

Isolation of avian infectious bronchitis coronavirus from domestic peafowl (*Pavo cristatus*) and teal (*Anas*)

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Coronavirus-like viruses, designated peafowl/China/LKQ3/2003 (pf/CH/LKQ3/03) and teal/China/LDT3/2003 (tl/CH/LDT3/03), were isolated from a peafowl and a teal during virological surveillance in Guangdong province, China. Partial genomic sequence analysis showed that these isolates had the S–3–M–5–N gene order that is typical of avian coronaviruses. The spike, membrane and nucleocapsid protein genes of pf/CH/LKQ3/03 had >99% identity to those of the avian infectious bronchitis coronavirus H120 vaccine strain (Massachusetts serotype) and other Massachusetts serotype isolates. Furthermore, when pf/CH/LKQ3/03 was inoculated experimentally into chickens (specific-pathogen-free), no disease signs were apparent. tl/CH/LDT3/03 had a spike protein gene with 95% identity to that of a Chinese infectious bronchitis virus (IBV) isolate, although more extensive sequencing revealed the possibility that this strain may have undergone recombination. When inoculated into chickens, tl/CH/LDT3/03 resulted in the death of birds from nephritis. Taken together, this information suggests that pf/CH/LKQ3/03 might be a revertant, attenuated vaccine IBV strain, whereas tl/CH/LDT3/03 is a nephropathogenic field IBV strain, generated through recombination. The replication and non-pathogenic nature of IBV in domestic peafowl and teal under field conditions raises questions as to the role of these hosts as carriers of IBV and the potential that they may have to transmit virus to susceptible chicken populations.

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INTRODUCTION

Coronaviruses (family *Coronaviridae*) belong to the order Nidovirales and contain a positive-stranded RNA genome that ranges from 27 to 31 kb in size (Cavanagh, 1997). Members of the family *Coronaviridae* infect a wide range of hosts and have been classified into three groups on the basis of antigenicity, genome organization and sequence similarity. Usually, coronaviruses infect only their normal target host species. It has, however, been reported that some strains of canine coronavirus and human coronavirus 229E can infect other, non-target species without causing disease (Barlough *et al.*, 1984, 1985). The recent emergence of severe acute respiratory syndrome coronavirus (SARS-CoV), which has been classified tentatively into group 2, has focused a great deal of interest on this virus family (Holmes, 2003). It was reported that SARS-CoV-like viruses

were isolated from Himalayan palm civets (Guan *et al.*, 2003) and ferrets (*Mustela furo*). Moreover, domestic cats (*Felis domesticus*) are susceptible to infection by SARS-CoV, suggesting that the reservoir for this pathogen might involve a range of animal species (Martina *et al.*, 2003).

Infectious bronchitis virus (IBV), together with genetically related coronaviruses of turkey and pheasant, belongs to the group 3 coronaviruses. IBV is a pleomorphic, enveloped virus with club-shaped surface projections (spikes) and a single-stranded, positive-sense RNA genome of >27 kb in length (Bournsnel *et al.*, 1987). Upon virus entry into cells, a 3'-coterminal nested set of six mRNAs is produced. About 74% of the genome at the 5' end encodes two overlapping replicase genes that are expressed from the genomic RNA, or mRNA1, in the form of polyproteins 1a and 1a/b. The four structural proteins, the spike (S) glycoprotein, the envelope (E) glycoprotein, the membrane (M) glycoprotein and the nucleocapsid (N) protein, are encoded by subgenomic mRNAs (sgRNAs) 2, 3, 4 and 6, respectively (Stern & Sefton, 1982; Lai & Cavanagh, 1997). Four small,

The GenBank/EMBL/DDBJ accession numbers for the sequences of isolates pf/CH/LKQ3/03 and tl/CH/LDT3/03 are AY702085 and AY702975, respectively.

non-structural proteins, 3a, 3b, 5a and 5b, are also encoded by sgRNAs 3 and 5. One of the distinguishing features of group 3 coronaviruses is that the third open reading frame (ORF) (3c) of the tricistronic mRNA 3 encodes the E protein, which, together with the M protein, plays an essential role in virus-particle assembly (Bos *et al.*, 1996; Vennema *et al.*, 1996).

With the exception of isolates from chicken, turkey and pheasant, only a small amount of experimental work has been carried out to study the host range of avian coronaviruses. On the basis of antigenicity and partial sequence data from isolated viruses, there are at least two additional avian species (pigeons and guinea fowl) that are susceptible to IBV-like coronaviruses (Barr *et al.*, 1988; Ito *et al.*, 1991). From both practical and academic viewpoints, it is important to understand the extent to which an avian coronavirus from one species can replicate in another. Although it has been shown that an avian coronavirus from one species can replicate in other avian species, no clinical signs are observed in most instances (Lister *et al.*, 1985; Guy, 2000; Ismail *et al.*, 2003).

In order to investigate the extent to which coronaviruses can replicate in bird species beside chickens, turkeys, pheasants, pigeons and guinea fowl, 55 specimens from four avian species were collected from apparently healthy domestic bird flocks in Guangdong province, China, in 2003. These specimens were tested for the presence of coronaviruses by using RT-PCR (Stephensen *et al.*, 1999) and virus isolation in 9- to 11-day-old specific-pathogen-free (SPF) chicken embryos. This report describes the isolation and characterization of two avian coronaviruses that were isolated by using these protocols.

METHODS

Sampling. Fifty-five lung and heart specimens from four avian species [20 from pheasant (*Phasianus colchicus*), two from peacock (*Pavo cristatus*), three from sheldrake (*Tadorna*) and 30 from teal (*Anas*)] were collected from apparently healthy domestic bird flocks in Guangdong province, China, in 2003. These avian species had not been immunized with any IBV vaccines. The heart and lung tissue samples from each bird were pooled and homogenized and 10% (w/v) suspensions were made in 0.1% PBS without calcium or magnesium.

Detection of coronaviruses by RT-PCR. Total RNA was prepared by mixing 500 µl tissue suspension, 100 µl 10 mM EDTA (Gibco-BRL), 100 µl 10% SDS (Gibco-BRL) and 15 µl proteinase K (20 mg ml⁻¹; TaKaRa). This mixture was incubated for 1 h at 55 °C. RNA was extracted by using TRIzol reagent (Gibco-BRL) and isolated according to the protocol of the manufacturer. The RNA was air-dried for 2–10 min, redissolved in 15 µl RNase-free water and used immediately or stored at –70 °C.

Oligonucleotide 4Bm, 5'-TCACA(C/T)TT(A/T)GGATA(G/A)TCC-CA-3', was used for reverse transcription and cDNA was synthesized in 20 µl reaction mixtures, as described by Stephensen *et al.* (1999).

For detection purposes, a genome-sense oligonucleotide (2Bp), 5'-ACTCA(A/G)(A/T)T(A/G)AAT(T/C)TNAATA(T/C)GC-3', and an

antigenome-sense oligonucleotide (4Bm), designed to amplify a 250 bp replicase gene fragment from most coronaviruses (Stephensen *et al.*, 1999), were used in PCRs. Specimens of cDNA were amplified by PCR as described by Stephensen *et al.* (1999).

Cloning, sequencing and analysis of PCR products. PCR products were cut from 1.0% agarose gels and purified by using an agarose gel DNA extraction kit (Boehringer Mannheim). Purified PCR products were cloned into the TA cloning vector (TaKaRa). Sequencing of three clones of each PCR product was performed with the M13 forward and reverse primers. The BLAST program (Altschul *et al.*, 1990) was used to search GenBank for homologous gene sequences. Nucleotide sequences of PCR products were aligned and compared with six strains of avian coronaviruses and seven strains of mammalian and human coronaviruses by using the MEGALIGN program (DNASStar).

Virus isolation and propagation. A domestic peafowl specimen and a domestic teal specimen that were coronavirus-positive by RT-PCR were used for virus isolation. Homogenized tissue samples, supplemented with 100 U penicillin and 100 µg streptomycin ml⁻¹, were used for this isolation. After 12 h at 4 °C, 200 µl aliquots of the homogenates were inoculated into the allantoic cavity of 9- to 11-day-old SPF embryonated chicken eggs. Five eggs were used for each sample. The inoculated eggs were incubated at 37 °C and candled daily to check for embryo viability. Five blind serial passages were performed in a similar fashion. All of the allantoic fluids of inoculated eggs were harvested and tested for the presence of IBV by using electron microscopy (EM).

EM. For virological investigation, samples of allantoic fluids were submitted for EM examination. After low-speed centrifugation at 1500 g for 10 min (AllegraTM 21R centrifuge; Beckman), 1.5 ml clarified allantoic fluid was centrifuged at 12 000 g for 30 min. The resulting pellet was resuspended in a minimal volume of deionized water and examined by negative-contrast EM (JEM-1200-EX; JEOL).

RNA extraction and partial genome 'walking' by RT-PCR. RNA was extracted from EM-confirmed virus-positive allantoic fluid samples by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The RNA was air-dried for 2–10 min, redissolved in 30 µl RNase-free water and stored at –70 °C until used.

Reverse transcription was carried out in a 40 µl reaction mixture containing 20 µl RNA by using a consensus IBV oligonucleotide [N(–), 5'-TGTACCCTCGATCGTACTCCGCGT-3'] specific for the 3' untranslated region (UTR), as well as random hexamers (Liu & Kong, 2004).

The N gene was used as the start point for partial sequencing of the viral genomes. Two primers, N(–) and N(+), 5'-GACGCCCC-AGCGCCAGTCATATAA-3', were designed from the consensus sequence of the IBV N gene. These primers amplify a fragment approximately 1600 bp in size. By using an additional set of four primer pairs (Table 1), 'targeted gene-walking PCR' (Parker *et al.*, 1991; Chang *et al.*, 2001) was used to amplify further viral RT-PCR products. The general conditions for RT-PCR have been described previously (Liu & Kong, 2004).

Cloning, sequencing and sequence analysis. PCR products were excised from 1.0% agarose gels and purified by using an agarose gel DNA extraction kit (Boehringer Mannheim). Purified PCR products were cloned into a TA cloning vector (TaKaRa) by following the manufacturer's instructions or cloned as described previously (Liu & Kong, 2004). Three independent clones of each PCR product were sequenced by using M13 sequencing primers. Sequences were compiled and ORFs were predicted by using the Gene Runner program, version 3.00 (<http://www.generunner.com>). The BLAST program was used to search GenBank for homologous

Table 1. Sequence and position of the oligonucleotides used for gene-walking RT-PCR

Name*	Sequence (5'→3')	Size (bp)†	Position in IBV genome‡
PM(K+)	GCTTTTGCCACTATTATCTTCATCTT	2287	23668–23693
PM(D+)	GTTTCCTAAGAACGGTTGGAATAA	1497	24458–24481
PM(K/D-)	CGACTTTAGGTGGTTTTGGTCCTCC		25930–25954
PE(K+)	TGGTCATATGCAGGAAGGTTTTAGA	1141	22779–22803
PE(K-)	TTAGTACAAGTTTACACCAAAGCAA		23895–23919
PE(D+)	TCATATGCAGGAAGGTTTTAGAAGT	1918	22782–22806
PE(D-)	TACTGCAATGTTAAGGGGCCAAAAG		24675–24699
PS2(K/D+)	TAATTTTGAATGTGGACTGT	1331	21540–21559
PS2(K/D-)	TTTCAGTAAGAATAGCACTC		22851–22870
PS1(K+)§	TGAAAACCTGAACAAAAGACA	1284	20302–20321
PS1(D+)	CCCAATTTGAAAACCTGAACA	1288	20298–20314
PS1(K/D-)	GCCACCGCTCTTAGTAAC		21568–21585

*D, tl/CH/LDT3/03; K, pf/CH/LKQ3/03; +, genome-sense oligonucleotides; -, antigenome-sense oligonucleotides.

†Predicted from the sequence.

‡Relative to the genome of IBV Beaudette strain.

§PS1(K+) = S1Oligo5' (Kwon *et al.*, 1993).

||PS1(D+) = Suni2+ (Adzhar *et al.*, 1997).

gene sequences (Altschul *et al.*, 1990) and multiple alignments and phylogenetic trees were made by using the MEGALIGN program (DNASStar).

Nucleotide and amino acid sequences of the S1 part of the S protein gene of the two coronavirus isolates were assembled, aligned and compared with other reference IBV strains and turkey coronavirus by using the MEGALIGN program (DNASStar). The sequences used for comparison and phylogenetic analysis in the present study were obtained from GenBank. Reference IBV strains were mainly isolated in China, because the two avian coronavirus strains in the present study were isolated in China and we wanted to determine the relationships between the two isolates and the China IBV strains. The accession numbers of these IBV isolates are shown in Fig. 1. Several IBV vaccine strains, H52, H120, Ma5, 4/91, D41 and W93, were also compared with the two isolates, because these vaccines were used widely for many years on poultry farms in China. In addition, two strains of turkey coronaviruses, Gh and G1, were used.

In addition, the nucleotide and amino acid sequences of the M and N protein genes of the two coronavirus isolates were assembled, aligned and compared with other reference IBV strains and turkey coronaviruses by using the MEGALIGN program (DNASStar). The sequences used for comparison in the present study were also obtained from GenBank. The accession numbers for the M genes of IBV and turkey coronaviruses were: BJ, AY319651; LX4, AY326960; SAIB14, AY302744; QXIBV, AF221667; M41, AF286184; H52, AF286185; Connecticut, AF286182; and Gray, AF363607. The accession numbers for the N genes of IBV and turkey coronaviruses were: BJ, AY319651; LX4, AY338732; SAIB14, AY121091; X, AY043315; ZJ971, AF352308; Beaudette, AJ311362; H52, AF352310; N, AF352309; N1/88, U52599; Q3/88, U52600; V18/91, U52601; Vic S, U52594; NC95, AF111997; Indiana, AF111995; and Minnesota, AF111996.

Virulence studies in chickens. Three groups of 10 White Leghorn SPF chickens (Harbin Veterinary Research Institute, China) were housed in isolators under negative pressure. At 15 days of age, groups of 10 chickens were inoculated intranasally with either pf/CH/LKQ3/03 or tl/CH/LDT3/03 ($1 \times 10^{4.5}$ and $1 \times 10^{5.0}$ median

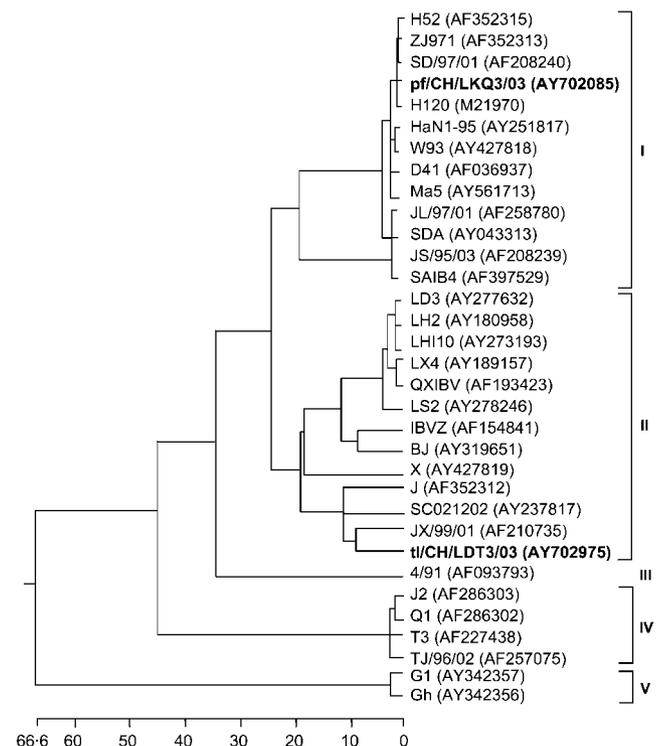


Fig. 1. Phylogenetic relationships, based on the S1 part of S protein gene sequences, of the isolates pf/CH/LKQ3/03 and tl/CH/LDT3/03, reference IBV strains and two turkey coronaviruses (the first 1686 nt, starting at the AUG translation start codon, of the S protein genes) using the MEGALIGN program in DNASStar with the Jotun Hein method (Higgins & Sharp, 1988). GenBank accession numbers are shown in parentheses.

embryo infectious doses per chick, respectively). The chickens in the remaining group were mock-inoculated with sterile allantoic fluid and served as a control. The chicks were examined daily for signs of infection for 30 days post-inoculation.

RESULTS

Detection of coronavirus by RT-PCR

Two of the 55 collected tissue specimens were coronavirus-positive by RT-PCR. The two positive samples originated from a peafowl and a teal. The other specimens produced no specific PCR products.

The specificity of the RT-PCR was confirmed by cloning and sequencing of the PCR products. The two RT-PCR products showed high sequence similarity to each other and to other avian coronaviruses (IBV and turkey coronavirus). Lower similarities were seen between the two products and canine coronavirus, murine hepatitis virus and other coronaviruses, including SARS-CoV. The amplified products had 90% gene 1 nucleotide sequence identity with the corresponding gene from IBV and turkey coronavirus, but <71% identity with the corresponding gene from other coronaviruses.

Virus characterization

Virus isolation from the two RT-PCR-positive samples was attempted by inoculation of embryonated chicken eggs. By the third passage, dwarfing, stunting, curling and embryo death were observed in eggs inoculated with aliquots from both RT-PCR-positive specimens. Analysis of day 3–5 allantoic fluids by EM showed the presence of virus particles with typical coronavirus morphology. No other agents were detected. The two virus isolates were designated peafowl/China/LKQ3/2003 (pf/CH/LKQ3/03) and teal/China/LDT3/2003 (tl/CH/LDT3/03).

Partial genome organization of the two coronavirus strains

In total, five overlapping cDNA clones, covering the 'unique regions' of mRNA2–mRNA6 (Cavanagh *et al.*, 1990), were obtained from both viruses by using RT-PCR. Sequence analysis of these clones revealed that both newly isolated avian coronaviruses had the S–3–M–5–N gene order that is typical of group 3 coronaviruses from chicken (IBV) (Bournsnell *et al.*, 1987), turkey (Breslin *et al.*, 1999a; Cavanagh *et al.*, 2001; Lin *et al.*, 2002) and pheasant (Cavanagh *et al.*, 2002).

S gene

S gene ORFs of 3489 and 3498 nt were found in the genomes of pf/CH/LKQ3/03 and tl/CH/LDT3/03, respectively. These ORFs are similar in size to the S gene of IBV Beaudette, which is 3489 nt in length (Bournsnell *et al.*, 1987). Compared with pf/CH/LKQ3/03, tl/CH/LDT3/03 had codon insertions at nucleotide positions 67–69, 351–353 and

365–367, all of which are located in the S1 region. Both tl/CH/LDT3/03 and pf/CH/LKQ3/03 contained a spike glycoprotein cleavage recognition site, Arg–Arg–Phe–Arg–Arg, which is identical to that found in the IBV Beaudette, H120, Mass41 and KB8523 strains and some Korean IBV isolates (Cavanagh *et al.*, 1986; Jackwood *et al.*, 2001; Lee *et al.*, 2004).

Composition of the S1 part of the S gene has been used widely to type and investigate serotypic variation in IBV at the molecular level (Lin *et al.*, 1991). Correspondingly, comparisons were made between the predicted S1 part of S proteins of pf/CH/LKQ3/03 and tl/CH/LDT3/03 and those of 23 Chinese field IBV strains, six IBV vaccine strains and two strains of turkey coronaviruses. The S1 part of the S proteins of pf/CH/LKQ3/03 and tl/CH/LDT3/03 had between 76.0 and 99.7% amino acid identity to those of the IBV strains, but not more than 42% to those of the turkey coronaviruses. Among the IBV strains, pf/CH/LKQ3/03 shared >99.5% S1 nucleotide and amino acid identity with the Massachusetts-type IBV vaccine strains H52, H120 and Ma5. Nucleotide and amino acid identity between tl/CH/LDT3/03 and Massachusetts-type IBV vaccine strains was not more than 82%. Two IBV isolates isolated in southern China, J and JX/99/01, shared >91% nucleotide and amino acid identity with tl/CH/LDT3/03.

Phylogenetic trees produced by using the available S1 part of S protein sequences showed that the 33 avian coronaviruses grouped into five distinct clusters (Fig. 1). pf/CH/LKQ3/03 formed a cluster with five IBV vaccine strains (H52, H120, Ma5, W93 and D41), whereas tl/CH/LDT3/03 formed another with J, JX/99/01 and other Chinese field IBV strains. Two other clusters of Chinese field IBV strains were present, with the two turkey coronaviruses, Gh and G1, forming the fifth cluster (Fig. 1).

Gene 3

Similar to the genetic organization of IBV strains (Cavanagh *et al.*, 1990), the mRNA3 (gene 3) 5'-terminal 'unique regions' of pf/CH/LKQ3/03 and tl/CH/LDT3/03 contain three separate ORFs, 3a, 3b and 3c. ORFs 3a and 3c of pf/CH/LKQ3/03 and tl/CH/LDT3/03 were 174 and 327 nt, respectively, comparable to the homologous ORFs of IBV Beaudette. ORF 3b of tl/CH/LDT3/03, however, was 189 nt in length, 6 nt shorter than the corresponding ORF from pf/CH/LKQ3/03 and IBV Beaudette. The 6 nt deletion was located at positions 172–177. BLAST searches revealed significant sequence similarity between gene 3 of pf/CH/LKQ3/03, tl/CH/LDT3/03 and IBV strains (up to 99% identity).

M gene

The M genes of pf/CH/LKQ3/03 and tl/CH/LDT3/03 encoded a predicted protein of 226 aa, equivalent in size to the homologous protein of most IBV strains (Bournsnell *et al.*, 1987; Cavanagh *et al.*, 2001). Although only limited IBV M gene sequences were available for comparison,

similarities of up to 99.6% were seen between the M genes of pf/CH/LKQ3/03 and Massachusetts-type strains M41 and H52. A 29 nt overlap was also seen between the 3' end of ORF 3c and the 5' end of the M protein genes of pf/CH/LKQ3/03 and tl/CH/LDT3/03, another feature in common with IBV Beaudette.

Gene 5

pf/CH/LKQ3/03 was found to have a 434 nt non-coding region between the 3' end of the M protein gene and the 5' end of gene 5. This non-coding region is 346 nt in length in tl/CH/LDT3/03 and 305 nt in length in IBV Beaudette. Gene 5 of pf/CH/LKQ3/03 contained two ORFs, 5a and 5b, which were 198 and 249 nt long, respectively. These sizes are consistent with ORFs 5a and 5b of IBV Beaudette. ORF 5a of tl/CH/LDT3/03 was identical in length to that of pf/CH/LKQ3/03, but ORF 5b contained a 24 nt insertion at the 5' end. This 24 nt insertion is also found in three Chinese field IBV isolates, QXIBV, GD/S14/2003 and LX4 (GenBank accession nos AF199412, AY646283 and AY338732, respectively). BLAST searches of the gene 5 sequences of pf/CH/LKQ3/03 and tl/CH/LDT3/03 again showed that they were most similar to IBV strains.

N gene

A 58 nt overlap was observed between the 5b and N protein ORFs in pf/CH/LKQ3/03 and IBV Beaudette, compared with an 82 nt overlap in the corresponding region of tl/CH/LDT3/03. Both pf/CH/LKQ3/03 and tl/CH/LDT3/03 N protein ORFs were 1230 nt in length, within the typical range for most IBV strains and turkey coronaviruses (Boursnell *et al.*, 1987; Breslin *et al.*, 1999b).

Nucleotide and amino acid sequence identities of the N genes of pf/CH/LKQ3/03 and tl/CH/LDT3/03 to IBV strains ranged from 64.0 to 99.9%. Corresponding identities to the turkey coronaviruses ranged from 86.3 to 94.5%.

Virulence studies

Clinical signs were observed in all tl/CH/LDT3/03-infected chicks from 3 to 10 days post-inoculation. These clinical signs included listlessness, huddling, ruffled feathers and dark, shrunken combs. Eight of the 10 chicks died during the experiment. Gross lesions in the organs of the dead chicks were confined mainly to the kidneys. The kidney parenchyma of the dead birds was pale, swollen and mottled; tubules and urethras were distended with uric acid crystals. Clinical signs of the surviving birds tended to disappear gradually and were absent by day 20 post-inoculation. No overt disease was observed in chicks that had been inoculated with pf/CH/LKQ3/03.

DISCUSSION

During 2003, 55 specimens were taken from four avian species in Guangdong province, China, as part of a wider

animal surveillance programme for identifying potential reservoirs of SARS-CoV and other animal coronaviruses. The specimens were collected from domestic bird flocks that showed no clinical signs. By using an RT-PCR assay based on a replicase gene consensus sequence (Stephensen *et al.*, 1999), we were able to identify individual samples from a teal and a peafowl that were coronavirus-positive. These results add teal and peafowl to the list of avian species from which coronaviruses have been detected. The remaining members of this avian host list are chicken (IBVs), turkeys, pheasants, pigeons and guineafowl. We were also able to propagate viable coronaviruses from the RT-PCR-positive samples by passage in chick embryos. Although coronaviruses from chickens, turkeys, pheasants, pigeons and guineafowl can be propagated in chick embryos (Lister *et al.*, 1985; Barr *et al.*, 1988; Ito *et al.*, 1991; Gough *et al.*, 1996), there can be differences in yields between isolates. For example, the turkey coronaviruses replicate to about 10⁴-fold higher titres in turkey embryos than in chick embryos (Adams & Hofstad, 1971). The chick-embryo titres of the two viruses isolated in this study were similar to those of IBV strains (De Wit, 2000), as were the effects that virus replication had on embryo development. From the third to the fifth passage, the coronaviruses (as confirmed by EM) from the teal and peafowl induced embryo dwarfing, stunting, curling and death, characteristics that are typical of 'field' IBV strains (Clarke *et al.*, 1972; De Wit, 2000).

Some of the primary criteria by which coronavirus species are delineated are genome organization and sequence (Cavanagh, 1997). The gene order of IBV is 5'-replicase-S-3-M-5-N-3' UTR. We have established in this study that both pf/CH/LKQ3/03 and tl/CH/LDT3/03 have the same S-3-M-5-N gene order (the replicase and 3' UTR sequences were not determined in this study), as is the case for coronaviruses from turkeys (Breslin *et al.*, 1999a, b; Cavanagh *et al.*, 2001) and pheasant (Cavanagh *et al.*, 2002). Consequently, the coronaviruses from domestic peafowl and teal are related closely to avian coronaviruses that are in group 3, and are distinct from mammalian coronaviruses that are in groups 1 and 2 (Lai & Cavanagh, 1997).

So far, no genetic features have been discovered that would mark a coronavirus as having originated from a particular host species. Comparison of the S, gene 3, M, gene 5 and N sequences showed that isolate pf/CH/LKQ3/03 was very similar in nucleotide and deduced amino acid sequences to IBV Massachusetts-type strains and Chinese IBV vaccine strains W93 and D41. In addition, by phylogenetic analysis based on the S1 part of S protein genes, pf/CH/LKQ3/03 clustered closely with the coronavirus strains Ma5, H52, H120, W93 and D41. IBV vaccines based on Massachusetts strains, such as Ma5, H52 and H120, have been used for many years on poultry farms in China. W93 and D41 were commercial, live-attenuated vaccine strains derived from field cases of infectious bronchitis in China. In addition, virulence studies showed that pf/CH/LKQ3/03 was of low virulence to 15-day-old chicks. On the basis of virus

characterization, virulence studies, partial genome organization and gene sequence of the virus, we can postulate that isolate pf/CH/LKQ3/03 may be a chicken infectious bronchitis coronavirus strain and that the widespread use of IBV vaccines in chickens is probably involved in the emergence and evolution of this virus.

tl/CH/LDT3/03, in contrast to pf/CH/LKQ3/03, was more similar to field IBV isolates from China than to the vaccine strains. Virulence studies revealed that this isolate is a nephropathogenic coronavirus strain and it caused morbidity and mortality of 100 and 80%, respectively, upon infection of 15-day-old chicks. It has been reported that nephropathological IBVs have been circulating widely in recent years in vaccinated and non-vaccinated flocks in China (Wu *et al.*, 1998; Li & Yang, 2001; Liu & Kong, 2004). Sequence analysis showed that, although tl/CH/LDT3/03 contained a 6 nt deletion at the 3' end of gene 3 compared with most other IBV strains, this deletion has also been reported in other IBV isolates (Cavanagh & Davis, 1988; Cavanagh *et al.*, 1992; Jia & Naqi, 1997). Similarly, tl/CH/LDT3/03 has an additional 24 nt at the very 5' end of ORF 5b, which has also been seen in three Chinese field IBV isolates, QXIBV, GD/S14/2003 and LX4. Interestingly, the nucleotide sequences of the tl/CH/LDT3/03 replicase gene, gene 3, gene 5 and N gene had a high degree of identity to the BJ strain (99.5, 99, 99 and 94.1%, respectively), whereas sequence similarities in the M gene and the S1 part of the S gene between these two viruses were substantially lower (89 and 77.9%, respectively). The M gene and the S1 part of the S gene of tl/CH/LDT3/03 shared high nucleotide and amino acid sequence identity with field IBV isolates from China (Fig. 1), suggesting that recombination is occurring in IBV-like viruses in the region (Cavanagh & Davis, 1988; Wang *et al.*, 1993; Jia *et al.*, 1995; Lee & Jackwood, 2001; Brooks *et al.*, 2004).

The data from this study show that, under field conditions, IBV-like viruses can replicate in domestic peafowl and teal without any overt signs of disease. Although, to our knowledge, this is the first report of IBV isolation from either teal or peafowl, the isolation from teal is of special note. The potential for fowl to act as a host reservoir for IBV, with possible transmission of the virus from this reservoir to chickens, warrants further investigation. Such an alternative reservoir would have major implications for vaccination and control programmes for IBV prevention.

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