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Coronaviruses from pheasants (*Phasianus colchicus*) are genetically closely related to coronaviruses of domestic fowl (infectious bronchitis virus) and turkeys

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Reverse-transcriptase polymerase chain reactions (RT-PCRs) were used to examine RNA extracted from mouth/nasal swabs from pheasants exhibiting signs of respiratory disease. The oligonucleotides used were based on sequences of infectious bronchitis virus (IBV), the coronavirus of domestic fowl. A RT-PCR for the highly conserved region II of the 3′ untranslated region of the IBV genome detected a coronavirus in swabs from 18/21 estates. Sequence identity with the corresponding region of IBVs and coronaviruses from turkeys was > 95%. A RT-PCR for part of the S1 region of the spike protein gene was positive with 13/21 of the samples. Sequence analysis of the RT-PCR products derived from nine of the pheasant viruses revealed that some of the viruses differed from each other by approximately 24%, similar to the degree of difference exhibited by different serotypes of IBV. Further analysis of the genome of one of the viruses revealed that it contained genes 3 and 5 that are typical of IBV but absent in both the transmissible gastroenteritis virus and murine hepatitis virus groups of mammalian coronaviruses. The nucleotide sequences of genes 3 and 5 of the pheasant virus had a similar degree of identity (approximately 90%) with those of coronaviruses from turkeys and chickens, as is observed when different serotypes of IBV are compared. This work: (a) confirms that coronaviruses are present in pheasants (indeed, commonly present in pheasants with respiratory disease); (b) demonstrates that their genomes are IBV-like in their organization; and (c) shows that there is sequence heterogeneity within the group of pheasant coronaviruses, especially within the spike protein gene. Furthermore, the gene sequences of the pheasant viruses differed from those of IBV to similar extents as the sequence of one serotype of IBV differs from another. On the genetic evidence to date, there is a remarkably high degree of genetic similarity between the coronaviruses of chickens, turkeys and pheasants.

Introduction

Viruses that morphologically resemble coronaviruses, with and without serological identity to infectious bronchitis virus (IBV), have been isolated from pheasants, sometimes associated with respiratory disease and/or nephritis (Spackman & Cameron, 1983; Lister *et al.*, 1985; Gough *et al.*, 1996; Pennycott, 2000), as can be exhibited by IBV in domestic fowl (Cook *et al.*, 2001b). Some of the viruses have been successfully isolated and propagated in embryonating domestic fowl eggs (Lister *et al.*, 1985; Gough *et al.*, 1996).

On one game farm from which a coronavirus was isolated (phUK438/94), 15% of breeding pheasants died rapidly with the only clinical sign being

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Table 1. *Coronaviruses from pheasants*[a]

Virus sample: complete name	Abbreviated name	Virus sample: complete name	Abbreviated name
Pheasant/UK/27/B287–4/99	phUK1/99	Pheasant/UK/21/B376–4/99	phUK14/99
Pheasant/UK/24/B295–11/99	phUK3/99	Pheasant/UK/24/B547–9/99	phUK15/99
Pheasant/UK/24/B204–11/99	phUK4/99	Pheasant/UK/24/B171–3/99	phUK17/99
Pheasant/UK/24/B287–10/99	phUK5/99	Pheasant/UK/28/B415–7/99	phUK18/99
Pheasant/UK/24/B505–10/99	phUK6/99	Pheasant/UK/24/B118–11/98	phUK19/98
Pheasant/UK/24/B363–9/99	phUK7/99	Pheasant/UK/24/B250–11/98	phUK20/98
Pheasant/UK/14/B278–6/99	phUK9/99	Pheasant/UK/24/B156–01/99	phUK21/99
Pheasant/UK/24/B646–3/99	phUK10/99		
Pheasant/UK/24/B88–4/99	phUK11/99	Pheasant/UK/750/83[b]	phUK750/83
Pheasant/UK/24/B307–12/98	phUK12/98	Pheasant/UK/438/94[b]	phUK438/94
Pheasant/UK/24/B114–4/99	phUK13/99	Pheasant/UK/602/95[b]	phUK602/95

[a] Complete and abbreviated names are based on the nomenclature suggested by Cavanagh (2001). Samples from 21 pheasant estates were analysed; coronaviruses were detected in 18 of them. UK, United Kingdom.

[b] These three were isolates that had been replicated in embryonated domestic fowl eggs.

sneezing (Gough *et al.*, 1996). Egg production and hatchability was reduced but egg quality was unaffected. In another group of pheasant poults from which a coronavirus was isolated (phUK750/83) there were no respiratory signs, although by 10 weeks of age mortality had reached 45%. The sick birds were reported as being hunched up, with drooping wings (Lister *et al.*, 1985).

Specific lesions that have been described are visceral gout and urolithiasis, with gross swelling and pallor of the kidneys (Lister *et al.*, 1985; Gough *et al.*, 1996; Pennycott, 2000). Histopathological examination of kidneys from affected pheasant poults revealed a moderately severe interstitial nephritis (Lister *et al.*, 1985; Pennycott, 2000). One of the pheasant isolates whose genes we have examined, phUK602/95, was associated with egg peritonitis and renal lesions. Depressed egg production has been observed in several pheasant flocks from which coronaviruses have been isolated (Gough *et al.,* 1998).

Coronaviruses isolated from pheasants are poorly cross-reactive in haemagglutination inhibition (HI) and virus neutralization tests using sera raised against serotypes of IBV, the coronavirus of the domestic fowl (Spackman & Cameron, 1983; Gough *et al.*, 1996). Recently, it has been shown in the US (Breslin *et al.*, 1999a,b; reviewed by Guy, 2000) and the UK (Cavanagh *et al.*, 2001) that coronaviruses isolated from turkeys are genetically similar to IBV, having approximately 90% nucleotide identity in the genes downstream from the spike protein gene. We have used reverse transcription polymerase chain reactions (RT-PCRs), initially designed to detect IBV, to investigate the presence of coronaviruses in pheasants. We have used this approach to examine oropharyngeal swabs from birds on 21 pheasant rearing estates and with three potential coronaviruses from pheasants that had been propagated in domestic fowl embryos. The results show that the pheasant viruses have a similar degree of identity with IBV strains as do IBV serotypes among each other, likewise in respect of coronaviruses from turkeys.

Materials and Methods

Origin of the pheasant coronaviruses

Live pheasants, from field outbreaks of respiratory disease, together with some healthy birds were submitted by gamekeepers via their private veterinary surgeons from sites in England, Wales and Scotland during 1998 and 1999. No stipulation had been made as to the age of the birds, which ranged from 6 weeks to 14 months. Swabs were collected from the oropharynx and turbinates of freshly killed birds and forwarded to regional laboratories of the Veterinary Laboratories Agency, as part of a study of pheasant respiratory disease (D. de B. Welchman, J.M. Bradbury, D. Cavanagh and N.J. Aebischer, in preparation). Some of the swabs were then sent to the Institute for Animal Health, Compton Laboratory (Table 1).

Pheasant coronaviruses ph/United Kingdom/750/83 (Lister *et al.*, 1985), ph/United Kingdom/438/94 (Gough *et al.*, 1996) and ph/United Kingdom/602/95 have been isolated and replicated in embryonated domestic fowl eggs.

Extraction of RNA

Allantoic fluid (250 μl) containing phUK/750/83, phUK/438/94 or phUK/602/95 was mixed with 250 μl guanidinium isothiocyanate denaturation solution (Chomczynski & Sacchi, 1987; Li *et al.*, 1993). Swabs (two to four per farm) were dipped into 0.5 ml denaturation solution. These mixtures were freeze-thawed before the addition of 50 μl of 3 M sodium acetate (pH 4.1) and 500 μl phenol-chloroform-isoamylalcohol (25 : 24 : 1, pH 6.7; Amresco). The RNA from the aqueous phase was precipitated by the addition of an equal volume of isopropanol and pelleted by centrifugation at 4°C, 13 000 × *g* for 30 min. The RNA was air-dried for 2 to 5 min and re-dissolved in 20 μl water (Sigma) containing 20 U RNasin (Promega).

RT-PCR, general procedure

All RT procedures were performed using 2 μl RNA in a 10 μl reaction volume containing 20 U RNaseH$^-$ M-MLV reverse transcriptase

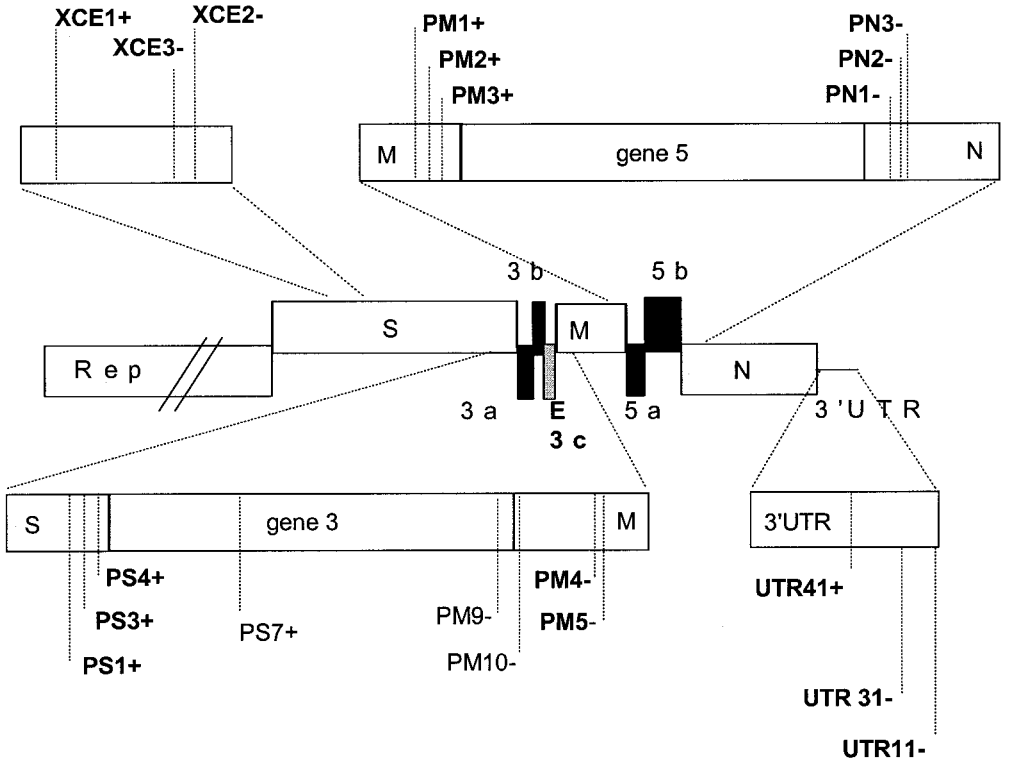

Figure 1. *Approximate positions of the oligonucleotides used for RT-PCRs on RNA from pheasant coronaviruses, relative to the genome of IBV. Oligonucleotides in bold font were used for RT-PCR and for sequencing; the other oligonucleotides were used only for sequencing. Rep, Replicase gene; S, spike glycoprotein gene; E, small envelope protein gene (part of gene 3); M, integral membrane glycoprotein gene; 5, gene 5; N, nucleocapsid protein gene; 3′ UTR, untranslated region at the 3′ end of the genome. Gene 3 comprises three ORFs, 3a, 3b and 3c; the latter encodes the E envelope protein. Gene 5 comprises two ORFs, 5a and 5b.*

(Superscript II; Life Technologies), 5 pmol anti-sense oligonucleotide, 0.5 mM dNTPs, 10 mM dithiothreitol, 50 mM Tris-HCl, 75 mM KCl and 3 mM $MgCl_2$. This was overlaid with 50 µl mineral oil and incubated at 45°C for 1 h followed by 72°C for 10 min.

The total volume of RT mix was used in the subsequent PCR by the addition of PCR components, including 10 pmol positive and negative oligonucleotide primer, as previously described (Capua *et al.*, 1999; Cavanagh *et al.*, 1999). The PCRs were performed in a Touchdown thermocycler (Hybaid) using the conditions 94°C for 1 min, 48°C for 1 min, and 72°C for 2 min, for 30 cycles.

All PCR products were analyzed by electrophoresis in 2% agarose gels (LE, analytical grade; Promega) in which the DNA was stained using ethidium bromide and detected by ultraviolet transillumination. The approximate positions of the oligonucleotides used in the RT-PCRs are shown in Figure 1, and the sequence of these oligonucleotides and of others used in the sequencing reactions are presented in Table 2.

RT-PCR and sequencing within the 3′ untranslated region

Oligonucleotides were designed within the 3′-terminal part of the 3′ untranslated region (UTR) and are universal to all known types of IBV, as this region of the UTR is highly conserved amongst IBVs (Figure 1) (Cavanagh *et al.*, 2001). The oligonucleotide, UTR11–, used in the RT reaction, was used with UTR41+ in a RT-PCR to generate a 266 base pair (bp) product. This product was directly sequenced using these two oligonucleotides. The oligonucleotides UTR11– and UTR41+ (Figure 1 and Table 2) were modifications of oligonucleotides UTR1– and UTR4+ of Adzhar *et al.* (1996). They had been modified in the light of new sequence data made available from Australian isolates of IBV (Sapats *et al.*, 1996a) and turkey coronavirus isolates (Breslin *et al.*, 1999b). Four of the pheasant samples were negative with this RT-PCR. A hemi-nested PCR was performed on these four samples using oligonucleotides UTR41+ and UTR31–; the results remained negative.

RT-PCR and sequencing within the spike protein gene

Oligonucleotides had been designed, for studies of IBV, within a relatively conserved region of the S1 region of the spike gene (Figure 1) (Capua *et al.*, 1999; Cavanagh *et al.*, 1999). Oligonucleotide XCE2– was used for both the RT reaction and subsequently the PCR with oligonucleotide XCE1+. A product, detectable by ethidium bromide staining, of 463 bp was generated with isolates phUK/750/83, phUK/438/94 and phUK/602/95 (all grown in embryonated eggs) but no product was seen with the swab samples. Therefore, a hemi-nested PCR was performed for the swab samples, using 5 µl initial PCR product with oligonucleotides XCE1+ and XCE3–, to generate a 380 bp product. The PCR products were directly sequenced using the oligonucleotides that had been used for in the PCR.

RT-PCR of gene 3

Gene 3 of IBV is located between the spike protein (S) gene and the membrane protein (M) gene (Figure 1). Pheasant isolate phUK/602/95 was analysed for the presence of gene 3.

Oligonucleotides were designed after comparing IBV sequences of the end of the spike protein gene (oligonucleotides PS1+, PS3+ and PS4+) and the beginning of the M protein gene (oligonucleotides PM4– and PM5–; Table 2) (Cavanagh *et al.*, 2001).

The negative sense primers, when used in combination with each of the positive sense primers in RT-PCRs, gave products of approximately 0.9 kbp. The products were sequenced directly using the same oligonucleotides plus oligonucleotides PS7+, PM9– and PM10– (Table 2).

RT-PCR of gene 5

Gene 5 is located between the M protein gene and the nucleocapsid protein gene in the IBV genome (Figure 1). Three oligonucleotides had been designed after comparison of IBV sequences for the end of the M

Table 2. *Sequence and position of the oligonucleotides used for RT-PCRs and sequencing*

Oligonucleotide[a]	Sequence	Gene	Position in genome[b]
XCE1+	ACTGGTAATTTTTCAGATGG	S1	21 070 to 21 089
XCE3–	CAGATTGCTTACAACCACC	S1	21 433 to 21 451
XCE2–	CCTCTATAAACACCCTTACA	S1	21 508 to 21 527
PS1+	TATATTAAGTGGCCTTGGTATGT	S2	23 632 to 23 654
PS3+	TTGTTGTTGTGGATGCTTTGG	S2	23 733 to 23 753
PS4+	TGAGTAAGTGTGGTAAGAAATC	S2	23 768 to 23 789
PS7+	AATAGTAACTTACAGTCTAGAC	3	23 982 to 24 003
PM9–	TACTACCCATGTATACCA	3	24 348 to 24 365
PM10–	GGAAACTCGTTAACAATAAC	3	24 444 to 24 463
PM1+	CTGGCGAGCTAGAAAGTGTA	M	25 133 to 25 152
PM2+	GGTGGAAGTAGCCTTTACAC	M	25 159 to 25 178
PM3+	ATAAATGTGTGTGTGTAGAGAG	M	25 179 to 25 200
PM4–	CAAAAGCACCATAACACTATCAT	M	24 658 to 24 680
PM5–	CAATGTTAAGGGGCCAAAAGCA	M	24 673 to 24 694
PN1–	GAAGAACCAACTTTAGGTGG	N	25 942 to 25 961
PN2–	TGAAACCAAGATGCATTTCC	N	25 963 to 25 982
PN3–	GCTTTTATTGCTTGAAACCAAGAT	N	25 971 to 25 994
UTR41+[c]	ATGTCTATCGCCAGGGAAATGTC	3′ UTR	27 342 to 27 364
UTR 31–[c]	GGGCGTCCAAGTGCTGTACCC	3′ UTR	27 501 to 27 520
UTR11–[c]	GCTCTAACTCTATACTAGCCTA	3′ UTR	27 586 to 27 607

[a] + represents positive and – represents negative sense oligonucleotides.

[b] The nucleotide positions correspond to those in the sequence of the IBV Beaudette genome, GenBank accession number M95169.

[c] UTR41+, UTR31– and UTR11– are similar to but not identical to UTR4+, UTR3– and UTR1–, respectively, of Adzhar *et al.* (1996). Minor changes were made at the ends of the oligonucleotides, taking into account sequence of the 3′ UTRs of Australian isolates of IBV (Sapats *et al.*, 1996) and of coronaviruses from turkeys (Breslin *et al.*, 1999b).

protein gene (gene 4; oligonucleotides PM1+, PM2+ and PM3+) and the beginning of the nucleocapsid protein gene (gene 5; oligonucleotides PN1–, PN2– and PN3–; Table 2) (Cavanagh *et al.*, 2001). Each pair of negative and positive oligonucleotides yielded a product of approximately 0.8 kbp, although the pair PN2–/PM3+ gave the most DNA with RNA from phUK602/95. As in the case of the corresponding PCR product for tyUK/412/00 (Cavanagh *et al.*, 2001), problems were encountered when attempting to sequence this product directly. Therefore, the 0.8 kbp product derived from the pheasant RNA was cloned as previously described for gene 5 of the turkey virus (Cavanagh *et al.*, 2001).

Cloning gene 5

DNA generated by PCR amplification was cloned using a T-tailed vector, pGEM-T (Promega), and transformed using JM109 competent cells (Promega) according to the manufacturer's instructions. Plasmid DNA was extracted from an overnight culture using the Qiagen midi kit and sequenced using PM3+ and PN2– oligonucleotides as primers.

Sequence analysis

The PCR products were sequenced either directly or from cloned DNA according to the instructions of the Thermosequenase dye terminator cycle sequencing kit, version 2.0 (Amersham), using 1 μg PCR product or 2 μg plasmid DNA. Sequences were aligned using ClustalX version 1.64b (Thompson *et al.*, 1994) and compared using GeneDoc Multiple Sequence Alignment Editor and Shading Utility version 2.5.000 (http://www.psc.edu/biomed/genedoc).

Accession numbers of nucleotide sequences

The sequences established in this paper will be deposited in the nucleotide databanks.

Results

Pheasant coronaviruses

All of the pheasant coronaviruses examined were from the UK. Three of the viruses (phUK750/83, phUK438/94 and phUK602/95) were isolates that had been replicated in embryonated domestic fowl eggs (Table 1). The other 18 pheasant coronaviruses detected in the 21 field samples were not actually virus isolates; RNA was extracted directly from swabs of the oropharynx and nasal region. The swabs had been collected from pheasants exhibiting respiratory clinical signs during late 1998 and throughout 1999 on game bird estates in southern England, Wales and Scotland.

The pheasant viruses have a 3′ UTR like that of IBV and coronaviruses from turkeys

Most (16/21) of the swab samples, and all three of the pheasant viruses grown in eggs, were positive in the single RT-PCR corresponding to the conserved region (Williams *et al.*, 1993; Dalton *et al.*, 2001) of the 3′ UTR. The four swab samples that were negative in the single RT-PCR remained negative in a nested PCR using UTR41+ and UTR31–.

The PCR products from 13 of the swab samples and the three viruses grown in eggs were

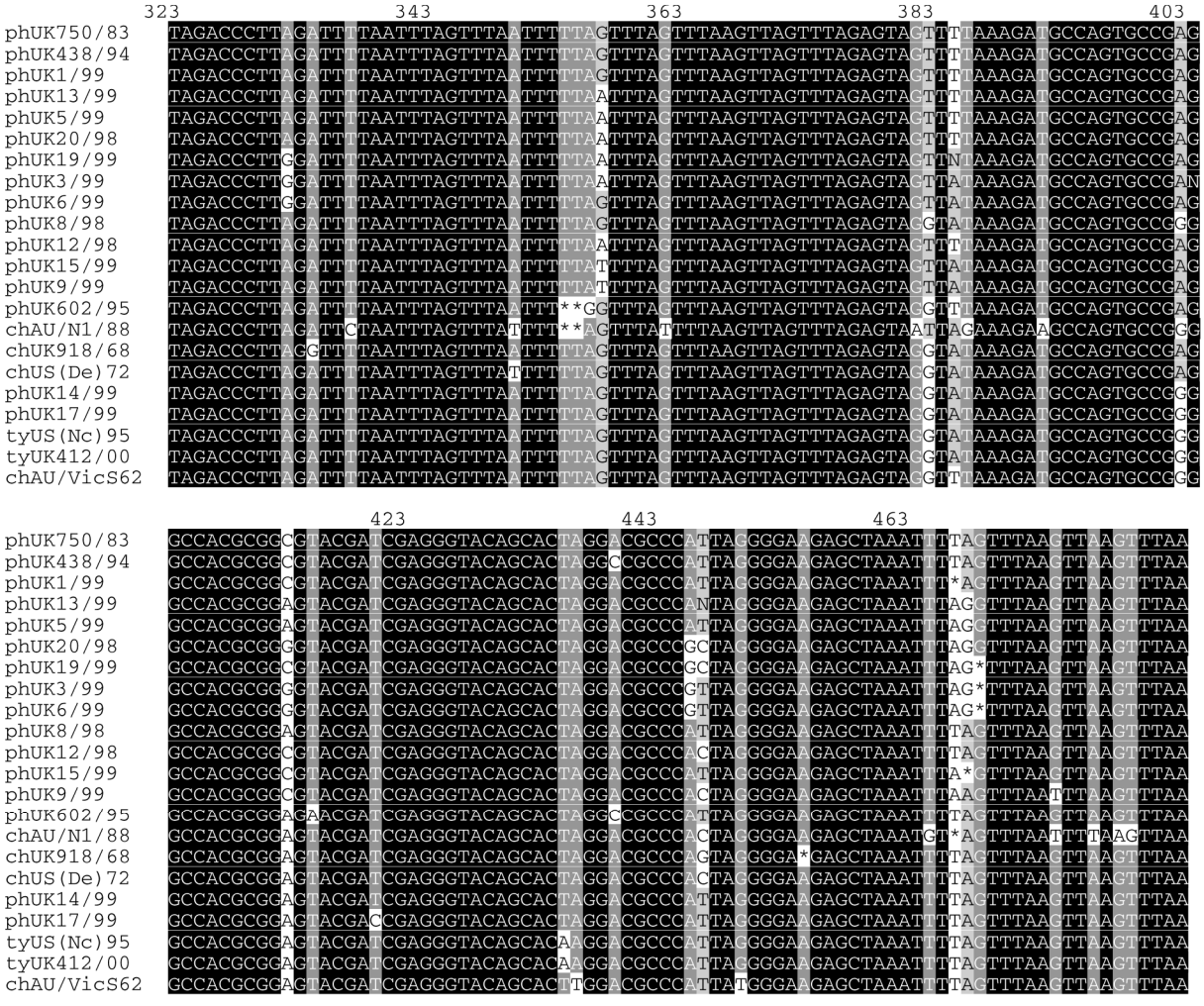

Figure 2. *Comparison of part of the 3′ UTR of 16 coronaviruses from pheasants (prefixed 'ph') with that of two coronaviruses from turkeys (prefixed 'ty') and four IBVs (prefixed 'ch', chicken). The numbers are relative to the first nucleotide of the 3′ UTR of IBV H120, which has the longest known 3′ UTR of avian coronaviruses (Williams* et al.*, 1993; Dalton* et al.*, 2001). Black shading indicates that all the viruses had the same nucleotide at that position; grey and white shading shows the nucleotides possessed by the majority and minority, respectively, of isolates at a given position. Deletions are marked *. chAU/88, chicken/Australia/N1/88 (Sapats* et al.*, 1996a; accession number U52599); chUK68, chicken/United Kingdom/918/68 (Dalton* et al.*, 2001; accession number AJ278334); chUS(De)72, chicken/United States(De)/DE072/72 (Lee & Jackwood, 2000; AF203002); tyUS(Nc)95, turkey/United States(Nc)/NC95/95 (Breslin* et al.*, 1999b; accession number AF111997); tyUK412/00, turkey/United Kingdom/412/00 (Cavanagh* et al.*, 2001; accession number AJ310642); chAU/VicS62, chicken/Australia/VicS/62 (Sapats* et al.*, 1996a; accession number U52594). De, Delaware; Nc, North Carolina.*

sequenced. The sequences have been compared with each other and with those of isolates of IBV (from Australia, the UK and the US) and coronaviruses from turkeys (from the UK and the US); some comparisons are shown in Figure 2. Very high sequence conservation was demonstrated. For example, phUK750/83 had 95.7 to 99.4% identity with the other pheasant coronaviruses, 96.3 to 97.0% identity with the turkey viruses, and 96.3 to 97.6% identity with the IBVs. The exception was in comparison with chicken/Australia/N1/88 (chAU/N1/88), where the identity was only 89%; this Australian isolate also differs extensively from other IBVs (Sapats *et al.*, 1996a). There were no features unique to the pheasant coronaviruses as a group.

Spike protein gene sequencing revealed heterogeneity among pheasant coronaviruses

Oligonucleotides XCE2– and XCE1+ were used in an attempt to amplify a region of the S1 part of the spike protein gene (Figure 1), as these oligonucleotides had been used successfully to amplify several genotypes of IBV (Capua *et al.*, 1999; Cavanagh *et al.*, 1999; Meulemans *et al.*, 2001). This was successful for the three pheasant isolates that had been grown in eggs, generating a product of 463 bp that was visible in an agarose gel after staining with ethidium bromide. Such a band was not observed using RNA extracted from swabs. Therefore, a hemi-nested PCR was performed using 5 µl initial PCR product, using oligonucleotides XCE1+ and XCE3– in the second PCR, to generate a 380 bp

Table 3. *Percentage nucleotide identities between part[a] of the S1 protein gene of nine coronaviruses from pheasants[b] and four[c] serotypes of IBV*

	Virus													
Virus	ph750	ph438	ph6	ph5	ph1	ph602	ph7	ph15	ph20	D207	123/82	M41	VicS	7/91
ph750	–	95	95	93	92	92	79	79	78	80	80	82	80	78
ph438		–	95	94	91	93	77	79	78					
ph6			–	92	91	93	76	76	75					
ph5				–	92	89	78	78	76					
ph1					–	89	77	77	77					
ph602						–	77	78	77	80	80	80	79	78
ph7							–	98	95					
ph15								–	97					
ph20									–					
D207[c]										–	97	79	81	84

[a] The region of S1 compared corresponds to oligonucleotide position 794 to 1008 in Figure 1 of Adzhar *et al.* (1997), and oligonucleotide position 21134 to 21348 in the IBV Beaudette complete genome sequence (GenBank accession number M95169).

[b] Pheasant viruses are prefixed 'ph'.

[c] The IBV D207 (chicken/Netherlands/D207/78) and 123/82 (chicken/United Kingdom/123/82) isolates are of the same serotype, which is distinct from the M41 (chicken/United States(Ma)/M41/41), VicS (chicken/Australia/VicS/62) and 7/91 (chicken/United Kingdom/7/91) strains that represent three other serotypes.

product. Six of these PCR products were selected for sequencing, plus the three pheasant viruses grown in eggs. The number of nucleotides compared for all viruses was 214.

In this region of S1, serotypes of IBV commonly have approximately 80% nucleotide identity (Table 3, bottom row, and Figure 3). Isolates of the same serotype have approximately 95% or greater nucleotide identity; for example, chNL/D207/78 has 97% identity with chUK123/82 (Table 3, bottom row), and chUK7/91 has 95% identity with isolate chFR/CR94047/94 (unpublished observation).

Three of the pheasant viruses (phUK/20/98, phUK/7/99 and phUK/15/99) were clearly closely related, having 95% or greater identity (Table 3) and clearly identifiable short sequences that distinguished them as a group from the other pheasant viruses (Figure 3).

In contrast, the other six pheasant viruses differed by 21 to 25% from these three viruses (Table 3 and Figure 3). Within the group of six viruses, some were very closely related; phUK/438/94 had 95% identity with phUK/750/84 and phUK/6/99. Most other comparisons within this group revealed identities of 89 to 94% (Table 3 and Figure 3).

Comparison of the pheasant sequences with those of IBV isolates showed identity of approximately 80% (and much less with IBVs that differ greatly from other IBVs, e.g. chNL/D1466/78). This is illustrated by a comparison of phUK/750/83 and phUK602/95 with four serotypes of IBV (Figure 3 and Table 3).

Thus, the pheasant viruses had identities with IBVs in this part of the S1 gene that were similar to the identities between most serotypes of IBV (i.e. approximately 80%). Among the pheasant viruses there was clearly heterogeneity, some of them differing from the others to the same extent as one serotype of IBV differs from another (i.e. approximately 80%).

The pheasant viruses have genes 3 and 5 like IBV and turkey coronaviruses

A feature of IBV and turkey coronaviruses, which are genetically close to each other (Breslin *et al.*, 1999a,b; Cavanagh *et al.*, 2001), is that they have a gene 3 and gene 5 that do not have homologues in the mammalian coronaviruses. We chose phUK/602/95 to look for the presence of IBV-like genes 3 and 5 in coronaviruses from pheasants.

RT-PCR using several pairs of oligonucleotides corresponding to sequences near the 3′ end of gene 2 (encoding the spike protein) and the 5′ end of gene 4 (encoding the membrane protein, M) of IBV did result in DNA products of a predicted 0.9 kb. Sequencing confirmed that the pheasant virus had a gene 3 analogous to that of IBV and coronavirus from turkeys (Figure 4). Thus, the pheasant gene 3 had three open reading frames (ORFs), 3a, 3b and 3c (the latter encoding the E envelope protein), of lengths very similar to those of other avian coronaviruses (Figure 4) (Cavanagh & Davis, 1988; Liu *et al.*, 1991). Some nucleotides were absent from the 3′ end of ORF 3c (Figure 4), as has been observed for tyUK412/00 (Cavanagh *et al.*, 2001) and some IBVs (Cavanagh & Davis, 1988; Liu *et al.*, 1991). The nucleotide identities of gene 3 of the

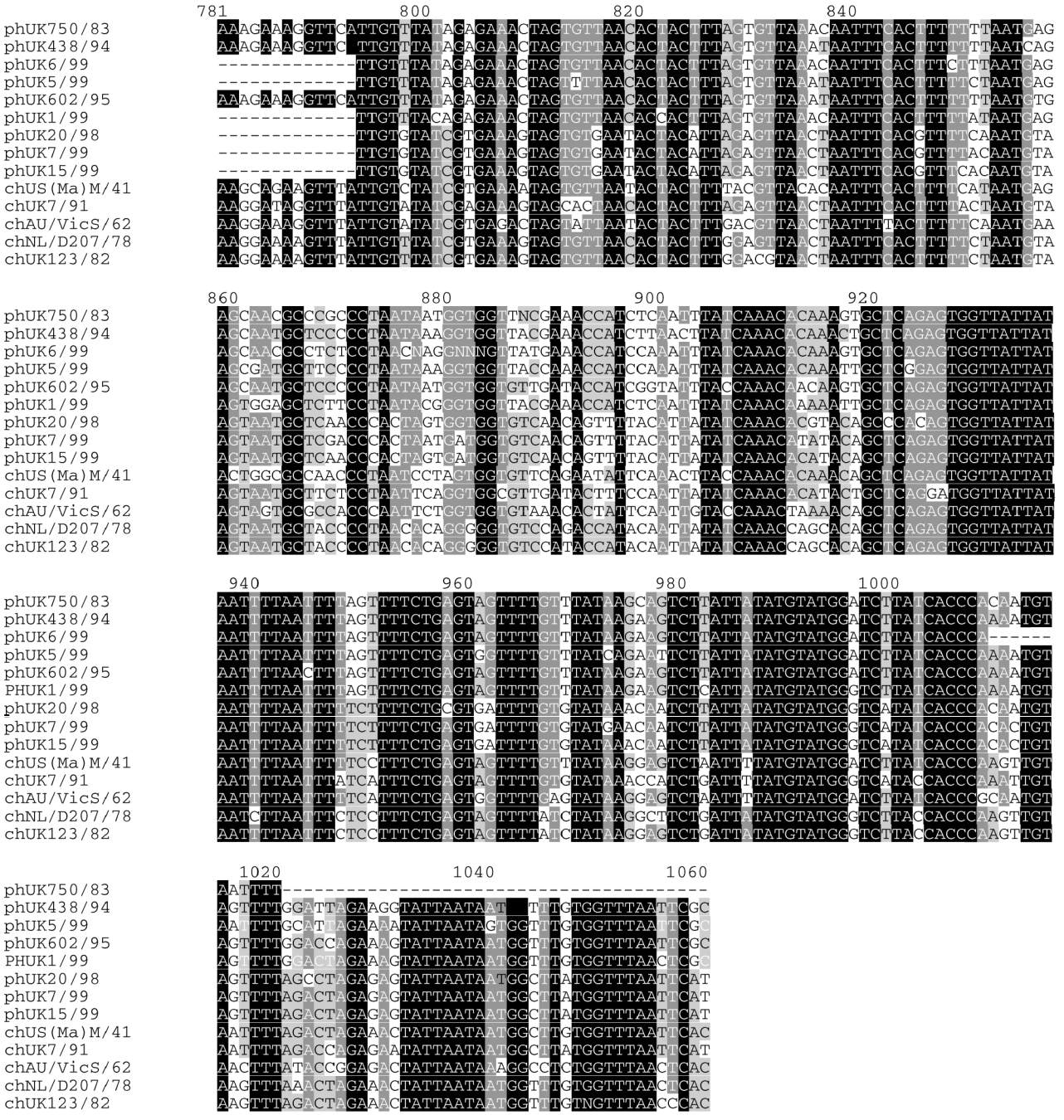

Figure 3. *Comparison of part of the S1 region of the spike protein gene of nine coronaviruses from pheasants (prefixed 'ph') with that of five IBVs (prefixed 'ch'). The first nucleotide corresponds to position 794 in the sequence comparison of figure 1 in Adzhar* et al. *(1997). Shading is as described for Figure 2. Unavailable data is shown by short lines (–––). chUS(Nd)M41, chicken/United States(Ma)/M41/41 (Binns* et al.*, 1986; accession number Z04722); chUK7/91, chicken/United Kingdom/7/91(Adzhar* et al.*, 1997; accession number Z83975); chAU/VicS62, chicken/Australia/VicS/62 (Sapats* et al.*, 1996b; accession number U29519); chNL/78, chicken/Netherlands/D207/78 (Kusters* et al.*, 1989; accession number M21969; J04329); chUK123/82, chicken/United Kingdom/123/82 (Cavanagh* et al.*, 1992; accession number X58067). Ma, Massachusetts.*

pheasant virus and those of tyUK/412/00 and three serotypes of IBV were similar to those among the tyUK/412/00 and IBV sequences (Table 4).

The transcription-associated sequences of gene 3 and the downstream gene 4 (M) (CTGAACAA and CTTAACAA, respectively) were identical to those of tyUK/412/00 and IBVs.

The pheasant virus also had a gene 5 that was homologous to that of turkey coronaviruses and IBVs (Figure 5), having two ORFs (5a and 5b). This gene was amplified using oligonucleotides corresponding to sequences near the 3′ end of gene 4 and near the 5′ end of gene 6 (encoding the nucleoprotein gene, N). The nucleotide identities of gene 5 of the pheasant virus and those of tyUK/412/00 and three serotypes of IBV were similar to those among the tyUK/412/00 and IBV sequences (Table 5).

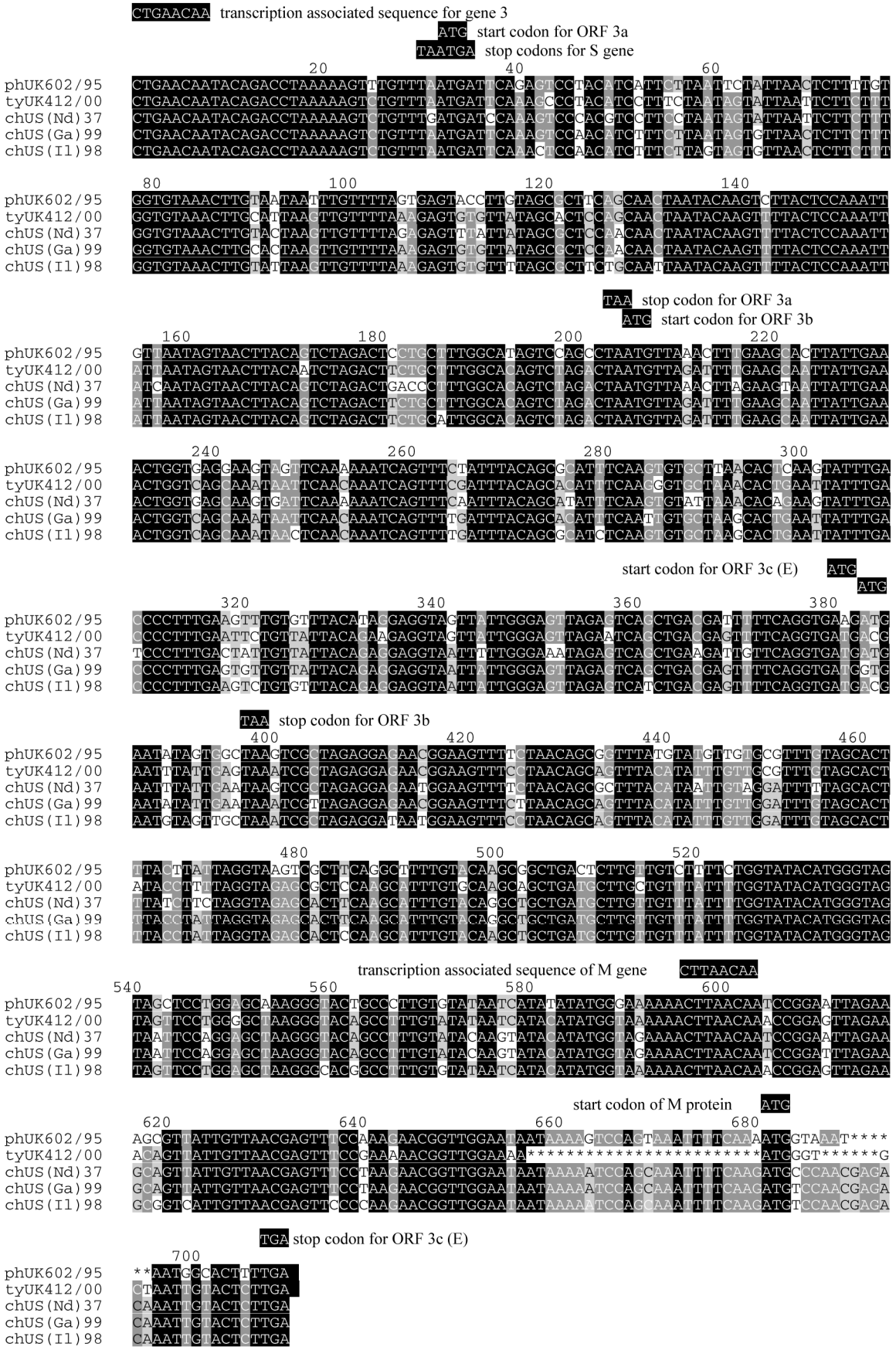

Figure 4. *Comparison of the end of the spike glycoprotein (S) gene, gene 3 and beginning of the M gene of phUK602/95 with that of one coronavirus from a turkey (prefixed 'ty') and three IBV isolates (prefixed 'ch'). Shading is as described for Figure 2. Deletions are marked ***. tyUK412/00, turkey/United Kingdom/412/00 (Cavanagh* et al.*, 2001; accession number AJ310640); chUS(Nd)37, Beaudette, chicken/United States(Nd)/Beaudette/37 (Boursnell* et al.*, 1985; accession number M95169); chUS(Ga)/99, chicken/United States(Ga)/GA5381/99 (Lee* et al.*, 2001; accession number AF206261); chUS(Il)/98, chicken/United States(Il)/IL2831/98 (Lee* et al.*, 2001; accession number AF206260). Nd, North Dakota; Ga, Georgia; Il, Ilinois.*

Table 4. *Nucleotide identity of gene 3 of coronaviruses from pheasant, turkey and chickens*

	Identity (%)				
Virus	phUK/602/95	tyUK/412/00	chUS(Nd)37[a]	chUS(Ga)/99[b]	chUK(Il)/98[c]
phUK/602	–	85	83	85	86
chUS(Nd)37[a]	83	88	–	91	87

[a] chUS(Nd)37, chicken/United States(Nd)/Beaudette/37.
[b] chUS(Ga)/99, chicken/United States(Ga)/GA5381/99.
[c] chUS(Il)/98, chicken/United States(Il)/IL2831/98.

Discussion

The gene order of IBV, the coronavirus of the domestic fowl, is 5′-replicase-S-3-M-5-N-3′UTR. We have established that coronaviruses from pheasants have the same S-3-M-5-N-3′UTR gene order, as is the case for coronaviruses from turkeys (Breslin *et al.*, 1999a,b; Cavanagh *et al.*, 2001); we have not analysed the replicase gene. Consequently, the coronaviruses from pheasants would be in the same group (group 3) as IBV and coronaviruses from turkeys, distinct from mammalian coronaviruses that

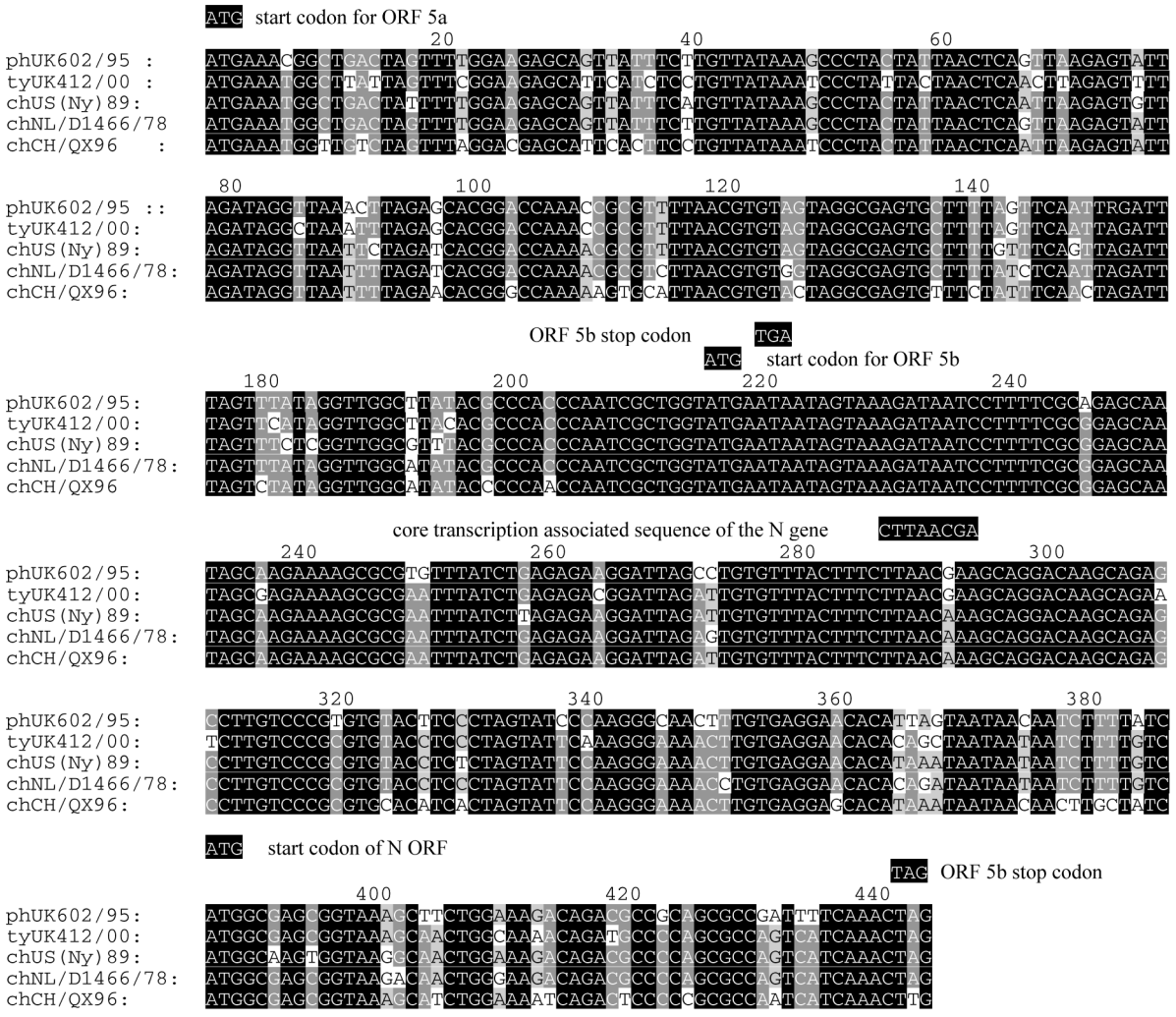

Figure 5. *Comparison of gene 5 of phUK602/95 with that of one coronavirus from a turkey (prefixed 'ty') and three IBVs (prefixed 'ch'). Shading is as described for Figure 2. tyUK412/00, turkey/United Kingdom/412/00 (Cavanagh* et al.*, 2001; accession number AJ 310641); ch/US(Ny)/89, chicken/United States(Ny)/CU-T2/89 (Jia* et al.*, 1995; accession number U46037); chNL/D1466/78, chicken/Netherlands/D1466/78 (Lee & Jackwood, 2000; accession number AF203005); chCH/QX96, chicken/China/QX/96 (Wang* et al.*, 1998; accession number AF221667; J. Pan, D. Chen, P. Chen & B. Cai, unpublished work). Ny, state of New York.*

Table 5. *Nucleotide identity of gene 5 of coronaviruses from pheasant, turkey and chickens*

	Identity (%)				
Virus	phUK/602/95	tyUK/412/00	chUS/(Ny)89[a]	chNL/1466/78[b]	chCH/QX/96[c]
phUK/602	–	89	90	91	87
chUS(Ny)89[a]	89	90	–	95	89

[a] ch/US(Ny)/89, Chicken/United States(Ny)/CU-T2/89. Ny, New York.
[b] chNL/D1466/78, Chicken/Netherlands/D1466/78.
[c] chCH/QX/96, Chicken/China/QX/96.

are in either group 1 or group 2 (Lai & Cavanagh, 1997; Enjuanes *et al.*, 2000; Enjuanes & Cavanagh, 2001). Furthermore, nucleotide sequencing has shown that the gene sequences of the coronaviruses of the three avian species are very similar. No genetic features have yet been discovered that would mark a coronavirus as coming from one of the host species rather than from the others. If genome organization and gene sequence were to be the primary criteria by which avian coronavirus species were defined, the pheasant, turkey and domestic fowl coronaviruses would, on current data, probably be considered as being one virus species. However, biological criteria might suggest otherwise. Turkey coronaviruses are associated with enteric disease and growth in the bursa of Fabricius (Goodwin *et al.*, 1995; Barnes & Guy, 1997; Nagaraja & Pomeroy, 1997) while IBV is largely associated with respiratory disease and reduced egg-laying performance (Cavanagh & Naqi, 1997), although IBV has been shown to replicate in explants of bursa of Fabricius, proventriculus, caecal tonsil, rectum, kidney (Bhattachargjee & Jones, 1997) and oviduct (Dhinakar Raj & Jones, 1996, 1997), and some are nephropathogenic (Lambrechts *et al.,* 1993; Pensaert & Lambrechts, 1994; Li & Yang, 2001). Pheasant coronaviruses have been associated with both respiratory and kidney disease in the field (Lister *et al.*, 1985; Gough *et al.*, 1996; Pennycott, 2000), in addition to egg production problems (Gough *et al*., 1998). Although isolation of coronaviruses from pheasants using domestic fowl eggs has been successful, there have been occasions when the presence of coronavirus was suspected, on clinical grounds, but no virus was isolated, perhaps indicating heterogeneity within the pheasant coronavirus population with regard to growth in fowl embryos.

A small amount of experimental work has been carried out to study the host range of the avian coronaviruses. Inoculation of 3-week-old specific pathogen free (SPF) chickens intranasally with phUK/750/83 did not result in any clinical signs or immunoprecipitating antibodies to the homologous virus (Lister *et al.*, 1985). When 1-day-old SPF chicks were inoculated both orally and intratracheally with a turkey coronavirus, there were no adverse effects on the chicks. However, the birds did seroconvert and virus was detected in intestinal tissues and bursa of Fabricius, as in turkeys (Guy, 2000).

Coronaviruses antigenically related to IBV have been reported from other avian species. Barr *et al.* (1988) isolated a coronavirus from racing pigeons in Australia, using embryonated chicken eggs. The pigeons had exhibited ruffled feathers, dyspnoea and excessive mucus at the commissures of the beak; some died. HI and virus neutralization tests (de Wit, 2000) revealed antigenic relationships with IBV (Barr *et al.*, 1988). When SPF chicks were inoculated with the pigeon virus, marked respiratory rales were observed and IBV-reactive HI antibodies were produced. In the same year, a coronavirus was isolated from guinea fowl in Sao Paolo, Brazil (Ito *et al.*, 1991) that were thin, dehydrated and showing enteritis, pancreatitis and nephritis. Kidney homogenates were inoculated into the allantoic cavity of SPF chicken embryos, which produced dwarfing and curling of the embryos. Electron microscopy revealed coronavirus-like particles. Pooled sera from commercial layer breeders, which would have been infected in the field by IBV (vaccinal and field strains, leading to broadly IBV-reactive sera), reacted strongly in HI tests using the guinea fowl isolate as antigen. Experimental inoculation of chicks with the guinea fowl virus was not reported. Work in Israel and the US has established antigenic relatedness between turkey coronaviruses and IBV (Weismann *et al.*, 1987; Guy *et al.*, 1997; Loa *et al.*, 2000).

The antigenic analyses described, taken with the sequence data of pheasant, turkey and chicken coronaviruses, are evidence that at least five avian species are susceptible to IBV-like coronaviruses. It is important to know, from both practical and academic viewpoints, the extent to which an avian coronavirus from one species can replicate in and, most importantly, cause disease in birds of other species. The question of the species status of avian coronaviruses from different bird species has been discussed at greater length by Cavanagh (2001).

Our sequence analysis of part of the S1 region of the spike protein gene of nine pheasant coronaviruses

has revealed extensive heterogeneity. At least two genotypes are represented by this collection of viruses, three viruses in one group differing by > 20% from the viruses in the other group of six viruses. Some viruses in the latter group differed by up to 11% in the region sequenced. Viruses phUK/7/99, phUK/15/99 and phUK/20/98, which had ≥ 95% identity in the S1 region that was sequenced (a value commonly observed for IBV strains of a given serotype), were from different estates, although all were in the same county (Hampshire). Viruses phUK/20/98 and phUK/5/99, which had S1 sequences differing by > 20%, were from the same estate, although obtained 10 months apart (November and September, respectively). This shows that an estate can be infected with at least two genotypes of coronavirus within 1 year, by entry of infected birds from the neighbouring area or by the introduction of new stock. Co-circulation of two or more coronaviruses in pheasants within a region might not be unexpected, as this has been observed with IBV in chickens (Capua *et al.*, 1999). Avian pneumovirus has been detected in pheasants (D. de B. Welchman, J.M. Bradbury, D. Cavanagh and N.J. Aebischer, in preparation). The presence of a coronavirus in pheasants might adversely affect the growth and detection of avian pneumovirus in this species, as has been observed in domestic fowl (Cook *et al.*, 2001a).

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RÉSUMÉ

Coronavirus des faisans *(Phasianus colchicus)* génétiquement proches du virus de la bronchite infectieuse des poules domestiques et des coronavirus des dindes

Les réactions de transcription inverse et d'amplification en chaîne par polymérase (RT-PCRs) ont été utilisées pour étudier l'ARN extrait des écouvillons nasaux et bucaux des faisans montrant des symptômes respiratoires. Les oligonucléotides utilisés ont été choisis à partir des séquences du virus de la bronchite infectieuse (IBV), le coronavirus des poules domestiques. Une RT-PCR réalisée à partir de la région II hautement conservée de la région 3' non traduite du génome de l'IBV a détecté un coronavirus dans les écouvillons de 18/21 sujets. L'identité de la séquence comparée à celle de la région correspondante des IBVs et des coronavirus de la dinde a été supérieure à 95%. Une RT-PCR d'une partie de la région S_1 du gène de la protéine de spicule a été positive pour 13/21 des échantillons. L'analyse de la séquence des produits de la RT-PCR dérivés de neuf des virus du faisan a révélé que quelques uns des virus différaient des autres d'environ 24%, ce qui est similaire à la différence observée entre les différents sérotypes d'IBV. Des analyses complémentaires du génome d'un de ces virus ont révélé qu'il contenait les gènes 3 et 5 qui sont typiques de l'IBV mais qui sont tous les deux absents dans le virus de la gastroentérite transmissible et du virus de l'hépatite murine appartenant au groupe des coronavirus de mammifères. Les séquences nucléotidiques des gènes 3 et 5 du virus du faisan avaient des degrés similaires d'identité (approximativement 90%) à ceux des coronavirus de la dinde et du poulet, comme il a été observé lors de la comparaison de différents sérotypes d'IBV. Ce travail : a) confirme que les coronavirus sont présents chez le faisan –très souvent présents chez les faisans présentant des symptômes respiratoires; b) démontre que leurs génomes sont très proches de celui de l'IBV dans leur organisation; et (c) montre qu'il y a une hétérogénéité dans la séquence au sein du groupe des coronavirus du faisan, surtout au niveau du gène de la protéine de spicule. De plus, la séquence génomique des virus du faisan diffère de celle de l'IBV, dans les mêmes proportions que la séquence d'un sérotype d'IBV diffère d'un autre. De toute évidence, il y a un haut degré de similarité génétique entre les coronavirus des poulets, des dindes et des faisans.

ZUSAMMENFASSUNG

Coronaviren von Fasanen (*Phasianus colchicus*), die mit dem Bronchitusvirus des Haushuhns und mit Coronaviren von Puten eng verwandt sind

Reverse-Transkriptase-Polymerase-Kettenreaktionen (RT-PCRs) wurden verwendet, um RNA zu untersuchen, die aus Schnabel-/Nasentupfern von Fasanen mit Symptomen einer Respirationskrankheit extrahiert worden war. Die verwendeten Oligonukleotide basierten auf Sequenzen des Bronchitisvirus (IBV), dem Coronavirus des Haushuhns. Eine RT-PCR für die hoch konservierte Region II der untranslatierten 3′-Region des IBV-Genoms entdeckte ein Coronavirus in Tupferproben von 18/21 Anwesen. Die Sequenzidentität mit der entsprechenden Region von

IBVs und Puten-Coronaviren betrug >95%. Eine RT-PCR zum Nachweis eines Teils der S1-Region des Spikeprotein-Gens war mit 13/21 der Proben positiv. Die Sequenzanalyse der aus neun der Fasanenviren stammenden RT-PCR-Produkte ließ erkennen, dass sich einige der Virusstämme um etwa 24% voneinander unterschieden, ähnlich wie der Grad der Differenz zwischen unterschiedlichen IBV-Serotypen. Die weitere Analyse des Genoms eines dieser Viren ergab, dass es die Gene 3 und 5 enthielt, die typisch für IBV sind, aber weder im transmissiblen Gastroenteritisvirus noch im Mäuse-Hepatitisvirus als Vertretern der Säuger-Coronaviren vorhanden sind. Die Nukleotidsequenzen der Gene 3 und 5 des Fasanenvirus hatten einen ähnlichen Grad der Identität (etwa 90%) mit denen der Coronaviren von Puten und Hühnern wie er beim Vergleich verschiedener IBV-Serotypen beobachtet wird. Diese Arbeit (a) bestätigt, dass Coronaviren bei Fasanen vorhanden sind – tatsächlich allgemein bei Fasanen mit Atemwegserkrankung vorhanden; (b) weist nach, dass ihre Genome in ihrem Aufbau IBV-ähnlich sind; und (c) zeigt, dass es eine Sequenzheterogenität innerhalb der Gruppe der Fasanen-Coronaviren gibt, insbesondere im Spikeprotein-Gen. Ferner unterschieden sich die Gensequenzen der Fasanenviren von denen des IBV in ähnlichen Ausmaßen wie sich die Sequenz eines IBV-Serotyps von der eines anderen unterscheidet. Nach den bis heute bekannten genetischen Hinweisen gibt es einen bemerkenswert hohen Grad von genetischer Ähnlichkeit zwischen den Coronaviren von Hühnern, Puten und Fasanen.

RESUMEN

Coronavirus de faisanes (Phasianus colchicus) que están genéticamente relacionados con los virus de bronquitis infecciosa en aves domésticas y con los coronavirus de pavos

Se utilizó una técnica de transcriptasa reversa-reacción en cadena de la polimerasa (RT-PCRs) para examinar el ARN extraído de hisopos nasales y bucales de faisanes que mostraban síntomas de enfermedad respiratoria. Los oligonucleótidos utilizados se basaron en las secuencias del virus de bronquitis infecciosa (IBV), el coronavirus de las aves domésticas. Mediante una técnica de RT-PCR de la región muy conservada II de la región no traducida 3' del genoma de IBV se detectaron coronavirus en hisopos provenientes de 18/21 estados. La identidad de la secuencia con la región correspondiente de los IBVs y de los coronavirus del pavo fue de >95%. Una técnica de RT-PCR para parte de la región S1 del gen de la proteína espícula fue positiva en 13/21 de las muestras. El análisis de la secuencia de los productos de PCR derivados de nueve de los virus de faisanes reveló que algunos de estos virus diferían entre ellos en aproximadamente un 24%, similar al porcentaje de diferencias exhibidas por diferentes serotipos de IBV. Los análisis posteriores de uno de estos virus reveló que contenía los genes 3 y 5 típicos de IBV pero que no se encuentran en los virus de los grupos de la gastroenteritis transmisible y de la hepatitis murina de los coronavirus de mamíferos. Las secuencias de nucleótidos de los genes 3 y 5 del virus de los faisanes presentaban un grado de identidad similar (aproximadamente del 90%) con los de los coronavirus de pavos y pollos, como se observa al comparar diferentes serotipos de IBV. Este trabajo: (a) confirma que los coronavirus están presentes en faisanes- además en faisanes se asocian frecuentemente con enfermedad respiratoria; (b) demuestra que sus genomas son similares a los del IBV en cuanto a organización; y (c) muestra que hay una cierta heterogeneidad en cuanto a secuencia entre los grupos de coronavirus de faisanes, especialmente por lo que se refiere al gen de la proteína espícula. Además, las diferencias en las secuencias del gen entre los virus de faisanes fueron similares a las observadas entre las secuencias de los diferentes serotipos de IBV. Según las evidencias genéticas que existen hasta el momento, se observa un remarcable grado de similitud entre los coronavirus de pollos, pavos y faisanes.