

## A Massachusetts prototype like coronavirus isolated from wild peafowls is pathogenic to chickens

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### Abstract

Coronavirus infection was investigated in apparently healthy wild peafowls in Guangdong province of China in 2003, while severe acute respiratory syndrome (SARS) broke out there. No SARS-like coronavirus had been isolated but a novel avian coronavirus strain, Peafowl/GD/KQ6/2003 (KQ6), was identified. Sequence analysis revealed that KQ6 was an avian coronavirus infectious bronchitis virus (IBV), a member of coronavirus in group 3. The genome sequence of KQ6 had extremely high degree of identity with that of a Massachusetts prototype IBV M41. KQ6 was pathogenic to chickens but non-pathogenic to peafowls under experimental conditions. Seventeen out of fifty-four (31.48%) peafowl serum samples were tested positive for specific antibodies against IBV. Present results indicate that the peafowl isolate KQ6 is a Massachusetts prototype like coronavirus strain which undergoes few genetic changes and peafowl might have acted as a natural reservoir of IBV for very long time. © 2007 Elsevier B.V. All rights reserved.

**Keywords:** Coronavirus; Peafowl; Infectious bronchitis virus; Massachusetts

### 1. Introduction

Coronaviruses have a wide host range including avian and mammalian. The known coronavirus species are classified into three groups. The recently emerging agent for severe acute respiratory syndrome (SARS) has been identified as a new member of coronavirus in group 2. The exact origin of this novel coronavirus is still not clear, but current molecular epidemiological evidence favors an animal origin of the virus (Guan et al., 2003). So how coronavirus breaks the host species barrier, causes inter-species infection and becomes zoonotic would be interesting questions to be noticed in public. Infectious bronchitis virus (IBV) is the prototype member of coronavirus in group 3, which also consists of turkey coronavirus (TCoV), pheasant coronavirus (PhCoV), goose coronavirus (GCoV), duck coronavirus (DCoV) and pigeon coronavirus (PiCoV). Although chickens

were believed to be the only natural hosts for IBV, pheasants were also reported to be susceptible to IBV infection (Gough et al., 1996; Spackman and Cameron, 1983). In addition, it had been described that some galliform birds (guinea fowl, partridge and peafowl) and non-galliform birds (teal) were infected by coronaviruses that were very similar to IBV (Ito et al., 1991; Liu et al., 2005). Since IBV could infect some birds, there was a high possibility that IBV strains might be transmitted over long distances through the migration.

IBV is a major pathogen to chicken population, causing an acute and highly contagious disease that mainly affects the respiratory system or sometimes urogenital tracts (King and Cavanagh, 1991). Avian infectious bronchitis has caused severe economic losses to the poultry industry all over the world (Estola, 1966; Calnek, 1997). The genome of IBV is a single-stranded, positive-sense RNA ranging from 27 to 31 kb in size (Cavanagh, 1997). Gene 1 encodes many proteins associated with RNA replication and transcription. In addition to four structural protein genes (S, E, M, and N), IBV has two genes encoding four non-structural proteins (3a and 3b, 5a and 5b) (Cavanagh, 2005).

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In present study, coronavirus infection was investigated in apparently healthy wild peafowls in Guangdong province of China in 2003 during SARS broke out. Three hundred and fifty-one laryngotracheal swabs and 54 serum samples were collected. A new avian coronavirus strain, Peafowl/GD/KQ6/2003 (KQ6), was isolated and characterized in detail.

## 2. Materials and methods

### 2.1. Sample collection

Three hundred and fifty-one laryngotracheal swabs and 54 serum samples were collected from the apparently healthy wild peafowls of different age in Guangdong province in 2003. All peafowls were free of IBV vaccination. These samples were stored at  $-20^{\circ}\text{C}$ .

### 2.2. Virus propagation

Laryngotracheal swabs were treated in 0.1% PBS buffer containing antibiotics (penicillin and streptomycin, 2000 IU/ml) for 12 h at  $4^{\circ}\text{C}$ . Then 200  $\mu\text{l}$  of supernatant from the suspension was inoculated into the allantoic cavity of 11-day-old SPF embryonated chicken eggs. Five eggs were used for each sample. The inoculated eggs were incubated at  $37^{\circ}\text{C}$  and candled daily. Eggs died within 24 h of inoculation were discarded. At 2–3 days after inoculation, surviving eggs were placed at  $4^{\circ}\text{C}$  overnight, and the allantoic fluid was collected. Then three blind serial passages were performed and all the allantoic fluid was harvested for further studies.

### 2.3. Detection of coronavirus by RT-PCR

Viral RNA was extracted from 200  $\mu\text{l}$  of infectious allantoic fluid using Trizol reagents (GIBCO-BRL). The RNA pellet was resuspended in 10  $\mu\text{l}$  of DNase-free, RNase-free double-distilled water and was used as the template for RT-PCR. Reverse transcription was performed using the SuperScript II kit (Invitrogen, San Diego, CA, USA). Three pairs of primers were designed to detect coronavirus. The universal primers for coronavirus were P3 (5'-ACTCARWTRAATYTWAARTAYGC-3') and P4 (5'-TCACAYTTWGGATARTCCCA-3') (R = A/G, W = A/T, Y = T/C), the specific primers designed according to the conservative region of SARS virus polymerase 1b were R13 (5'-GCGCAAGTATTAAGTGAGATG-3') and R14 (5'-GAAGTGCATTTACATTGGCTG-3'), and the primers designed according to the 3'-UTR of IBV were UTR-1 (5'-CTAACTCTATACTAGCCTAT-3') and UTR-2 (5'-GGAAGATAGGCATGTAGCTT-3'). The cDNA of all specimens were amplified by PCR using these three pairs of primers, respectively.

### 2.4. Hemagglutination assay (HA)

The positive allantoic fluid was centrifuged at 4000 rpm for 5 min, and treated with equal volume of 1% trypsin for 3 h at  $37^{\circ}\text{C}$  before HA test by the commonly used procedures (Estola,

1966; Calnek, 1997). Trypsin-free allantoic fluid was used as negative control.

### 2.5. Embryo infection test

The positive allantoic fluid was inoculated into the allantoic cavity of 11-day-old SPF embryonated chicken eggs. The inoculated eggs continued to be incubated for 120 h at  $37^{\circ}\text{C}$  before evaluating embryo dwarfism. Inoculated eggs with an equal volume of PBS served as negative controls.

### 2.6. Virus interference test

The positive allantoic fluid was inoculated (0.1 ml/egg) into the allantoic cavity of 11-day-old SPF embryonated chicken eggs and incubated for 10 h at  $37^{\circ}\text{C}$ . The Newcastle disease virus (NDV) B1 strain was diluted to 1:10,000 with PBS and inoculated (0.1 ml/egg) into the same location where the peafowl virus strain was inoculated. Eggs were incubated for 48 h at  $37^{\circ}\text{C}$  before allantoic fluids were harvested and tested by a standard hemagglutination assay. Inoculated eggs with PBS or NDV B1 served as the negative and positive controls, respectively.

### 2.7. Neutralization test

Chicken-anti-IBV polyclonal serum (polyclonal antibodies against M41 strain, ELISA titer = 7988) and IBV-antibody-free serum were heat-inactivated for 30 min at  $56^{\circ}\text{C}$  before use in neutralization test. The positive allantoic fluid was 10-fold serially diluted ( $10^{-1}$ – $10^{-6}$ ) before mixing with an equal volume of chicken-anti-IBV polyclonal serum or IBV-antibody-free serum. The virus-serum mixtures were placed for 30 min at  $25^{\circ}\text{C}$  prior to inoculating embryonated eggs (0.2 ml/egg, seven eggs per group). The chicken-anti-IBV polyclonal serum inoculated group, IBV-antibody-free serum inoculated group and peafowl virus strain inoculated group were designated as the positive serum control, negative serum control, and virus control, respectively. After inoculation, eggs were continuously incubated for 7 days at  $37^{\circ}\text{C}$ . The EID<sub>50</sub> of the chicken-anti-IBV polyclonal serum and IBV-antibody-free serum inoculated groups were determined. A neutralization index was calculated from the EID<sub>50</sub> value as: EID<sub>50</sub> of chicken-anti-IBV polyclonal serum inoculated group/EID<sub>50</sub> of IBV-antibody-free serum inoculated group. A neutralization index greater than 50 was considered as Massachusetts serotype positive.

### 2.8. Pathogenicity in chickens and peafowls

Three groups of White Leghorn SPF chickens ( $n = 10$ ) and three groups of IBV negative peafowls ( $n = 5$ ) were housed in isolators. At 7 days old, one group of chickens and peafowls were inoculated intranasally with  $10^6$  EID<sub>50</sub> of the positive allantoic fluid, another group of chickens and peafowls were inoculated intranasally with  $10^6$  EID<sub>50</sub> of M41 strain, respectively. Some chickens and peafowls served as mock-infected controls with 0.1 ml of allantoic fluid from normal SPF eggs. Chickens and peafowls were examined daily for clinical signs for 14 days post-

Table 1  
Primer pairs for genomic amplification

No.	Primers (5'–3')		Size (bp)
1	1-F <sup>a</sup>	CAA GCC TAA AAC AGG GAG TAT C	940
2	2-F	GGC ATG CGA AAT GAC TTA AC	941
3	3-F	GAG AAA ATG TGT GAC GAC CT	1421
4	4-F	TGG ATG AGT TTA AAG AGT TCT G	988
5	5-F	ATG GTG TTA AAT ACC GCT C	800
6	6-F	GCA GAA CAC CTT AAT AAA CCT	677
7	7-F	GCC ATC TAT TAT GCG CAA	1034
8	8-F	TCC TAC TTG GTT TAA TAG AGA AA	962
9	9-F	GGC AAT GAG TTT GTT GGT AAT	926
10	10-F	TGC ATT ACA CAC TGG AAC TGA	1150
11	11-F	TTT GAC TAA GCT TAA TGT TGA A	1730
12	12-F	CAT ATT CAA CAG TTG TTT GGA	1628
13	13-F	AAT ACC TAC TAC GGA RAA AGA CC	741
14	14-F	CTT TAA GTA CTG GGA TCA AGA GTA	1324
15	15-F	GAT AAG AGT GTC GGC TAY CC	453
16	16-F	TGT TCW CAA CAY ACA ATG CTA GT	696
17	17-F	GTA TCT AAT GGT ACY GTT TTT GG	1298
18	18-F	GTC MTT TAT TAA TGG TAA GAT AAA	481
19	19-F	GTT CTT GTG GTT MTA GAG CTA CAA C	1326
20	20-F	AA Y ATT CGC ACA CTG CCA AA	871
21	21-F	TTG AAA ACT GAA CAA AAG ACA	1372
22	22-F	CAG TTT GTA GTT TCT GGT GG	840
23	23-F	TTG TCW AAA TGG TGG AAT GA	657
24	24-F	CCT AAG AAC GGT TGG AAT	632
25	25-F	TGA CCA ARG CGG AAA TAA GA	1290
26	26-F	GAT GGT ATA GTG TGG GTT	1644
27	27-F	GAT GAC AAG ATG AAT GAG GA	1010
28	5'RACE-F	CAT TTA GAA GAC ATC TTT GGT GTC	604
29	3'RACE-F	GGA AGA TAG GCA TGT AGC TT	332
	1-R <sup>b</sup>	GTT AAG TCA TTT CGC ATG CC	
	2-R	AGG TCG TCA CAC ATT TTC TC	
	3-R	CAG AAC TCT TTA AAC TCA TCC A	
	4-R	GAG CGG TAT TTA ACA CCA T	
	5-R	AGG TTT ATT AAG GTG TTC TG	
	6-R	TTG CGC ATA ATA GAT GGC	
	7-R	TTT CTC TAT TAA ACC AAG TAG GA	
	8-R	ATT ACC AAC AAA CTC ATT GCC	
	9-R	TCA GTT CCA GTG TGT AAT GCA	
	10-R	TTC AAC ATT AAG CTT AGT CAA A	
	11-R	TCC AAA CAA CTG TTG AAT ATG	
	12-R	GGT CTT TYT CCG TAG TAG GTA TT	
	13-R	TAC TCT TGA TCC CAG TAC TTA AAG	
	14-R	GGR TAG CCW GCA CTC TTA TC	
	15-R	ACT AGC ATT GTR TGT TGW GAA CA	
	16-R	CCA AAA ACR GTA CCA TTA GAT AC	
	17-R	TTT ATC TTA CCA TTA ATA AAK GAC	
	18-R	GTT GTA GCT CTA KAA CCA CAA GA	
	19-R	TTT GGC AGT GTG CGA ATR TT	
	20-R	TGT CTT TTG TTC AGT TTT CAA	
	21-R	CAT AAC TAA CAT AAG GGC AAT	
	22-R	TCA TTC CAC CAT TTW GAC AA	
	23-R	ATT CCA ACC GTT CTT AGG	
	24-R	TAC TCT CTA CAC ACA CAC	
	25-R	AAT GAA RTC CCA ACG GAA AT	
	26-R	CTC CTC ATT CAT CTT GTC	
	27-R	AAG CTA CAT GCC TAT CTT CC	
	5'RACE-R	TGT GAC GTA TAG AAA AAC AAA GCG TCA C	
	3'RACE-R	CTG ATC TAG AGG TAC CGG ATC C	

<sup>a</sup> F represents forward primer.

<sup>b</sup> R represents reverse primer.

inoculation. Necropsies were performed immediately after death and the infected birds were sacrificed by intravenous injection with barbiturate for examination of gross lesions. Lung, trachea, kidney and proventriculus were collected. These tissue samples were fixed with 10% neutral buffered formalin for 7 days. The fixed tissues were then cut into four pieces and immersed in 98% formic acid for 1 h before routine processing and paraffin wax embedding. Five-micrometer thick sections were cut and stained with hematoxylin and eosin for routine histopathological examination.

## 2.9. Genome sequencing

Twenty-nine pairs of primers (Table 1) were designed for the amplification, cloning, and sequencing of genomic segments of the peafowl coronavirus. Reverse transcription-polymerase chain reaction (RT-PCR) was used to amplify the genomic segments. Viral RNA was extracted from 200 µl of infectious allantoic fluid using Trizol reagents (GIBCO-BRL), then reverse transcription, 3'-RACE and 5'-RACE were performed. PCR products were excised from 1.0% agarose gels, and purified using the gel extraction kit (Omega Bio-tek, USA). The purified PCR products were cloned into the pMD18-T Vector (TaKaRa). Plasmid DNA for sequencing was prepared with the plasmid mini kit (Omega Bio-tek). Sequencing of three independent clones of each PCR product was performed in order

to eliminate errors resulting from the RT-PCR or cloning steps. Genomic segments were sequenced by Shanghai BioAsia Biological Technology Co., Ltd.

## 2.10. Nucleotide sequence accession numbers

The GenBank accession number of the sequence reported in this paper is AY641576.

## 2.11. Sequence analysis

The sequence lengths of the whole genome, 5'-UTR, 3'-UTR and protein1ab, S, 3a, 3b, E, M, 5a, 5b and N of the peafowl isolate were compared with those of other avian coronavirus isolates available in the GenBank database. These viruses include the partridge IBV strain partridge/GD/S14/2003 (S14, AY646283), the peafowl IBV strain Peafowl/China/LKQ3/2003 (LKQ3, AY702085), and the avian IBV strains M41 (AY851295), Beaudette (NC\_001451) and BJ (AY319651).

The nucleotide and amino acid sequences of three main structural protein genes S1, M and N of the peafowl IBV were compared with those of LKQ3 and reference avian IBV strains with MegAlign software (DNASTar 5.01). Four types of IBVs (respiratory, respiratory mutant, nephrogenic and proventriculosis) were selected for reference strains. These reference

strains were IBVs isolated from chickens in China and two M41 serotype IBV vaccine strains, M41 and H120, which had been widely used to control avian infectious bronchitis for many years in China. The sequences of reference IBV strains were obtained from GenBank.

To reveal the relationships between the peafowl isolate and other coronaviruses at the molecular level, the whole genome nucleotide sequences were compared with those of representative animals and human coronaviruses available in the GenBank database, including BCoV (bovine coronavirus, NC\_003045), HCoV-229E (human coronavirus 229E, NC\_002645), IBV (infectious bronchitis virus M41, AY851295), MHV (murine hepatitis virus strain A59, NC\_001846), PEDV (porcine epidemic diarrhea virus, NC\_003436), TGEV (transmissible gastroenteritis virus, NC\_002306) and SARS-CoV (severe acute respiratory syndrome coronavirus, NC\_004718). The radialized phylogenetic tree was drawn with the MEGA 3.1 program by using neighbor-joining analysis.

### 2.12. Serological survey

Since IBV was isolated from peafowls, it would be possible that peafowls could carry anti-IBV antibodies. To investigate into the IBV infections in peafowls, we examined the serum samples collected from the wild peafowls in 2003 for the presence of anti-IBV antibodies using an ELISA kit per manufacturer's instruction (IDEXX, Spain).

## 3. Results

### 3.1. A novel IBV strain from peafowls was biologically characterized

Only 1 sample from 351 laryngotracheal swabs was coronavirus positive and confirmed as IBV rather than SARS virus. No specific PCR products were detected from the rest of examined samples. This isolate from peafowl was named as Peafowl/GD/KQ6/2003 (KQ6). For further understanding of KQ6 strain, biological characteristics of KQ6 were determined systematically. In the HA test, the HA titer of KQ6 was greater than 64, whereas no detectable HA titer was observed in the non-treated control samples, which indicated that trypsinized virus was able to hemagglutinate chicken red blood cells as observed in many other strains of IBVs. In the embryo infection test, all of the embryos showed dwarfism post-infection with the peafowl isolate, losing at least 2 g of body weight compared with the negative controls. This observation clearly demonstrated that KQ6 was able to inhibit the growth of chicken embryos as many other IBV strains did. To further characterize the isolate, the virus interference test was performed. It was found that the reciprocal HA titers of KQ6+NDV B1 infected eggs were lower than 20, while those infected with NDV B1 only were greater than 256 and no HA titers in PBS inoculated controls, which clearly demonstrated the interference of the isolate to the growth of NDV. Moreover, we performed the neutralization test for further identification of the isolate. It was found that the EID<sub>50</sub> of chicken-anti-IBV serum-

treated group was 10<sup>-2.7</sup>, while the EID<sub>50</sub> of IBV-antibody-free serum-treated group was 10<sup>-4.5</sup>, giving out the neutralization index of 63.09. It indicated that the peafowl isolate KQ6 was Massachusetts serotype. These results showed that no SARS-like coronavirus had been isolated but a novel IBV strain with the typical biological characteristics was identified from peafowls.

### 3.2. IBV isolated from peafowl was pathogenic to chickens

Clinical signs were observed in all KQ6 and M41 infected chickens 5–14 days post-infection. These clinical signs included listlessness, huddling, dark shrunken combs and ruffled feathers. During the experiment, 3 of 10 chickens in M41 infected group died, however all chickens in KQ6 infected group survived. Gross lesions in the organs of the dead M41 infected chickens were primarily confined to the lungs with haemorrhagic changes. Microscopic examination demonstrated that there were severe haemorrhagic changes, epithelium degeneration and lymphocyte infiltration in the lungs of some M41 infected chickens, while the similar pathological changes were observed in both lungs and kidneys from some KQ6 infected chickens (Fig. 1). In contrast, mock-infected chickens showed no significant changes in the lung, kidney, trachea and proventriculus when examined microscopically.

### 3.3. IBV could be carried in peafowls causing no clinical sign

ELISA kit (IDEXX) was used to measure the titers of anti-IBV antibodies from the peafowl serum in nature infection. It was found that 17 out of 54 serum samples were anti-IBV positive (ELISA titer  $\geq$  227), which indicated that about one-third (31.48%) of peafowls carried IBV in Guangdong province.

Experimental infection was performed to evaluate the virulence of KQ6 in IBV negative peafowl. Interestingly, no peafowls inoculated with KQ6 or M41 strain displayed clinical signs and lesions, while the specific antibodies could be detected post-infection (data not shown).

### 3.4. Genome characterization

The genome of KQ6 was 27,434 bp in length, including the poly-(A) tail. As many IBVs, gene 1 of KQ6 accounted for approximately two-third of the genome. KQ6 also had four structural protein genes (S, E, M, and N) and two genes encoding four non-structural proteins (3a and 3b, 5a and 5b). Its genome organization was 5'-Gene1-S-3a-3b-E-M-5a-5b-N-3', which was typical for IBV. As shown in Table 2, there was a great diversity in the genome of different avian coronavirus strains in terms of the genome size (27,434–27,733 bp), 5'-UTR size (330–529 bp) and 3'-UTR size (322–521 bp), as well as the amino acid numbers of protein 1ab (6610–6641 amino acids), S (1153–1169 amino acids), 3b (62–64 amino acids), E (108–109 amino acids), M (225–226 amino acids) and 5b (81–82 amino acids). However, the amino acid numbers of protein 3a, 5a and N were invariable.

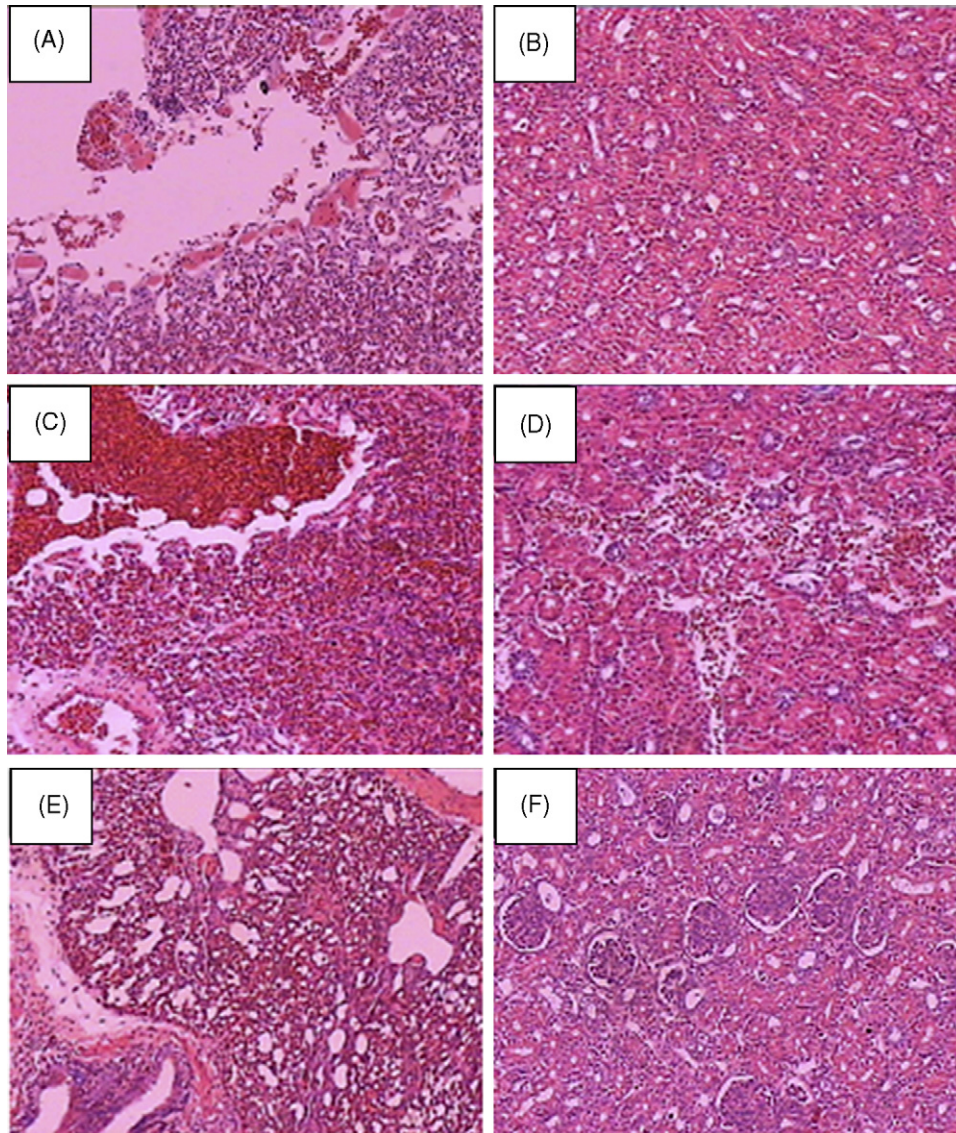


Fig. 1. Hematoxylin- and eosin-stained sections of lung and kidney from M41, KQ6 and mock-infected chickens. (A), (C) and (E) were lung sections of M41, KQ6 and mock infected chickens, respectively. (B), (D) and (F) were kidney sections of M41, KQ6 and mock-infected chickens, respectively. Severe haemorrhagic lesions, epithelium degeneration and lymphocyte infiltration were limited in lungs from some M41 infected chickens, while these changes were observed in both lungs and kidneys from some KQ6 infected chickens (original magnification  $\times 100$ ).

Table 2  
Genome size comparison among KQ6 and other coronavirus isolates

Strains	Accession numbers	Host	Genome (bp)	5'-UTR (bp)	3'-UTR (bp)	Numbers of amino acids (aa)								
						1ab	S	3a	3b	E	M	5a	5b	N
M41	AY851295	Chicken	27,475	528	322	6,631	1,162	57	64	109	225	65	82	409
Beaudette	NC_001451	Chicken	27,608	528	506	6,629	1,162	57	64	108	225	65	82	409
BJ	AY319651	Chicken	27,733	529	521	6,641	1,169	57	62	109	226	65	81	409
S14 <sup>a</sup>	AY646283	Partridge	27,503	330	518	6,639	1,165	57	62	108	225	65	82	409
LKQ3 <sup>b</sup>	AY702085	Peafowl	– <sup>c</sup>	–	–	–	1,162	57	64	108	226	65	82	409
KQ6	AY641576	Peafowl	27,434	528	335	6,610	1,153	57	64	109	225	65	82	409

<sup>a</sup> Partridge/GD/S14/2003.

<sup>b</sup> Peafowl/China/LKQ3/2003.

<sup>c</sup> Not available.

Table 3  
Sequence similarity comparison between KQ6 and other avian IBVs

Strains	Accession numbers			Types	Percent similarity (%)					
	S1	M	N		S1		M		N	
					nt	aa	nt	aa	nt	aa
M41	M21883	AF286184	M28566	Respiratory	99.6	98.9	99.9	99.6	95.2	95.6
GX1-98	AY319302	AY325727	AY278110	Respiratory	98.0	96.1	99.0	99.1	94.3	96.3
LKQ3	AY702085	AY702085	AY702085	– <sup>a</sup>	97.5	95.7	99.9	100	96.3	97.1
H120	M21970	AY028295	AY028296	Respiratory	97.4	95.9	96.0	99.1	93.8	95.4
HaN1-95	AY251817	AY325734	–	Respiratory	97.1	95.5	90.3	94.2	–	–
CU-T2	U04739	U46035	U04805	Respiratory mutant	77.0	74.9	85.3	81.8	91.9	93.2
JX1-99	AF210735	–	–	Nephrogenic	80.1	78.6	–	–	–	–
Gray	L14069	AF286180	S48137	Nephrogenic	79.5	78.0	89.3	94.6	93.7	94.9
BJ	AY319651	AY319651	AY319651	Nephrogenic	74.3	73.6	85.5	91.6	87.7	91.9
LX4	AY189157	AY326960	AY338732	Proventriculus	74.9	74.7	89.1	91.1	86.7	90.2
ZJ971	AF352311	–	AF352308	Proventriculus	97.1	94.8	–	–	93.4	93.9

<sup>a</sup> Not available.

The S1 subunit was identified as the major target of neutralizing antibodies and as the determinant gene altering cell tropism (Casais et al., 2003; Cavanagh et al., 1988; Ignjatovic and Galli, 1994). The S1 gene of KQ6 strain was 1611 bp in length with a G + C percentage of 35.88%, encoding 537 amino acids that contain 18 potential *N*-glycosylation sites. Hydrophilicity analysis of the deduced S1 amino acid sequence revealed that the first 20 amino acid residues formed a hydrophobic region that was presumed to be the signal sequence of S precursor protein. The amino acid sequences at the cleavage site of S precursor protein were RRFR.

The M gene was 678 bp in length, encoding 225 amino acids. There were two predicted *N*-glycosylation sites at the N-terminus. The first 11 amino acid residues were hydrophilic, forming the outer membrane sequence of the M protein. The subsequent 20–100 amino acids formed three hydrophobic regions which were thought to be three transmembrane structures.

The N gene was 1230 bp in length and encoded 409 amino acids, with a single predicted *N*-glycosylation site at the N-terminus. The deduced amino acid sequence of the N protein was hydrophilic, which was likely beneficial for the interaction between N protein and genomic RNA.

### 3.5. Phylogenetic analysis

The nucleotide and amino acid sequences of the S1, M and N genes of KQ6 were compared with those of reference avian IBV strains (Table 3). It showed that KQ6 was most closely related to Massachusetts serotype IBV strains, such as M41, GX1-98, LKQ3, H120, and so on. Interestingly, only 0.4% differences at nucleotide level and 1.1% differences at amino acid level were found when compared the S1 genes of KQ6 and M41.

Phylogenetic analysis based on the whole genome sequence was performed. As shown in Fig. 2, all coronaviruses were clearly divided into three groups. KQ6 and IBV (M41) were in the same cluster of group 3, and they were distant from SARS coronaviruses in group 2, which further confirmed that KQ6 was a member of IBV.

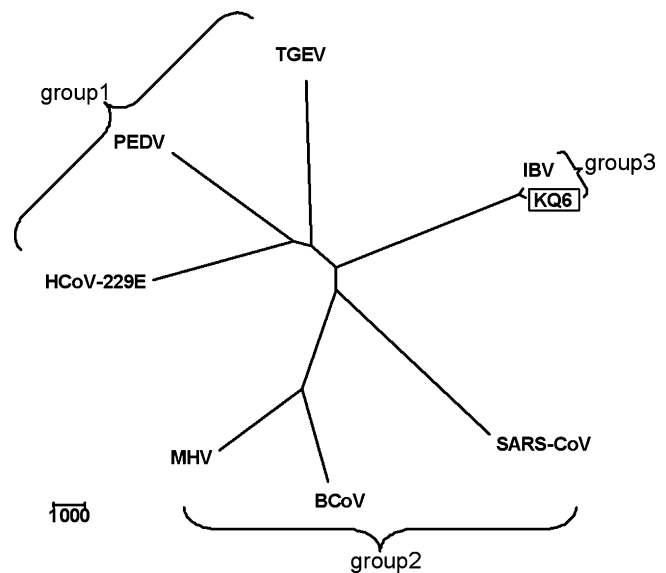


Fig. 2. Radialized phylogenetic tree for coronaviruses. The nucleotide sequences were analyzed with MEGA program (version 3.1) by using neighbor-joining analysis. The full-length genome sequences were used for the phylogenetic analysis. Scale bar: 1000 nucleotide changes. BCoV: bovine coronavirus; HCoV-229E: human coronavirus 229E; IBV: infectious bronchitis virus M41; MHV: murine hepatitis virus strain A59; PEDV: porcine epidemic diarrhea virus; TGEV: transmissible gastroenteritis virus; SARS-CoV: severe acute respiratory syndrome.

## 4. Discussion and conclusions

IBV is not a simple pathogen because of its heterogeneity with respect to its protein sequences, broad tissue tropism and pathogenicity in the wide host range. It has been shown that an avian coronavirus from one species can replicate in other species, but no clinical sign is observed in most cases (Lister et al., 1985; Ismail et al., 2003; Guy, 2000). It was not until 2005 that peafowl infected by IBV (LKQ3) was first reported in Guangdong province, Southern China (Liu et al., 2005). LKQ3 shared high degree of identity with the Massachusetts serotype IBV strains H120. But KQ6 was different from LKQ3 in terms

of its high sequence identity with M41 and its pathogenicity to chickens.

It is well known that IBV has a high error rate during the transcription of its genomes (Lai and Cavanagh, 1997) because its RNA polymerase lacks the 3'–5' exonuclease activity (editing function) of DNA polymerase. If point mutations, insertions, deletions or RNA recombinations occur in the antigenic S protein, the characters of antigen will change and a new type of virus might be born (Jia et al., 1995; Wang et al., 1993, 1994). Interestingly, the S1 gene of KQ6 had extremely high degree of identity with that of the Massachusetts prototype IBV, M41, which has undergone countless number of in vivo and in vitro host passages since 1940s. Only 0.4% differences at nucleotide level and 1.1% differences at amino acid level were found in their S1 genes. There were two explanations of their high degree of sequence identity. One was that propensity of frequent mutation was not necessarily intrinsic to all IBV strains, such as the Massachusetts serotype virus. It had been reported that a difference of only 2% in nucleotide sequence and 4% in amino acid sequence was found in the S1 genes of a Massachusetts serotype virus of the 1940s and the present-day Massachusetts prototype M41 strain (Jia et al., 2002). The other explanation was that IBV in peafowls had not undergone the immune pressure from vaccine like chickens. So the point mutations of IBV in peafowls were much less than those in other species. This high degree of sequence identity showed that KQ6 was a Massachusetts prototype like IBV strain which had undergone few genetic changes.

Although LKQ3 and KQ6 had high sequence identity and both belonged to Massachusetts serotype IBV strains, the results of their virulence studies in chickens were completely different. No overt disease was observed in chickens that had been inoculated with the first reported peafowl IBV strain LKQ3 (Liu et al., 2005). In contrast, KQ6 strain was able to cause clinical signs and pathogenic changes in chickens. Severe haemorrhagic lesions, epithelium degeneration and lymphocyte infiltration were observed in both lungs and kidneys of the KQ6 infected chickens. It was possible that the different experiment procedure, different environment and different criterion in the virulence studies might generate different experiment results, but we believed that KQ6 was pathogenic to chickens. Meanwhile, we found that KQ6 was non-pathogenic to peafowls.

We also found one-third of peafowls were anti-IBV positive in Guangdong province. It suggested that peafowl was susceptible to IBV infection and IBVs other than those previously reported in chickens and turkeys might be carried in peafowls causing no clinical sign. Up to date, there has been no more data about IBV infection in peafowls, so the exact origin of peafowl IBV is still not clear. Liu et al. speculated that LKQ3 might originate from the attenuated vaccine strain IBV H120. But peafowls, unlike many other birds on farms, are not immunized with vaccines in China and peafowls in nature are unlikely to be infected by IBV vaccine strain through direct contacting with immunized chickens. So we believed that peafowl was the natural carrier for IBV.

Since coronavirus can break the host species barrier, causes trans-species infection and becomes zoonotic, it is necessary to

pay attention to the coronavirus IBV infection in other species. KQ6 was pathogenic to chickens under experimental conditions, so the peafowl IBV had the potential ability to infect chickens in nature. Our investigations in peafowls indicate that KQ6 is a Massachusetts prototype like coronavirus strain which undergoes few genetic changes and peafowl might have acted as a natural reservoir of IBV for very long time.

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