Enhanced laboratory surveillance of group III coronaviruses in life poultry markets in Guangdong province, China, after the SARS outbreak

Mariette Ducatez, Yi Guan, Honglin Chen, Claude P. Muller

ENHANCED LABORATORY SURVEILLANCE OF GROUP 3 CORONAVIRUSES IN LIFE POULTRY MARKETS IN GUANGDONG PROVINCE, CHINA, AFTER THE SARS OUTBREAK

Ducatez M.F.[1, 3], Guan Y.[2, 3], Chen H.[2, 3], Muller C.P.[1]

[1] Institute of Immunology, National Public Health Laboratory,
20A rue Auguste Lumière, L-1950 Luxembourg
[2]State key Laboratory of Infectious Diseases, Department of Microbiology,
University of Hong Kong SAR, China and
[3]Joint Influenza Research Centre of Shantou University Medical College and
The University of Hong Kong, Shantou, China

SUMMARY

One hundred and seven chickens or silky chickens were tested for infectious bronchitis virus between August 2003 and December 2005 in Guangdong and Hunan Chinese provinces, from which SARS coronavirus has initially emerged. IBV was detected in 82 birds (prevalence: 77%). Limiting the IBV detection PCR to tracheal or cloacal swabs would have led to a considerable underestimation of virus prevalence of 50 to 66% only. 15 sequences of 362 bp of the spike 1 gene (S1) were obtained. 13 strains clustered with Chinese genotype IV strains, which were recently reported in South China too. Genotype IV also showed the larger evolutionary distances in comparison to other Chinese genotypes. IBV/CK(T)/GD.CH/05-04/3587 strain clustered with genotype III virus, showing that genotype III continues to circulate in Guangdong province at least. A vaccine strain was probably detected in a bird as IBV/CK(C)/HN.CH/05-06/2904 was identical to H120 and H52 vaccines which are commonly used in Chinese poultry farms. It is nevertheless not likely that the IBV strains which could not be sequenced were vaccine strains: since both detection and sequencing PCRs were equally sensitive for the vaccine strain, sequencing would rather overestimate vaccine strains than wild-type variants. Our results suggest that at life-bird markets almost all birds carry wild-type IBV and that these markets may be an important and so far underestimated source of infection for IBV.

INTRODUCTION

The recent emergence of a coronavirus variant causing severe acute respiratory syndrome (SARS) in humans has renewed interest in the *Coronaviridae* family of viruses. This enveloped virus was first found in the United States in 1930 and since has been reported from most countries

throughout four continents: America, (Johnson and Marquardt, 1975), Europe, (Capua et al., 1994; Cavanagh and Davis, 1993; Gough et al., 1992), Asia, (Wang et al., 1997) and Australia, (Ignjatovic and McWaters, 1991; Lohr, 1976). Coronaviridae form 3 genetic groups, two of which (group 1 and 2) are pathogenic in humans. Group 1 and 2 viruses cause upper respiratory infections that vary in frequency and severity in different outbreaks (Holmes, 2001). SARS CoV was classified with group 2 viruses but was highly lethal in humans. Group 1 and 2 viruses also infect canines, rodents, felines, porcines and bovines (Lai, 2001). Turkeys can be infected by group 2 as well as group 3 turkey coronaviruses (Lai, 2001). Group 3 viruses such as Infectious Bronchitis Virus (IBV) (Cavanagh, 2000; Enjuanes, 2000), occur only in birds. So far group 3 viruses have not been found in human but phylogenetic analysis of SARS-CoV showed that its genome contained sequences that seem to be of group 3 origin (Stavrinides and Guttman, 2004).

SARS coronavirus has initially emerged from the province of Guangdong in Southeast China. In an effort to understand the natural history of this virus, laboratory surveillance of different host species has been intensified. The Chinese horseshoe bat has been suggested as possible natural reservoir of SARS-like coronavirus (Lau et al., 2005). In a recent study, we showed that bat coronaviruses cluster in several distinct groups, emphasizing the complexity of this viral family and the special role of bats in its ecology and evolution (Tang, 2006).

In China, IBV strains have first been isolated in 1982 (Liu et al., 2006). Outbreaks have occurred frequently and they continue to damage the poultry industry, although vaccines such as H120 and H52 are in used since many years (Liu et al., 2006). IBV infects primarily the respiratory tract, kidney and oviduct (Cavanagh, 2003). Recent reports suggest a shift in tissue tropism (Liu and Kong, 2004; Liu et al., 2006; Yu et al., 2001; Zhou et al., 2004) and an extended host range into new bird species reared close to domestic fowl. For instance, IBV was recently detected in Chinese peafowl (*Pavo*), guinea fowl (*Numida meleagris*), partridge (*Alectoris*) and teal (*Anas*) (Cavanagh, 2005).

IBV is an enveloped and positive stranded RNA virus with a genome of about 27 kb. While the nucleoprotein is the most conserved gene of IBV, the spike 1 (S1) subunit of the spike protein gene is the most variable (Cavanagh, 2003). This subunit is responsible for inducing neutralizing and serotype-specific antibodies. Mutations within this genome region may therefore result in emergence of new variants against which vaccines do no longer protect (Moore et al., 1998). Here we present a surveillance study of group 3 coronavirus IBV in life poultry markets mainly in Guangdong province in Southeast China.

MATERIALS and METHODS

Field samples

Since July 2000, systematic influenza virus surveillance was carried out in Southeastern Chinese live birds markets: cloacal, tracheal and fecal swab samples were collected once every 7-10 days in each market from apparently healthy birds. For the present study, 107 chicken or silky chicken cloacal and tracheal swab samples collected between August 2003 and December 2005 in Guangdong and Hunan provinces were tested. Market-vendors buy their birds

from a wholesale market where poultry from both large and family farms are mixed. Meat chickens from large industrial farms are between 50 and 60 days of age while family farm chickens are usually older than 4 months of age. Old layers and breeders are also sold on these markets. The samples were stored at –80°C in virus transport medium (PBS + 6 antibiotics) and had either never been thawed or thawed just once (for AIV isolation attempt) before being processed within the framework of the present study.

RNA isolation and Reverse Transcription - Polymerase Chain Reaction (RT-PCR)

RNA was extracted using the QIAamp viral RNA mini kit (Qiagen, Hilden, Germany), following the instructions of the manufacturer. RNA was eluted in 50 µl elution buffer and processed immediately without intermediate freezing-thawing step. The extracted RNA was first reverse transcribed with random primers and SuperScript III (Invitrogen, Merelbeke, Belgium), following the instructions of the manufacturer. Complementary DNA was first screened for IBV genome using a highly sensitive nested detection PCR of a constant region of the nucleoprotein gene (Akin et al., 2001). The S1 gene was amplified from IBV-positive samples in a semi-nested format (Adzhar, 1997) for sequencing purposes. All PCRs were performed in 25 µl with 0.5 U Platinum® Taq DNA Polymerase (Invitrogen, Merelbeke, Belgium) per reaction for the 1st round PCR and 1 U of Platinum® Taq per reaction for the nested PCR. The equivalent of 0.5 µl of the reaction mix of the RT or of the first PCR was transferred to a new tube for the 1st round or the nested reactions, respectively. Primer concentrations were 0.5 µM and 1 µM for detection and sequencing PCRs, respectively. $MgCl_2$ concentrations were optimized and ranged between 1.5 and 5 µM. All PCRs counted 35 cycles, annealing temperatures were 55°C, 58°C, 53°C and 55°C for detection 1st round, detection nested, sequencing 1st round and sequencing semi-nested PCRs respectively. All programmed cycling was performed in a thermocycler (Mastercycler Gradient, Eppendorf, Hamburg, Germany). PCR amplicons were analyzed in a 1.5% agarose gel (Ultrapure, Invitrogen, Merelbeke, Belgium), using 1X TAE as electrophoresis running buffer and stained with ethidium bromide (15 µg in 100 ml of agarose gel). IBV vaccine strain H120 kindly provided by Dr. Palya and Penzes, Phylaxia Veterinary Biologicals Co. Ltd, Budapest, Hungary, was used as positive PCR control and to optimize the different PCRs.

Sequencing

PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer. 10-100 ng DNA were used for sequencing in both directions with the Big Dye Terminator v.3.1 cycle sequencing kit (Applied Biosystems, Foster city, USA) on a 16 capillary sequencer (model 3130xl**,** Applied Biosystems, Foster city, USA) using the PCR primers (Eurogentech, Seraing, Belgium) as sequencing primers. South-Eastern Chinese S1 sequences were submitted to GeneBank under the accession numbers AM262512 to AM262521. Identical sequences were submitted only once. Strains were designated as follows: IBV/host code(sample type code)/location.WHO country code/year-month/sample number. Hosts were

designed either with CK for chicken or with SCK for silky chicken. Sample types were either tracheal or cloacal swabs designed with T and C respectively.

Data Analysis

Sequences were analyzed using the Bioedit program (Hall, 1999). This program was also used to read the sequencing electropherograms and to exclude nucleotide ambiguity. Sequences were aligned with ClustalW (Chenna et al., 2003). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (Kumar S, 2001). Phylogenic analysis of nucleic acid and deduced amino acid sequences was done with the Neighbor Joining method, Kimura 2 parameters, pairwise deletion. Amino acid sequences were also analyzed with the Neighbor Joining method, with the Poisson correction. Bootstrap values (1000 replications) were indicated on the tree. Win Episcope 2.0 (Thrusfield et al., 2001) was used for the kappa test (Cohen, 1960).

RESULTS

Detection of IBV

107 chickens or silky chickens were tested for IBV genome both in cloacal and tracheal swab samples. 53 (50%) tracheal and 66 (62%) cloacal swabs were positive in the IBV detection PCR. Only 36 birds (34%) were positive for both swabs (Table 1). Kappa coefficient of agreement was poor (Landis and Koch, 1977) 0.123 (confidence interval: 95%, Table 1). The prevalence of IBV was 77%. Although IBV was detected in 82 birds, only 15 sequences of 362 bp of the spike 1 gene (S1) were obtained, corresponding to 18% of the positive birds. Samples from 2003 (1/3), 2004 (18/22) as well as from 2005 (63/82) were detected positive for IBV, from Guangdong (75/99) as well as from Hunan (7/8) province.

Phylogenetic analysis of nucleotide sequences

12 strains clustered with genotype IV strains while IBV/CK(T)/GD.CH/05-04/3587 clustered with genotype III strains and IBV/CK(C)/HN.CH/05-06/2904 with Massachusetts genotype V strains (genotype numbering according to Liu et al., 2006, Fig. 1). Within the genotypes III and IV proposed by Liu et al. (2006), several sub-groups IIIa and IIIb, IVa to IVd became apparent: the genotype III strain IBV/CK(T)/GD.CH/05-04/3587 seems to belong to cluster IIIb but was genetically essentially as close to cluster IIIa strains as to cluster IIIb strains. Its average genetic distance to cluster IIIa strains was 7.25%±0.35%, while the distance to cluster IIIb was 6.70%±0.52%. IIIa and IIIb strains - available on GenBank and for which the location is known- originated from Guangdong (IBV/CH/DQ167133-CK/CH/LGD/03I) or Tianjin (IBV/CH/AF257075-TJ/96/02 and IBV/CH/DQ167151-CK/CH/LTJ/95I) provinces.

Our genotype IV strains belonged to 2 sub-clusters: IBV/CK(C)/GD.CH/05-01/518, IBV/CK(T)/GD.CH/05-01/215 and IBV/CK(C)/HN.CH/05-01/388 were closest to genotype IVa, the predominant cluster, while IBV/CK(T-C)/HN.CH/05-01/316-387-388-like strains grouped with IBV/CH/AY837465-TA03 to genotype IVd (Fig. 1). The new strains slightly increase the genotype IV maximum diversity (on the 362 nt) from 19.2% (between IBV/CH/DQ167128-CK/CH/LAH/03I and IBV/CH/DQ167135-CK/CH/LGD/04III) to 20.6% (between IBV/CH/DQ167128-CK/CH/LAH/03I and IBV/SCK(T)/GD.CH/05-04/3495).

Strains from our study showed specific nt in only 2 positions in comparison to all other Chinese IBV strains: ^{176}G for IBV/CK(C)/GD.CH/05-11/19466 and ^{279}G for most genotype IVd strains (nt numbers according to sequenced bases: nt1 to nt362). Our genotype V strain IBV/CK(C)/HN.CH/05-06/2904 was identical with the H120 and H52 vaccines.

Amino acid sequence comparison

The maximal distance between aa sequences of our Chinese S1 gene fragments was 25.2% (between IBV/CH/AF352315-H52 and IBV/CH/DQ167137-CK/CH/LHB/96I, between IBV/CH/DQ167143-CK/CH/LHN/00I and IBV/CH/DQ075323-SH1, between IBV/CK(C)/HN.CH/05-06/2904 and IBV/CH/DQ167137-CK/CH/LHB/96I, between IBV/vaccine H120 and IBV/CH/DQ167137-CK/CH/LHB/96I). These strains did not increase the overall Chinese strain diversity at the aa level. There was only one unique aa found (^{60}R in IBV/CK(C)/GD.CH/05-11/19466, aa numbers: aa1 to aa121).

DISCUSSION

Limiting the IBV detection PCR to tracheal or cloacal swabs would have led to a considerable underestimation of virus prevalence of 50 to 66% only. Testing both tracheal and cloacal swabs brought the prevalence up to almost 80%. Recent studies reported a shift in tissue specificity (e.g. the proventricular, Liu and Kong, 2004; Liu et al., 2006; Yu et al., 2001) suggesting that in life-bird markets the prevalence of virus carriers may even be higher, if only enough tissues are sampled.

82 birds were positive in the nucleoprotein detection PCR, but only 15 sequences of S1 gene fragments were obtained, although both PCRs were equally sensitive for the H120 vaccine strain (10^{-3} $TCID_{50}$). The difficulties to amplify S1 gene fragments could be due to RNA degradation, although this would equally affect the nucleoprotein and the S1 PCR. Mutations at primer locations of the more variable spike protein gene used for detection (Cavanagh, 2003) seem to be a more likely explanation.

In China, the life-attenuated IBV vaccine (e.g. H120 or H52) is recommended for commercial meat chickens at 4, 14 and 40-45 days of age. IBV vaccination is common but not systematic in industrial farms in Southeastern China. Therefore, we cannot fully exclude that vaccine virus was detected in our tests. However, among 15 strains only one (7%) had a vaccine-like spike gene. Since both detection and sequencing PCRs were equally sensitive for the vaccine strain, sequencing would rather overestimate vaccine strains than wild-type variants. Moreover, as much as 50% of the birds tested may be from family farms which rarely vaccinate. Thus our results suggest that at life-bird markets almost all birds carry wild-type IBV and that life-bird markets may be an important and so far underestimated source of infection for IBV.

Our results further show that genotype III strains that have been found in China since 1995 continue to circulate at least in Guangdong province in 2005. Genotype IV strains seem to be of a more recent origin as genotype III strains as they have mostly been found after 2000 (Liu et al., 2006). Nevertheless this genotype showed a larger evolutionary diversity both in our as well as previous studies: distances within groups IVa and IVd were 5.59 and 4.91% respectively,

while the mean distance between the 2 groups reached about 20% both at the nt and aa level. The detection of IBV genome is not necessarily proof of current or former disease, but our strains are closely related to nephropathogenic and proventricular strains found since 2000 (Liu and Kong, 2004; Liu et al., 2006; Yu et al., 2001; Zhou et al., 2004). The low efficiency of the sequencing PCR may indicate that the diversity of IBV in Southeastern China may be greater than reflected by the sequences.

Our study shows that the prevalence of IBV is extremely high in life birds markets and the diversity is probably even higher than so far estimated. Stavrinides and Guttman (2004) showed that at least partial sequences of the matrix, nucleoprotein and spike genes of SARS-CoV may be of avian origin. It is therefore necessary further characterize the genes of these IBV strains as well as potential variants that may have gone undetected.

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Table 1. Detection of IBV tracheal and cloacal swabs of life-bird markets.

Kappa=0.123 (95% CI)	Tracheal swab positive	Tracheal swab negative	Total
Cloacal swab positive	36	30	66
Cloacal swab negative	17	24	41
Total	53	54	107

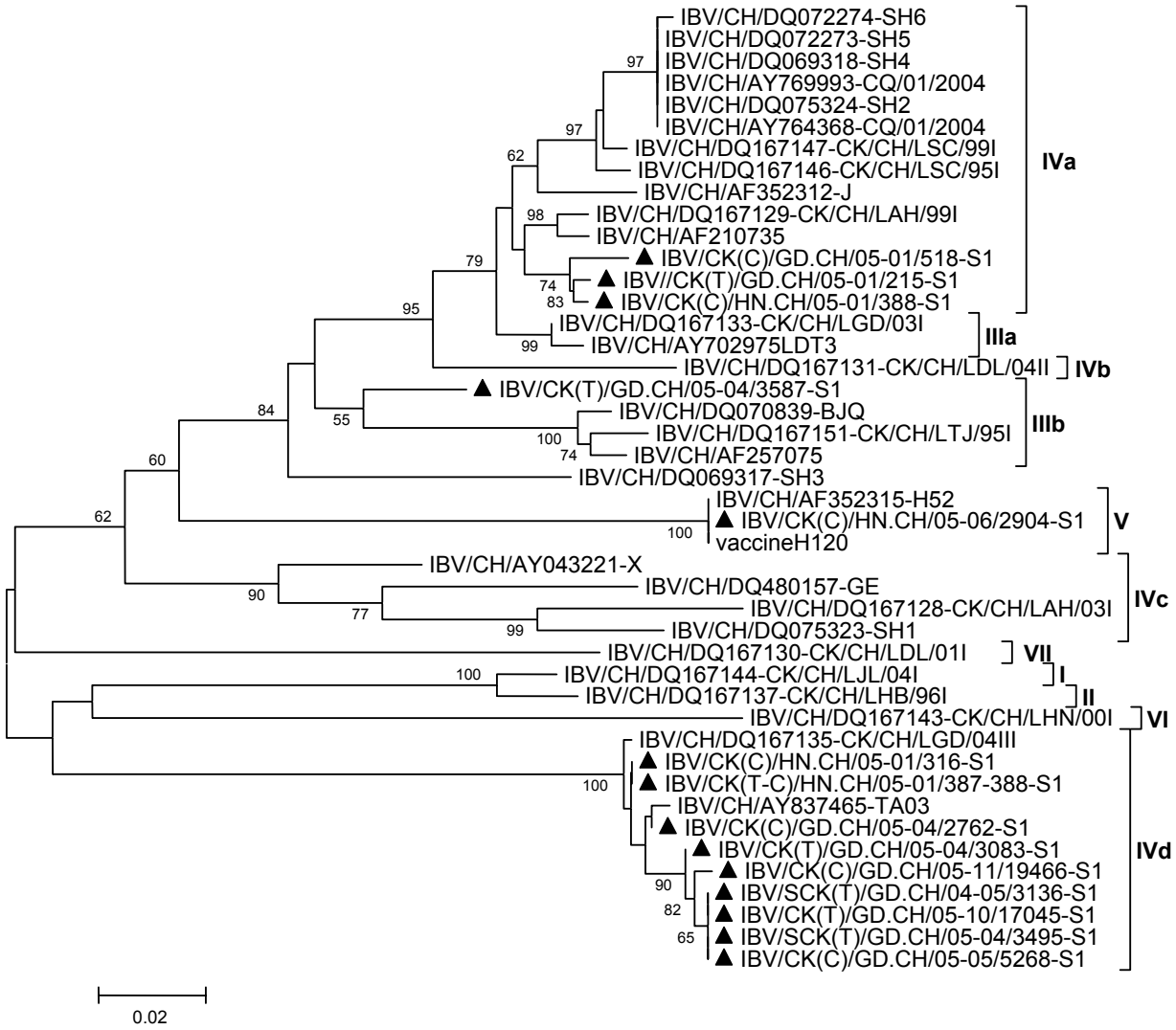

Fig. 1. Phylogenetic analysis of a 362 bp fragment of the spike 1 gene of IBV. Our 14 South-Eastern Chinese IBV strains are compared with 30 other Chinese strains whose sequences are available on GenBank. All Chinese genotypes III and IV strains were included while a single Chinese strain was randomly selected for genotypes I, II, VI and VII. Numbers at nodes correspond to bootstrap values >49. Closed symbols indicate strains reported in the present study. Genotypes were indicated on the right hand side with I, II, IIIa, IIIb, IVa, IVb, IVc, IVd, V, VI and VII.