimura 2-parameter model. The aa sequences were also analyzed with the neighbor-joining method, with the Poisson correction. Bootstrap values (1000 replications) were indicated on each tree. Nucleotides were numbered according to Meehan et al. (11). All relevant complete VP1 sequences available on GenBank were used for comparison. Their accession numbers are indicated on Figs. 1 and 2.

Omega (dN/dS) values correspond to nucleotide mutation rate ratios of nonsynonymous and synonymous mutations. They were calculated for each group of sequences using PAML version 3.14 program (25) (http://abacus.gene.ucl.ac.uk/software/paml.html). Another test was carried out with PAML version 3.14 to detect individual codons that are under positive natural selection (12,26). The model (M0-M13) best adapted to the data set was selected with likelihood ratio tests with $P < 0.01$ (1,18,26).

RESULTS

Nucleic acid alignment and phylogenic analysis. Ninety-three of 107 (87%) cloacal swabs from southeastern China were positive for CAV in the nested detection PCR. Sixty-five complete sequences

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nodes correspond to bootstrap values >49. Chinese strains from the present study are marked with a closed triangle, whereas other Chinese sequences from GenBank have open triangles. Chinese strains with identical aa sequences are shown only once. Mixed infected samples sequences are not included in the phylogenetic analysis.
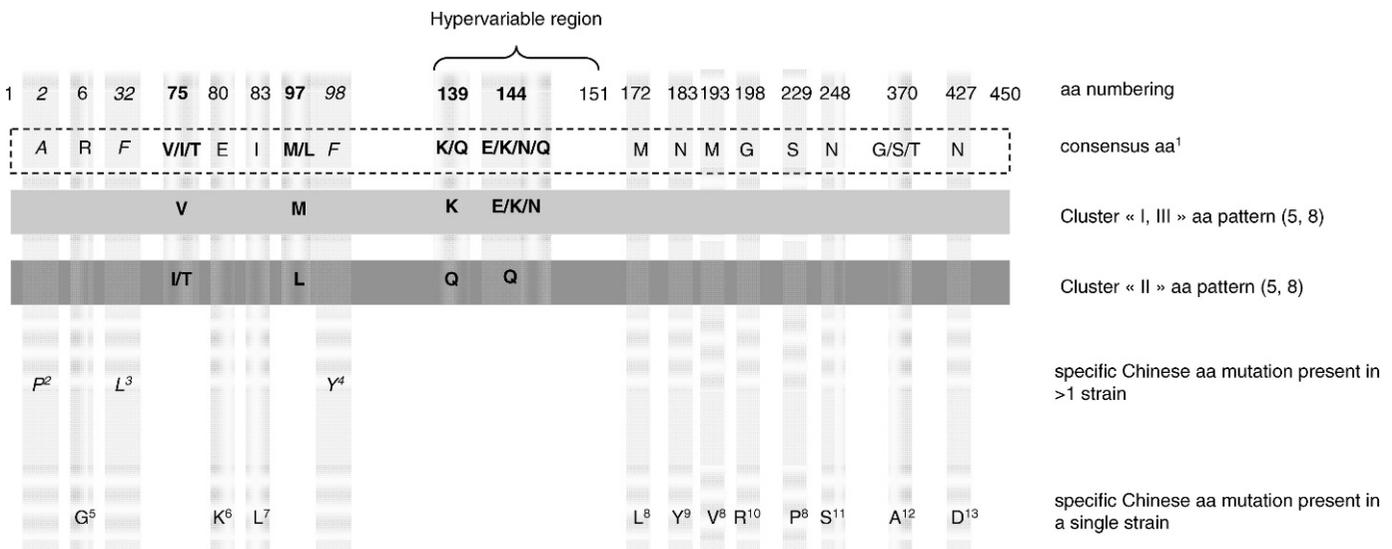
Fig. 2. Amino acid substitutions within VP1 protein of both cluster I and II strains. The hypervariable region (16) and the aa substitutions specific to Chinese strains are shown. Specific Chinese aa mutations: italic for mutations present in >1 Chinese strains; normal font for mutations present in a single Chinese strain; and bold: aa pattern of previously described clusters I, II, and III (5,8). [1] Most frequent aa. [2] aa substitution in CH683, CH5256, CH2768, CH4665, CH22035, CH4663, and CH 5840. [3] aa substitution in CH210, CH208, and CH7386. [4] aa substitution in CH-TJBD33 and CH-BJ0401. [5] aa substitution in CH5264. [6] aa substitution in CH4665. [7] aa substitution in CH-AY839944. [8] aa substitution in CH-BJ0401. [9] aa substitution in CH-SD24. [10] aa substitution in CH-HN9. [11] aa substitution in CH-AH4. [12] aa substitution in CH656. [13] aa substitution in CH-SH11.

of VP1 gene were obtained from Guangdong ($n = 61$) and Hunan ($n = 4$) provinces. All sequences were 1350 nucleotides, and they had no insertions or deletions. Chinese VP1 sequences presented nucleotide mutations that had not been observed before in strains from other parts of the world, as, for example, the following 23 mutations, which occurred in at least 3 Chinese strains: positions 4, 94, 250, 420, 426, 444, 522, 555, 570, 807, 876, 903, 930, 1065, 1122, 1125, 1215, 1221, 1248, 1266, 1282, 1293, and 1308. The reliability of the sequences was ensured by forward and reverse sequencing of a template amplified with a high-fidelity polymerase with proofreading capability.

The maximum Kimura distance between the 74 Chinese VP1 sequences available was 5.2% (between AH4-China and BJ0401) in comparison with 6.2% (between Pallister-Australia and NI118-Nigeria) for the world maximum Kimura distance calculated for 112 relevant CAV VP1 sequences (neighbor-joining method, Kimura 2-parameters model). The new South Chinese sequences did not increase the latter diversity nor the diversity of the previous Chinese sequences. Phylogenetic analysis of CAV strains did not allow easy grouping of sequences into distinct clusters at the nt level (Fig. 1).

Amino acid alignment and phylogenic analysis. When the amino acid sequences of 112 VP1 genes were aligned, the maximal Kimura distance was 4.1%. The new Chinese sequences did not increase the overall maximal amino acid diversity of CAV.

Only 3 variable amino acids positions specific to Chinese strains were detected in more than one of the Chinese sequences, and 11 additional substitutions were detected in a single strain only (Fig. 2). None of these mutations were found in the hypervariable region (aa 139–151; Fig. 2) (17).

The phylogenetic tree at the aa level also showed no obvious CAV clusters supported by significant bootstrap values (Fig. 3).

Mean Kimura distances among the strains collected first (January 2004) and last (December 2005), and the mean distance between these 2 groups at the aa level, did not significantly differ (1%, 0.8%,

and 0.7%, respectively). Mutations were, as expected, much more frequent in the "hypervariable" region (aa 139–151) (mutation probability: 62% or 8/13 positions) than in the whole VP1 aa sequence (mutation probability: 14% or 61/450 positions), confirming the hypervariable nature of this domain.

Analysis of divergence and selection. The CAV VP1 genes are highly conserved worldwide with an omega value (dN/dS) of 0.08, suggesting that the chance of a nonsynonymous mutation is only 8% of the chance of a synonymous mutation of becoming fixed in the population (Table 1). The calculation of omega ratios for China and for each continent demonstrated a negative (purifying) selection worldwide albeit with geographic differences. Omega values ranged from 0.04 (in China or in Nigeria) to 0.20 (in Europe). No single codon with positive (diversifying) selection could be detected in the Chinese strains.

DISCUSSION

Although CAV has been detected in some parts of China, this is the first molecular epidemiologic analysis of this virus in the country. As the detection PCR was more sensitive than the sequencing PCR, only 70% of the CAV-positive cloacal swabs (65 of the 93 positive CAV strains) were suitable for sequencing. We cannot exclude that some strains may also have been missed because of mutations within primer locations. About 40% of the sequences showed double peaks with degenerated nt in the electropherograms, suggesting mixed infections as was observed and confirmed by cloning by us and others (5,23).

The nt or aa sequences revealed no clear phylogenetic clusters, or potential genotypes that would be supported by high bootstrap values (Figs. 1 and 3) irrespective of the method used (neighbor joining, maximum parsimony, minimum evolution, or unweighted pair group method with arithmetic mean; data not shown). Three clusters were previously identified by us and others (5,8) at the aa
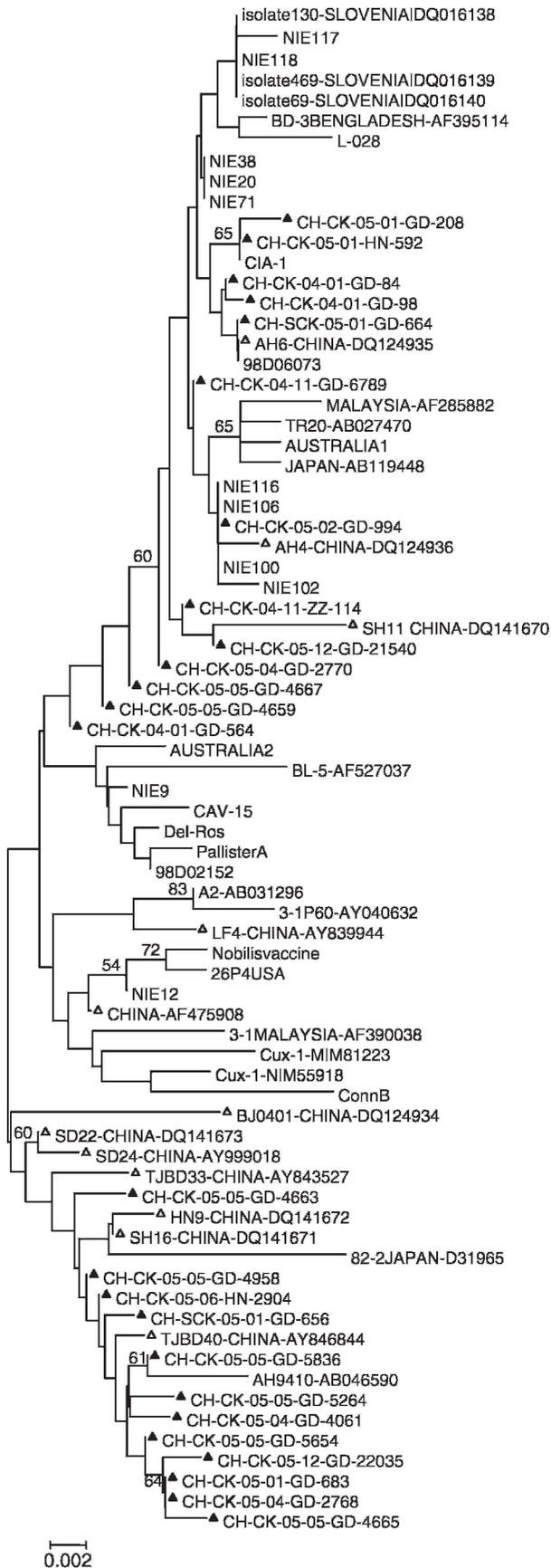
Fig. 3. Phylogenic analysis of the predicted aa sequences of the 74 CAV strains from Fig. 1. Symbols are as described in Fig. 1. Sequences of mixed infected samples are not included.

level: clusters I and III with the aa pattern [75]V, [97]M, [139]K, [144]E/K/N, and cluster II with the aa pattern [75]I/T, [97]L, [139]Q, [144]Q (Fig. 2). Maximum Kimura distances between (4.1% between 82-2Japan and Malaysia/AF285882) and within (3.4% and 2.0% between 82-2Japan and CH-BJ0401 and between Malaysia/AF285882 and CH-SH11, respectively) clusters were relatively low. Whether a 1.2-fold difference between inter- and intracluster maximum Kimura distances would be sufficient to define genotypes or whether current strains belong to a single genotype requires further attention.

Mutations associated with attenuation and a weaker reactivity with monoclonal antibody 2A9 (complete [75]I, [89]T, [125]L, [141]L, and [144]E pattern (22); or [394]H, (24)) were absent in the Chinese strains.

CAV VP1 genes are worldwide under strong negative selection (omega = 0.08; Table 1). In China and in Nigeria, even lower omega values were observed (0.04; Table 1), perhaps because of the absence of vaccination in both countries. However, omega calculations may be biased by the low number of sequences available on GenBank for most other countries and by a short collection period such as in this study. Omega values are also influenced by the number of passages of some strains such as Cux-1. The nt diversity of CAV is similar to the diversity of other DNA viruses, but very few of the mutated nt translate into aa. In addition to low virus survival rates with nonsilent mutations, other factors may slow down virus evolution such as its exquisite host adaptation (19), or the short generations in poultry.

In southeastern China most poultry from both family and commercial farms transit through wholesale markets, to retail live bird markets. CAV DNA was detected in 87% of all birds tested in this study. The birds were a cross-section of essentially healthy animals for sale at the participating live poultry markets in Guangdong and Hunan provinces, but it was not possible to trace the birds back to their farms. Although unlikely, it cannot be totally excluded that identical sequences from the same live poultry market are from the same farms (e.g., CH208-CH210 and CH4960-CH4964, collected on the same day, in the same live bird market; CH510-CH990 and CH3086-CH5262, collected within 3- and 6-wk intervals, at the same live bird markets). Therefore, our data suggest that the prevalence of CAV is very high in the region and that farms have a high risk of infection.

CAV DNA was detected for at least 40 wk after infection (2,3). After about 3 wk of age, immunocompetent chickens are resistant to disease, but they can acquire asymptomatic infections (15,19). The adult birds tested were probably subclinically infected. Because the virus was detected in cloacal swabs, all of these birds can contribute to the spread of the virus. Thus, the return of poultry or cages from markets to farms is likely to facilitate spill-back of virus to farms.

Previous studies suggested that coinfections with CAV and infectious bursal disease virus (IBDV) may result in increased mortality (8), but IBDV PCRs (14) were negative in all swabs (data not shown), probably because of widespread vaccination in China.

Although the high proportion of the virus suggests that young chickens may be partially protected by maternally derived antibodies, further studies are required to quantify losses in productivity caused by CAV-related immunosuppression and secondary infections by other pathogens. A complementary study at a farm rather than a market level would be warranted to further evaluate the prevalence of CAV in the region, the role of live bird markets in the spread of CAV, and the cost–benefit of vaccination. Wholesale and live birds markets may have a grossly underestimated role in the spread, evolution, or both of avian pathogens (10), as suggested previously for infectious bronchitis virus (6).

Table 1.   Mean genetic distances and omega values for CAV VP1 genes per country or continent

Geographic origin	No. of sequences	Mean nt genetic distance (%)[A]	Mean aa genetic distance (%)[A]	Omega values (dN/dS)
Worldwide	112	2.5	1.4	0.08
Europe (Germany and Slovenia)	5	1.4	1.4	0.20
America (U.S.A.)	9	2.9	1.7	0.09
Africa (Nigeria)	11	2.7	0.7	0.04
Asia	84	0.5	0.2	0.08
Asia without China	10	3.5	2.4	0.13
China	74	0.4	0.1	0.04
Australia	3	3.8	1.9	0.06

[A]Mean genetic distances were calculated with neighbor-joining model, nt distances with Kimura-2 parameters method, and aa distances with Poisson correction.

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