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Detection of a coronavirus from turkey poults in Europe genetically related to infectious bronchitis virus of chickens

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Intestinal contents of 13-day-old turkey poults in Great Britain were analysed as the birds showed stunting, unevenness and lameness, with 4% mortality. At *post mortem* examination, the main gross features were fluid caecal and intestinal contents. Histological examination of tissues was largely unremarkable, apart from some sections that showed crypt dilation and flattened epithelia. Negative contrast electron microscopy of caecal contents revealed virus particles, which in size and morphology had the appearance of a coronavirus. RNA was extracted (turkey/UK/412/00) and used in a number of reverse transcription-polymerase chain reactions (RT-PCRs) with the oligonucleotides based on sequences derived from avian infectious bronchitis virus (IBV), a coronavirus of domestic fowl. The RT-PCRs confirmed that turkey/UK/412/00 was a coronavirus and, moreover, showed that it had the same partial gene order (S-E-M-5-N-3′ untranslated region) as IBV. This gene order is unlike that of any known mammalian coronavirus, which does not have a gene analogous to the gene 5 of IBV. The gene 5 of the turkey virus had two open reading frames, 5a and 5b, as in IBV and the coronaviruses isolated from turkeys in North America. The turkey/UK/412/00 also resembled IBV, but not mammalian coronaviruses, in having three open reading frames in the gene encoding E protein (gene 3). The percentage differences between the nucleotide sequences of genes 3 and 5 and the 3′ untranslated region of turkey/UK/412/00 when compared with those of IBVs were similar to the differences observed when different strains of IBV were compared with each other. No sequences unique to the turkey isolates were identified. These results demonstrate, for the first time, that a coronavirus was associated with disease in turkeys outside of North America and that it is a Group 3 coronavirus, like IBV.

Introduction

To date, coronaviruses in turkeys have only been confirmed in the US and Canada (Nagaraja & Pomeroy, 1997). A disease of turkeys initially known as ‘mud fever’ or ‘bluecomb disease’ was first described in the state of Washington in the 1940s and rose to prominence following outbreaks in Minnesota in 1951. Some 20 years later, it was demonstrated that the causative agent was a coronavirus (Hofstad *et al.*, 1969; Adams & Hofstad, 1971, 1972a; Ritchie *et al.*, 1973). The disease is now most appropriately referred to as ‘coronaviral enteritis of turkeys’, and the virus is commonly referred to as turkey coronavirus.

Turkeys of any age can be infected by turkey coronaviruses, morbidity being close to 100%, and mortality varying from < 10 to 50% or more, being greatest in young birds. The virus has been detected only in epithelium of the intestinal tract (enterocytes lining the upper portion of intestinal villi) and bursa of Fabricius (follicular and interfollicular

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epithelium; Adams *et al.*, 1970; Nagaraja & Pomeroy, 1997). Birds huddle together for warmth, stop eating, lose weight and have wet droppings. Older birds are depressed, and their head and skin darkens. Wings may be seen to droop, the back arch and the head to retract (Nagaraja & Pomeroy, 1997). Clinical signs can be observed for up to 2 weeks and recovery of weight, if achieved, may take several weeks. Laying birds experience a drop in production. Infection of gnotobiotic poults with a turkey coronavirus alone produced only mild disease (Adams & Hofstad, 1972b) but addition of gut flora was reported to exacerbate the disease (Larsen, 1979).

Turkey coronaviruses are also one of a number of pathogens associated with poult enteritis and mortality syndrome (PEMS), of increasing economic importance in the US during the 1990s (Goodwin *et al.*, 1995; Barnes & Guy, 1997; Brown *et al.*, 1997; Guy *et al.*, 2000; Koci *et al.*, 2000; Schultze-Cherry *et al.*, 2000; Yu *et al.*, 2000a,b). The disease is essentially one of poults up to 1 month of age. Two manifestations of the syndrome have been described: 'spiking mortality of turkeys', and the less severe 'excess mortality of turkeys'. Yu *et al.* (2000a) have reported that turkey coronavirus by itself was able to cause mortality and clinical responses similar to those of natural PEMS. Guy *et al.* (2000) obtained only mild responses to a turkey coronavirus alone but produced severe disease and high mortality (79%) when poults were co-infected with the virus and an enteropathogenic *Escherichia coli*.

Prior to sequence analysis (reviewed by Guy, 2000), turkey coronaviruses had been assigned as the sole member of coronavirus Group 4, infectious bronchitis virus (IBV) being the only member of Group 3 (Siddell *et al.*, 1983). Group 1 comprised viruses related antigenically to transmissible gastroenteritis virus while Group 2 contained, among others, bovine coronavirus (BCoV). Further antigenic (Michaud & Dea, 1993) and gene sequence analyses showed that some US and Canadian turkey coronavirus isolates were closely related to BCoV. The M and N genes of the turkey viruses had > 99% identity with the corresponding genes of BCoV (Dea *et al.*, 1990; Verbeek & Tijssen, 1991; Verbeek *et al.*, 1991). This resulted in the turkey coronaviruses being transferred to coronavirus Group 2, concomitantly reducing the number of groups from four to three (Lai & Cavanagh, 1997). Ismail *et al.* (2001) have shown that a classical BCoV isolate, DB2, can cause enteritis in poults. This and other circumstantial evidence (Brown *et al.*, 1996) suggests that some turkeys in the field may be infected by BCoV.

More recently, other coronavirus isolates from turkeys have been found to be closely related to IBV (Guy *et al.*, 1997; Breslin *et al.*, 1999a,b; Stephensen *et al.*, 1999; Guy, 2000). The M and N protein genes of three coronaviruses, isolated from turkeys in Minnesota, North Carolina and Indiana at different times, had > 90% identity with the corresponding genes of IBV and had similar gene 5 (located between the M and N genes) sequences and 3′ untranslated regions (UTRs). The three turkey coronavirus isolates differed from each other to a similar extent as IBV isolates differ among each other.

Figure 1 shows the genome organization of human coronavirus 229E (representing Group 1 coronaviruses), murine hepatitis virus (Group 2) and IBV (Group 3) (Enjuanes *et al.*, 2000). A monocistronic and a dicistronic gene encode the small envelope protein (E) of Group 1 and 2 coronaviruses, respectively. In IBV (Group 3), the E protein is encoded by the third open reading frame (ORF 3c) of a tricistronic gene (gene 3) (Boursnell *et al.*, 1985; Liu *et al.*, 1991). The Group 1 and 2 coronaviruses have no ORFs analogous to the 3a and 3b ORFs of IBV. Similarly, gene 5, which has two ORFs (5a and 5b), is unique to Group 3 coronaviruses. Thus, the presence in a coronavirus genome of a tricistronic gene 3, located between the spike and membrane protein genes, and a dicistronic gene 5, located between the membrane protein and nucleoprotein genes, would be indicative of a Group 3 coronavirus.

In this report, we describe a coronavirus that was recovered from the caecal contents of 13-day-old turkey poults in Great Britain. The poults had exhibited stunting and 4% mortality, and had fluid caecal and intestinal contents. The virus had a genome organization characteristic of IBV and gene sequences very like those of IBV. This is the first report and description of a turkey coronavirus outside North America.

Materials and Methods

Origin of the turkey coronavirus

In May 2000, samples of caecal content from 13-day-old turkeys were received at VLA Weybridge for virological investigation. The poults were from a multi-age farm with approximately 10 000 birds on site. The samples were from a house containing 500 birds, of which approximately 100 showed stunting, unevenness and lameness, with 4% mortality.

Electron microscopy

For virological investigation, samples of caecal content were submitted for electron microscopy examination. Briefly, a 20% (w/v) homogenized suspension of caecal content was prepared in deionized water. After clarification of the suspensions by centrifugation at 720 × *g* for 5 min, the supernatant was concentrated by ultracentrifugation at 32 000 × *g* for 1 h at 4°C. The resulting pellet was resuspended in a minimal volume of deionized water and examined by negative contrast electron microscopy.

Extraction of RNA

The caecal sample (300 µl) from a turkey was added to 300 µl guanidinium isothiocyanate denaturation solution (Chomczynski & Sacchi, 1987; Li *et al.*, 1993). This mixture was freeze-thawed before the addition of 50 µl of 3 M sodium acetate (pH 4.1) and 600 µl phenol-chloroform-isoamylalcohol (25 : 24 : 1, pH 6.7; Amresco). The

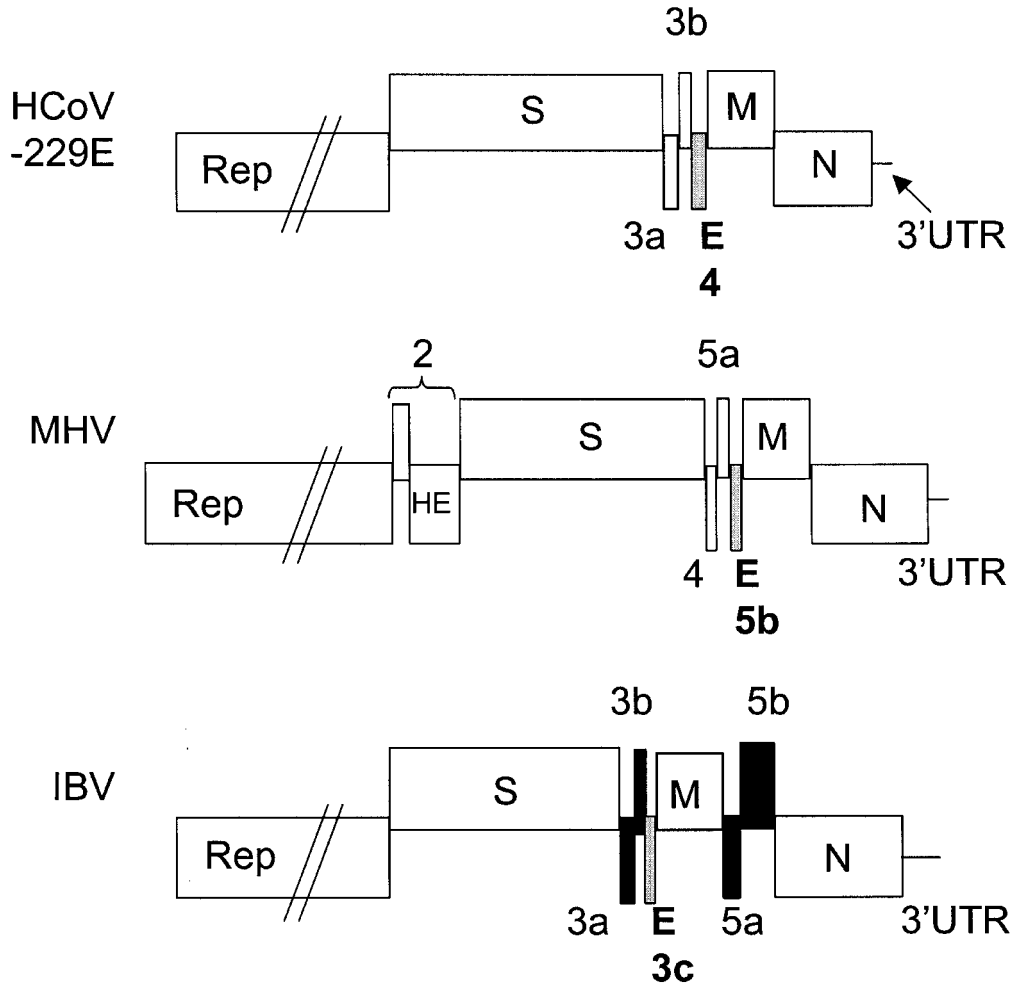

Figure 1. *Genome organization of human coronavirus 229E (HCoV 229E) (a Group 1 coronavirus), murine hepatitis virus (MHV) (Group 2) and IBV (Group 3). The E envelope protein is encoded by monocistronic gene 4 in Group 1, the second ORF of dicistronic gene 5 in Group 2 and in the third ORF of tricistronic gene 3 in IBV. Group 1 and 2 viruses do not have ORFs homologous with ORFs 3a and 3b of IBV. Gene 5 of IBV, which comprises two ORFs (5a and 5b) and is situated between the M and N genes, has no homologue in Group 1 and 2 viruses. Also, viruses in those groups do not have any gene between the M and N genes. Genes 3 and 5 are, therefore, discriminatory for IBV and perhaps for other avian coronaviruses.*

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).

Reverse transcriptase-polymerase chain reaction general procedure

All reverse transcriptase (RT) procedures were performed using 2 µl RNA in a 10 µl reaction volume containing 20 u RNaseH-M-MLV reverse transcriptase (Superscript II; Life Technologies), 5 pmol antisense 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 polymerase chain reaction (PCR) by the addition of PCR components to the RT mix as previously described (Capua *et al.*, 1999; Cavanagh *et al.*, 1999). The PCRs were performed in a Touchdown thermocycler (Hybaid) using the following 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 1% 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 2, and the sequence of these oligonucleotides and of others used in the sequencing reactions are presented in Table 1.

RT-PCR and sequencing within the 3′ UTR

Oligonucleotides were designed within the 3′-terminal part of the 3′ UTR and are universal to all known types of IBV, as this region of the UTR is highly conserved among IBVs. The oligonucleotide, UTR11–, used in the RT reaction, was used with UTR41+ in a RT-PCR to generate a 266 base pair product. This product was directly sequenced using these two oligonucleotides.

RT-PCR of gene 3

Comparison of the S gene sequences of IBV strains US/Beaudette/37, US(Ma)/M41/41, US(Ar)/Ark99/73, US(Ny)/CU-T2/89, Netherlands/D207/78 and Belgium/B1648/87 (named according to Cavanagh (2001)) resulted in the selection of four sequences, near the 3′ end of the S2 part of the S gene, that were identical in all six isolates; four positive sense oligonucleotides (PS1+, PS2+, PS3+ and PS4+) were synthesized (Table 1 and Figure 2).

The M gene sequences of IBV strains US/Beaudette/37, UK/6/82 and Netherlands/D1466/78 were compared, resulting in the selection of three sequences, near the 5′ end of the M gene, that were identical (negative-sense oligonucleotides PM4– and PM5–) or very similar (PM6–) among the three isolates (Table 1 and Figure 2).

Various combinations of these M gene negative-sense and S gene positive-sense oligonucleotides were used to see whether a gene analogous to gene 3 of IBV could be amplified from the turkey/UK/

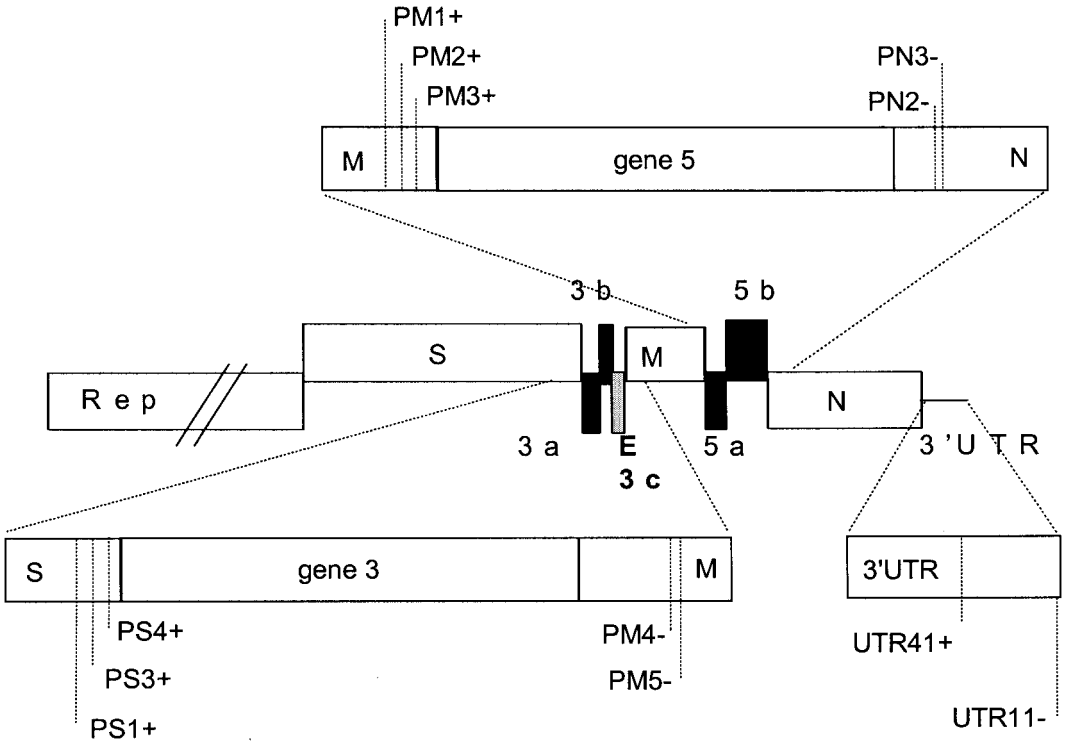

Figure 2. *Approximate positions of the oligonucleotides used for RT-PCRs of RNA from turkey/UK/412/00, relative to the genome of IBV. Rep, Replicase gene; S, spike glycoprotein gene; E, small envelope protein gene (part of gene 3); M, integral membrane glycoprotein gene; 5, gene 5; N, nucleocapsid protein gene; 3′ UTR, untranslated region at the 3′ end of the genome.*

412/00 RNA. Oligonucleotides PM5– and PM4– were used in the RT reactions. PCRs using PS1+, PS3+ or PS4+ with either negative-sense oligonucleotide routinely gave products of approximately 0.9 kilobase pairs (kbp). The products generated by PS1+ and PM5– or PM4– were directly sequenced using oligonucleotides PS7+, PS8+, PM9– and PM10– (Table 1 and Figure 2).

Table 1. *Sequence and position of the oligonucleotides used for RT-PCRs and sequencing*[a]

Oligonucleotide	Sequence	Gene	Position in genome[b]
PS1+[c]	TATATTAAGTGGCCTTGGTATGT	S2	23 632–23 654
PS3+	TTGTTGTTGTGGATGCTTTGG	S2	23 733–23 753
PS4+	TGAGTAAGTGTGGTAAGAAAT	S2	23 768–23 789
PS7+	AATAGTAACTTACAGTCTAGAC	3	23 982–24 003
PS8+	GTCCAGCCTAATGTTAAACTT	3	24 019–24 039
PM9–[d]	TACTACCCATGTATACCA	3	24 348–24 365
PM10–	GGAAACTCGTTAACAATAAC	3	24 444–24 463
PM1+	CTGGCGAGCTAGAAAGTGTA	M	25 133–25 152
PM2+	GGTGGAAGTAGCCTTTACAC	M	25 159–25 178
PM3+	ATAAATGTGTGTGTGTAGAGAG	M	25 179–25 200
PM4–	CAAAAGCACCATAACACTATCAT	M	24 658–24 680
PM5–	CAATGTTAAGGGGCCAAAAGCA	M	24 673–24 694
PM7+	TGTAACCTTCCAAGTTGACAATG	Upstream of 5	25 319–25 341
PM8–	TCAAGATTCTGCTTGTCCTGCT	5	25 780–25 801
PN2–	TGAAACCAAGATGCATTTCC	N	25 963–25 982
PN3–	GCTTTTATTGCTTGAAACCAAGAT	N	25 971–25 994
UTR11–[e]	GCTCTAACTCTATACTAGCCTA	3′ UTR	27 586–27 607
UTR41+[e]	ATGTCTATCGCCAGGGAAATGTC	3′ UTR	27 342–27 364

[a] The primers used for RT-PCRs are shown in Figure 2.

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

[c,d] Negative-sense and positive-sense oligonucleotides, respectively.

[e] UTR11– and UTR41+ are similar but not identical to UTR1– and UTR4+ of Adzhar *et al.* (1996). Minor changes were made at the ends of the oligonucleotides, taking into account sequence of the UTRs of Australian isolates of IBV (Sapats *et al.*, 1996)

RT-PCR of gene 5

Comparison of the M gene sequences of IBV strains USA/Beaudette/37, England/6/82 and Netherlands/D1466/78 resulted in the selection of three sequences, near the 3′ end of the M gene, that were identical (oligonucleotide PM3+) or very similar (oligonucleotides PM1+ and PM2+; Table 1 and Figure 2).

Comparison of the N gene sequences of Williams *et al.* (1992) for IBV strains USA/Beaudette/37, USA(Ma)/M41/41, USA(Ar)/Ark99/73, Netherlands/H120/55, USA(De)/Gray/60 and Japan/KB8523/83 resulted in the selection of three sequences, near the 5′ end of the N gene, that were very similar among the six strains and that were used to design negative sense oligonucleotides PN2– and PN3– (Table 1 and Figure 2).

Oligonucleotides PN2– and PN3–, used in the RT reactions, were used with PM1+, PM2+ or PM3+ for PCRs to determine whether turkey/UK/412/00 had a gene situated between the membrane protein and nucleoprotein genes. The PCR products of approximately 0.8 kbp, generated using PM3+ and PN2– or PN3–, were cloned as difficulties were experienced when sequencing the PCR product directly.

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 M13 forward and reverse 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, v2.0 (Amersham), using 1 µg PCR product or 2 µg plasmid DNA. The oligonucleotides used for sequencing are presented in Table 1. Sequences were compared using GeneDoc Multiple Sequence Alignment Editor and Shading Utility v 2.5.000 (www.psc.edu/biomed/genedoc).

Accession numbers

The gene sequences for turkey/UK/412/00 have been submitted to the EMBL database and have been assigned the following accession numbers: partial 3′ UTR sequence, AJ310642; gene 3, AJ310640; gene 5 and the preceding intergenic region, AJ310641.

Results

Case history

In May 2000, samples of caecal content from 13-day-old turkeys were received for virological investigation. The birds were from a multi-age farm with approximately 10 000 birds on site. In a house containing 500 turkeys, approximately 100 showed stunting, unevenness and lameness, with 4% mortality.

At *post mortem* examination, the main gross features were fluid caecal and intestinal contents. There was some evidence of uncharacterized osteo/chondrodystrophy in several of the birds. No significant bacteria were cultured. Three out of five sera examined by enzyme-linked immunosorbent assay (ELISA) gave positive values for *Mycoplasma meleagridis* and two gave positive results by rapid slide agglutination test. No mycoplasmas were cultured using routine methods, nor were detected by PCR. Histological examination of tissues was largely unremarkable, apart from some sections of small intestine that showed crypt dilation and flattened epithelia.

Negative contrast electron microscopy revealed virus particles with typical coronavirus morphology (data not shown).

Sequencing of the 3′ UTR confirmed coronavirus identity

The oligonucleotides (UTR41+ and UTR11–; Table 1) that were used to attempt the amplification of the 3′ UTR of the UK turkey virus corresponded to sequences with Region 2 of the UTR that are highly conserved among 22 isolates of IBV, isolated in Australia (Sapats *et al.*, 1996), Europe (Dalton *et al.*, 2001), Japan (Sutou *et al.*, 1988) and the USA (Williams *et al.*, 1993), and three genetically IBV-like coronaviruses from turkeys in the USA (Breslin *et al.*, 1999b). The RT-PCR was successful in amplifying a 266 bp cDNA from turkey/UK/412/00 RNA.

Sequence analysis of the 266 bp RT-PCR product revealed that turkey/UK/412/00 had 97.6 to 98.9% identity with the corresponding region of the 3′ UTR of most of the avian coronaviruses for which the 3′ UTR sequence is available, i.e. the 22 IBV isolates and the three IBV-like turkey coronaviruses of the USA (Figure 3). The exceptions were in comparison with some of the Australian IBV isolates (N1–88, Q3–88 and V18–91) that had only 89 to 91% identity with turkey/UK/412/00 and with the other IBVs (Figure 3).

There was nothing notable about the 3′ UTR sequences of isolate turkey/UK/412/00, other than the very close identity with the North American turkey isolates and most of the chicken IBV isolates.

The turkey virus had a gene 3 typical of IBV

Oligonucleotides corresponding to common sequences near the end of the S gene (gene 2) and the start of the M gene (gene 4; Figure 2) of several IBV isolates were used to successfully amplify gene 3 of turkey/UK/412/00. Sequence analysis revealed that turkey/UK/412/00 had a gene 3 typical of IBV (Figure 4).

The 3a and 3b ORFs of turkey/UK/412/00 were exactly the same length as those of several IBV isolates, as illustrated by the alignment of the translational start and stop codons (Figure 4). The translation start codon of the turkey/UK/412/00 ORF 3c was also at the same relative position as for the E gene (ORF 3c) in the IBV isolates, but the turkey virus ORF 3c was shorter than in some of the IBV isolates. This is not a unique finding; several IBV isolates of different serotypes and countries of origin have shorter ORF 3c sequences than other isolates (Figure 4; Cavanagh & Davis, 1988; Liu *et al.*, 1991).

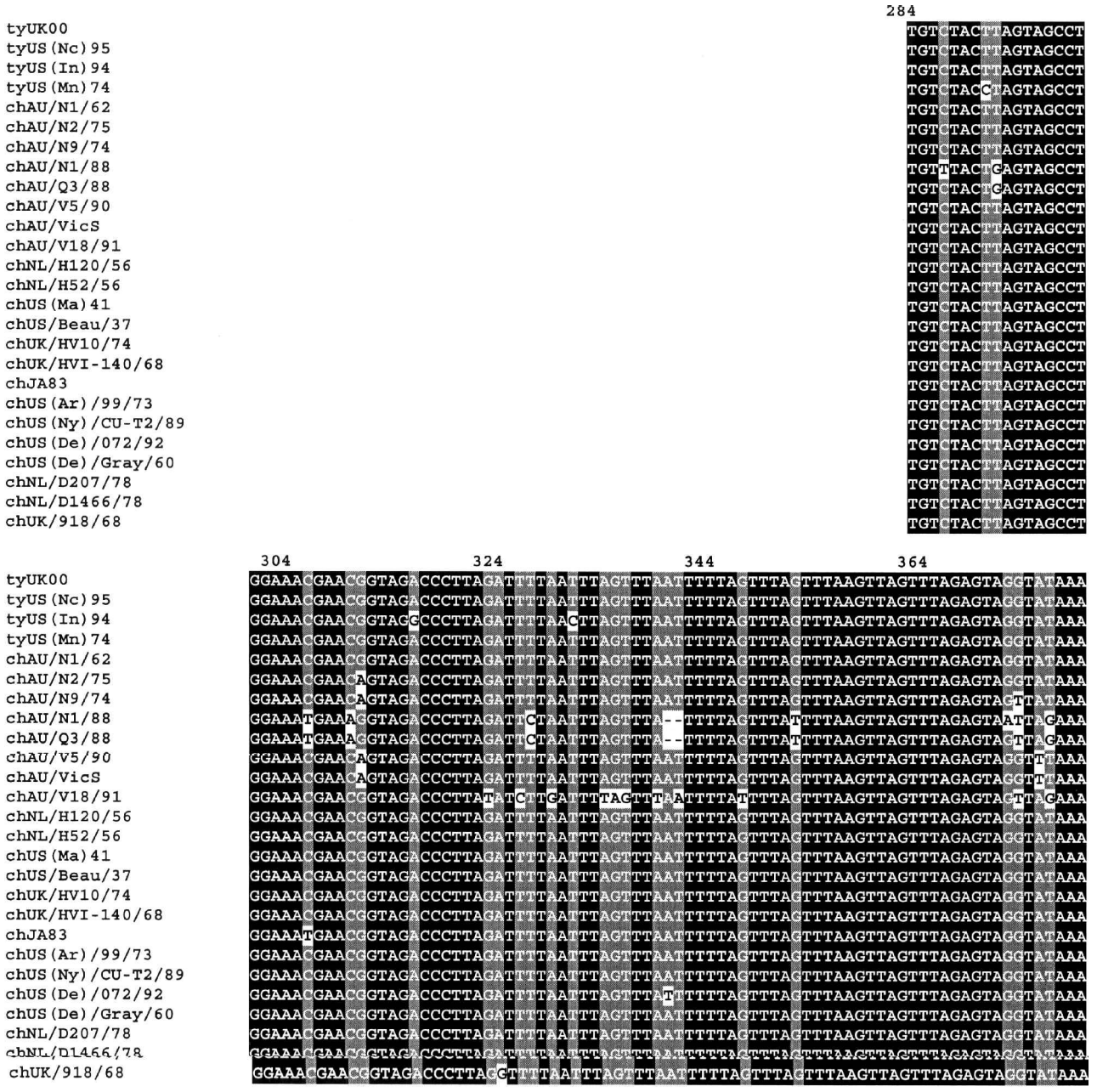

Figure 3. *Comparison of part of the 3′ UTR of turkey/UK/412/00 (tyUK00) with that of three coronaviruses from turkeys (prefixed 'ty') from North America and 22 IBV isolates (prefixed 'ch', for 'chicken') from Australia (AU), Europe (NL, Netherlands; and UK), Japan (JA) and North America (US). The numbers are relative to the first nucleotide of the 3′ UTR of IBV H120, which has the longest known 3′ UTR of avian coronaviruses. Black shading, All viruses had the same nucleotide at that position; grey and white shading, nucleotides possessed by the majority and minority, respectively, of isolates at a given position. Ar, Arkansas; Beau, Beaudette; De, Delaware; In, Indiana; Ma, Massachusetts; Mn, Minnesota; Nc, North Carolina; Ny, New York.*

The ORF 3c sequence terminates after the start of the M gene ORF in several IBV isolates, i.e. the genes overlap. The deletions within ORF 3c of turkey/UK/412/00 had the effect of reducing the first part of the M ORF by six nucleotides (two codons; Figure 4). This has also been observed among IBV isolates (Figure 4; Cavanagh & Davis, 1998).

Comparison of the ORF 3a, 3b and 3c sequences of turkey/UK/412/00 with those of IBV Beaudette revealed nucleotide identities of 90, 85 and 89%, respectively, and 91, 72 and 81% for amino acids, respectively. These differences are similar to those observed among IBV isolates (Liu *et al.*, 1991). The observation that the amino acid sequence of ORF 3b was the least conserved reflects the finding among IBV isolates (Liu *et al.*, 1991).

Other notable features of the data include: (a) the transcription associated sequence (TAS), CTGAA-CAA, for the gene 3 of IBV strains was present in the turkey/UK/412/00 sequence (Figure 4, nucleotides 57 to 64); (b) the pair of translation stop codons for the S gene of IBV strains were in the same relative position in turkey/UK/412/00 (Figure

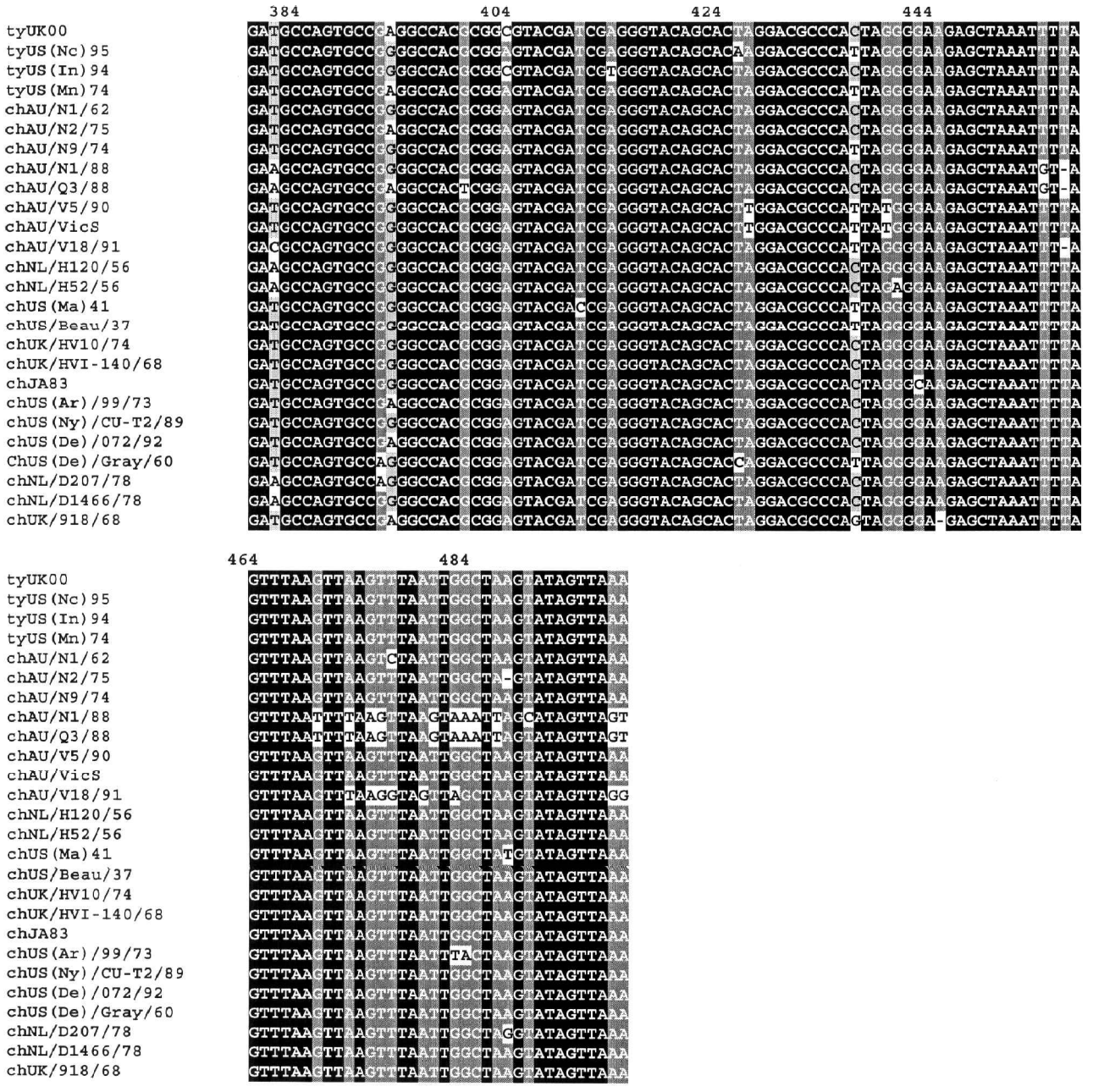

Figure 3. *(Continued)*

4, nucleotides 86 to 91); and (c) the TAS, CTTAACAA, for the M gene was also conserved in turkey/UK/412/00 (Figure 4, nucleotides 652 to 659).

In conclusion, there was nothing remarkable about gene 3 of turkey/UK/412/00 or the North American isolates.

The turkey virus had a gene 5 typical of IBV

There are no genes in the Group 1 and Group 2 coronaviruses that are homologous to gene 5 of IBV; this gene is unique to Group 3 coronaviruses (Figure 1). To determine whether turkey/UK/412/00 had a gene 5 homologous to that of IBV, RT-PCRs were performed. Oligonucleotides corresponding to common sequences near the 3′ end of the M gene (gene 4) and the 5′ end of the nucleocapsid protein gene (gene 6) of several IBV isolates (Figure 2) were used. PCR products of 0.8 kbp were obtained, indicative of a gene 5 sequence within turkey/UK/412/00.

Sequence analysis confirmed the presence of a gene 5, with ORFs 5a and 5b, homologous to that of IBV and the North American IBV-like turkey isolates (Figure 5). The ATG translational start codons and TGA/TAG translation stop codons of ORFs 5a and 5b lined-up exactly among all the isolates (Figure 5). Comparison of the gene 5 nucleotide sequences in Figure 5 showed that IBV chicken/Japan/KB8523/83 differed from the other IBV isolates by 6 to 11%; gene 5 of turkey/UK/

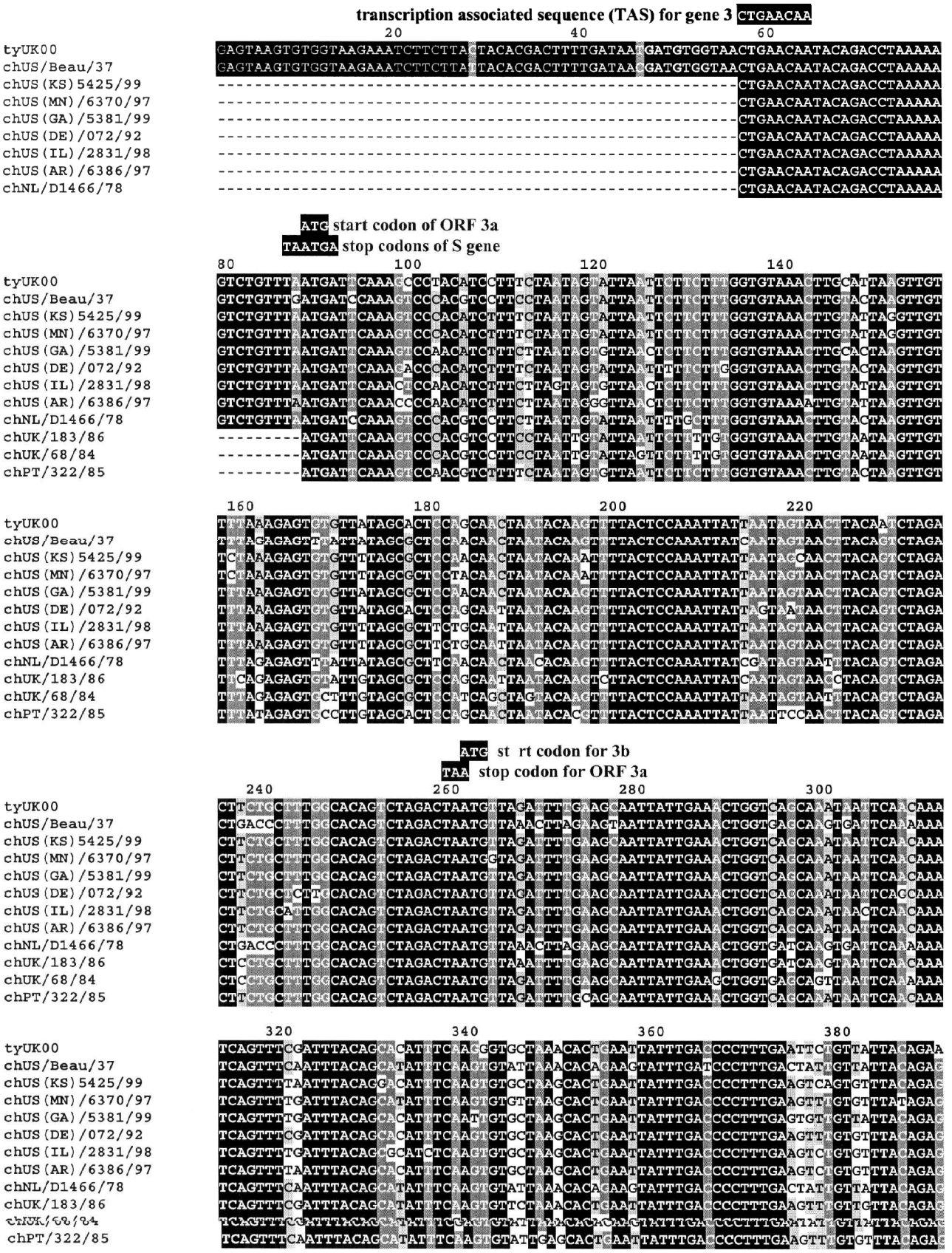

Figure 4. *Comparison of the end of the spike glycoprotein (S) gene, gene 3 and beginning of the M gene of turkey/UK/412/00 (tyUK00) with that of 11 IBV isolates (prefixed 'ch' for 'chicken') from, Europe (NL, Netherlands; PT, Portugal; and UK) and North America (US). (–) Deletions, and (. . .) sequences not available. Shading as described in Figure 3 caption. GA, Georgia; KS, Kansas; IL, Ilinois; MN, Minnesota; AR, Arkansas; DE, Delaware; Beau, Beaudette.*

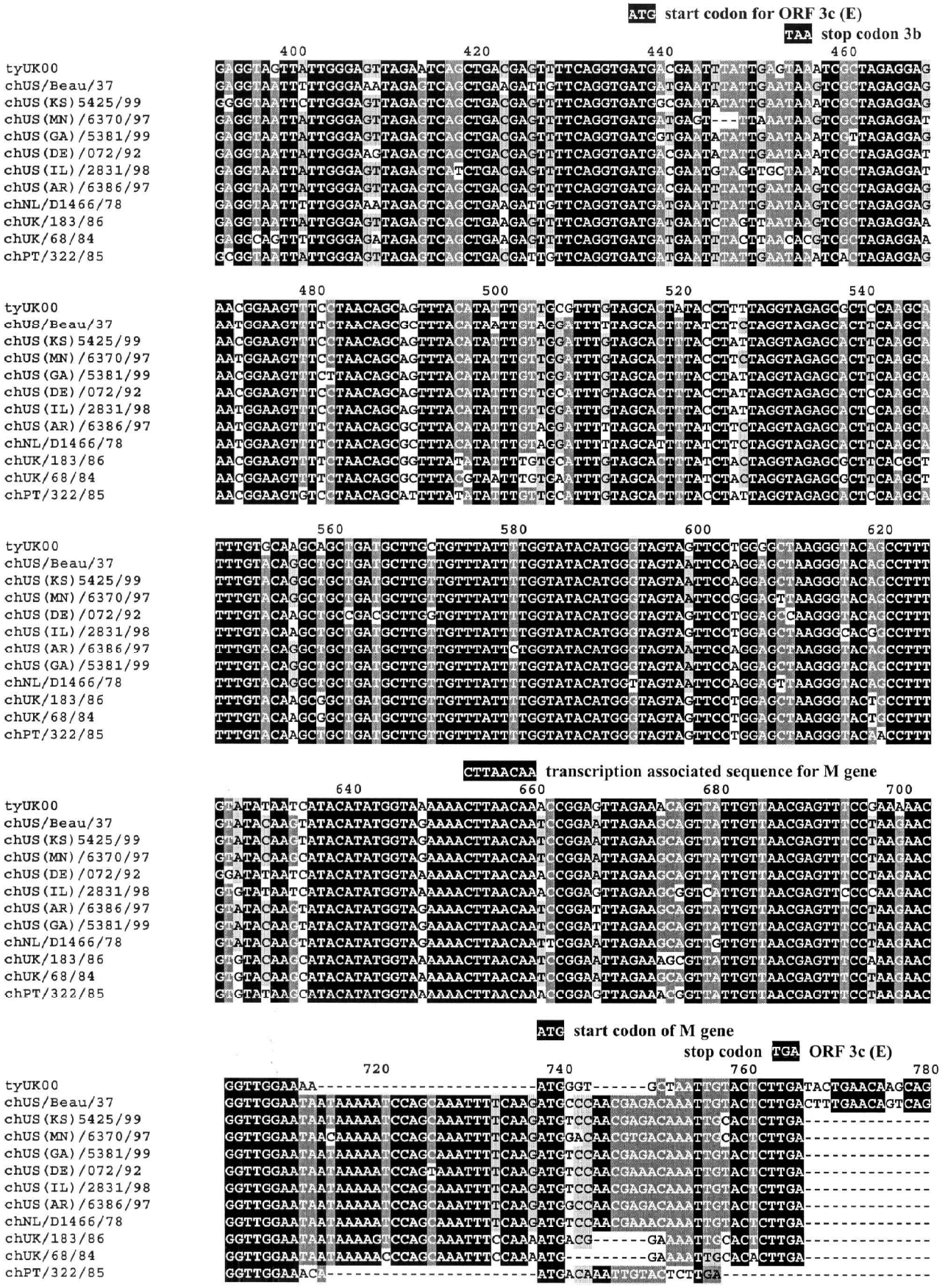

Figure 4. *(Continued)*

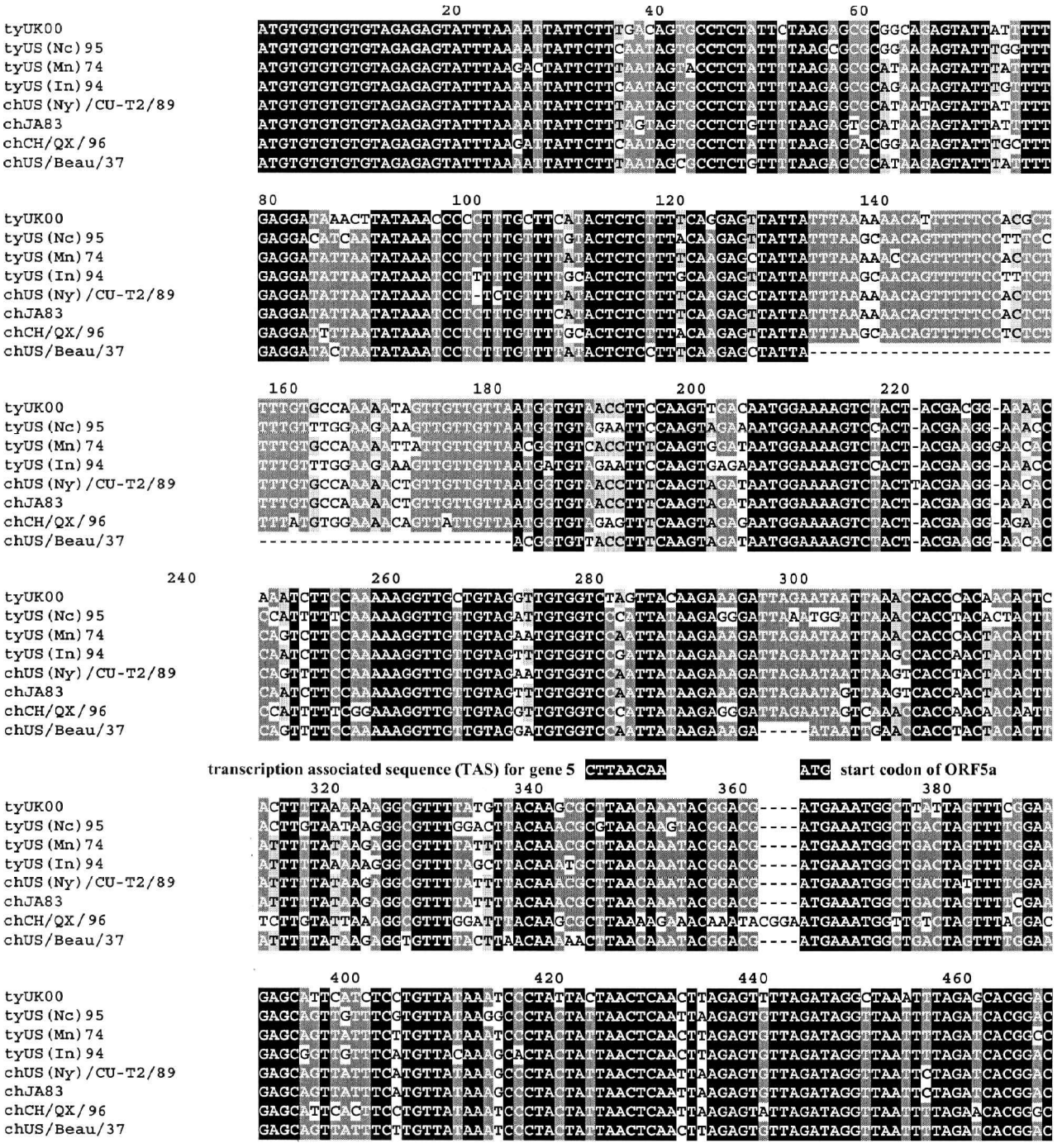

Figure 5. *Comparison of gene 5 of turkey/UK/412/00 (tyUK00) with that of three isolates of three coronaviruses from turkeys (prefixed 'ty') from North America (US) and four IBV isolates from China (CH), Japan (JA) and North America (US). IBV chicken/Japan/KB8523/83 is therefore represented as ChJA83. The first 340 or so nucleotides shown comprise an intergenic region between gene 5 and the upstream M gene. (–) Deletions. Shading as described in Figure 3 caption. In, Indiana; Mn, Minnesota; Nc, North Carolina; Ny, New York; Beau, Beaudette.*

412/00 was within this range, at 10%. The ORF 5a and 5b sequences of the IBV isolates differed from chicken/Japan/KB8523/83 by 7 to 16% and 1 to 9%, respectively, compared with 14 and 6%, respectively, for turkey/UK/412/00. The IBV isolate, chicken/China/QX/96 (Wang *et al.*, 1998), differed more from chicken/Japan/KB8523/83 than did any of the turkey isolates.

Most IBV genes, except for the M gene, overlap with the neighbouring downstream gene. Following the end of the M gene there is an intergenic sequence of approximately 360 nucleotides, depending on the isolate, before the start of gene 5. The M/gene 5 intergenic region of turkey/UK/412/00 was typical of that of IBV isolates and the three IBV-like North American turkey isolates in terms of length (Figure 5). The Beaudette strain of IBV has substantial deletions in this intergenic region. Among the isolates shown in Figure 5, the differences in the sequences of the intergenic region

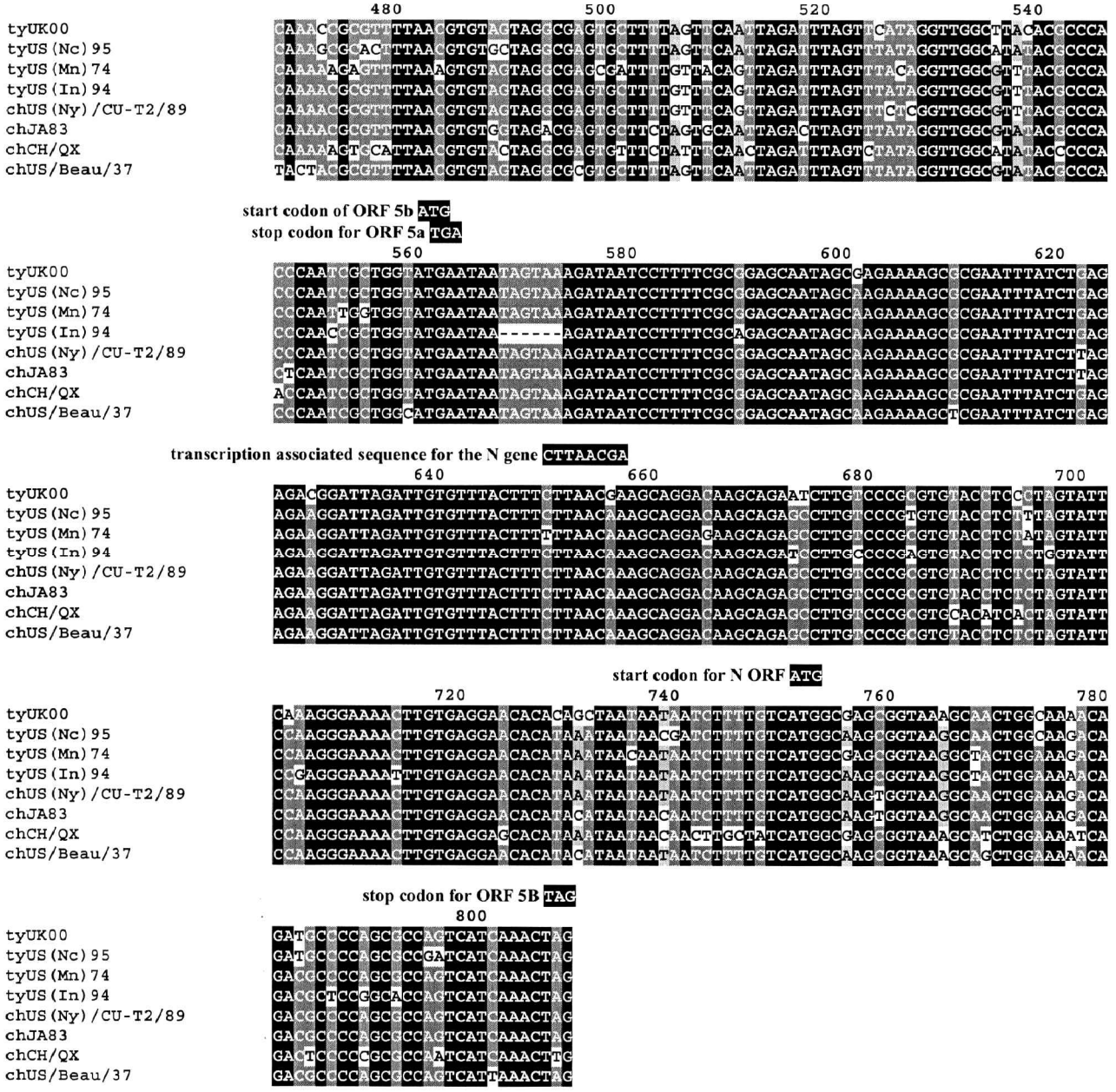

Figure 5. *(Continued)*

compared with chicken/Japan/KB8523/83 ranged from 4 to 17%, that of turkey/UK/412/00 being 12%.

The TAS, CTTAACAA, of gene 5 of turkey/UK/412/00 was identical to that possessed by most IBV strains (Figure 5). Interestingly, turkey/USA(Nc)/NC/95 and chicken/China/QX/96 did not have TASs identical to the other strains. The TAS of the N gene of turkey/UK/412/00, which commences before the end of the 5b ORF, was CTTAACGA, rather than the more common CTTAACAA. The sequence was obtained from two clones, derived from one RT-PCR product, and confirmed by direct sequencing of DNA arising from an independent RT-PCR product.

Discussion

To our knowledge, this is the first unequivocal demonstration of a coronavirus in turkeys outside North America. Electron microscopy of gut contents of a scouring turkey revealed the presence of a virus with morphology typical of that of a coronavirus. Gene sequencing confirmed that the virus, turkey/UK/412/00, was very similar to IBV. In North America, turkeys with enteritis, more

recently referred to as poult enteritis and mortality syndrome, have often been associated with the presence of a coronavirus (Barnes & Guy, 1997; Brown *et al.*, 1997; Guy *et al.*, 2000; Loa *et al.*, 2000), although an astrovirus (Koci *et al.*, 2000; Schultze-Cherry *et al.*, 2000; Yu *et al.*, 2000a,b) is also able to cause the disease. Therefore, it is quite possible that turkey/UK/412/00 was involved in the enteritis of the turkeys in which it was detected. However, the pathogenic characteristics of this virus remain to be determined, as does the incidence of coronaviruses in turkeys beyond North America.

Perhaps the most remarkable thing that can be said about turkey/UK/412/00, from the data reported herein, is that it has a genome organization and gene sequences typical of IBVs. So, too, do three North American coronavirus isolates from turkey (Guy *et al.*, 1997; Breslin *et al.*, 1999a,b; Stephensen *et al.*, 1999; Guy, 2000). These four turkey viruses possess no unique genetic features, on the basis of the data to date, that would distinguish them, as a group, from chicken isolates (IBV). This raises the question as to whether the genetically IBV-like turkey viruses should be considered to be a species distinct from IBV or whether the turkey viruses and IBV are simply variants of one coronavirus species.

Guy (2000) has proposed that the coronaviruses isolated from turkeys and domestic fowl coronaviruses (IBV) be considered as distinct species, at least until more data is available. The turkey viruses have a strict tropism for the epithelia of the intestines and bursa of Fabricius (Patel *et al.*, 1975; Naqi *et al.*, 1972). Although IBVs can grow in enteric tissues (Lambrechts *et al.*, 1993; Pensaert & Lambrechts, 1994; Dhinakar Raj & Jones, 1997), the initial target organ, and the most common site of clinical disease, is the respiratory tract (Cavanagh & Naqi, 1997). Guy *et al.* (1999) have reported that a turkey coronavirus (genetically related to IBV) grew, aclinically, in the intestinal tissues and bursa of Fabricius of domestic fowl chicks inoculated at 1 day of age, and the virus was not detected in respiratory tissues. These findings may be used to support the view that the genetically IBV-like turkey coronaviruses and IBV are distinct species.

On the other hand, the data from the chick experiments could be used to support the view that the two viruses are simply host range variants. The two viruses are antigenically related (Guy *et al.*, 1997), to the extent that commercial ELISAs incorporating serotypes of IBV were able to detect coronavirus antibodies in sera from field turkeys (Weisman *et al.*, 1987; Loa *et al.*, 2000). In a previous study in the UK, significant titres of IBV (M41) antibody were detected by haemagglutination inhibition (HI) test in the sera of 16-week-old turkeys in which coronavirus-like particles had been detected at 11 weeks of age. Interestingly, no infectious bronchitis HI antibodies were detected in the sera using other strains of infectious bronchitis antigen (R. Gough and S. Lister, unpublished observations). This does not mean that turkey/UK/412/00 will necessarily have a S protein with a sequence close to that of the M41 strain (Massachusetts serotype), as the serotype-specificity of the HI test is complex (De Wit, 2000).

As shown herein and by Breslin *et al.* (1999a,b), neither the genome organization nor specific gene sequences distinguished the turkey isolates as a group from IBVs as a group. The question of the species status of coronaviruses from different avian species has been discussed in more detail recently (Cavanagh, 2001).

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RÉSUMÉ

Détection d'un coronavirus, génétiquement apparenté au virus de la bronchite infectieuse du poulet, chez le dindonneau en Europe

Les contenus intestinaux de dindonneaux âgés de 13 jours, élevés en Grande-Bretagne appartenant à un lot hétérogène présentant un arrêt de croissance, des boiteries et 4 % de mortalité ont été analysés. A l'examen post mortem, ce sont les contenus intestinaux et cloacaux qui étaient liquides qui ont retenu l'attention. L'examen histologique des tissus n'a rien révélé, à l'exception de quelques coupes qui ont montré une dilatation des cryptes et un aplatissement de l'épithélium. Des examens en microscopie électronique par coloration négative des contenus caecaux ont révélé la présence de particules virales qui du point de vue taille et morphologie avaient l'apparence de coronavirus. L'ARN a été extrait (turkey/UK/412/00) et a fait l'objet de réactions de transcription inverse et d'amplification en chaîne par polymérase (RT-PCR) avec des oligonucléotides basées sur les séquences dérivées du virus de la bronchite infectieuse (IBV), un coronavirus de la poule. Les RT-PCRs ont confirmé que le turkey/UK/412/00 était un coronavirus et, de plus, qu'il avait les gènes dans le même ordre (S-E-M-5-N-3′ UTR) comme celui de l'IBV. L'ordre des gènes est différent de celui des coronavirus des mammifères qui n'ont pas un gène analogue au gène 5 de l'IBV. Le gène 5 du virus de la dinde possède deux cadres ouverts de lecture (ORFs) 5a et 5b comme l'IBV et les coronavirus isolés chez la dinde en Amérique du Nord. Le turkey/UK/412/00, comme l'IBV mais différent des coronavirus des mammifères, présente trois ORFs au niveau du gène codant pour la protéine E (gène 3). Les différences de pourcentage entre les séquences nucléotidiques des gènes 3, 5 et le 3′ UTR du turkey/UK/412/00 comparées à celles des IBVs sont similaires aux différences observées quand les différentes souches d'IBV sont comparées entre elles. Il n'a été identifié aucune séquence spécifique des isolats de dinde. Ces résultats montrent, pour la première fois, qu'un coronavirus est associé à une maladie chez les dindes, hors du continent Nord américain et qu'il appartient au groupe 3 des coronavirus, comme l'IBV.

ZUSAMMENFASSUNG

Nachweis eines Coronavirus von Putenküken in Europa, das mit dem Hühner-Bronchitisvirus verwandt ist

Der Darminhalt von 13 Tage alten Putenküken in Großbritannien wurde untersucht, da bei den Tieren Kümmern, Ungleichmäßigkeit und Lahmheit mit einer Mortalität von 4% zu beobachten waren. Bei der Sektion bestanden die hauptsächlichen makroskopischen Besonderheiten in flüssigem Zäkum- und Darminhalt. Die histologische Untersuchung der Gewebe ergab weitgehend unauffällige Befunde, abgesehen von einigen Schnitten, die eine Kryptendilatation und abgeflachtes Epithel zeigten. Die Negativkontrast-Elektronenmikroskopie von Blinddarminhalt ließ Viruspartikeln erkennen, die in Größe und Morphologie das Aussehen eines Coronavirus hatten. RNA wurde extrahiert (Turkey/UK/412/00) und in einer Reihe von Reverse-Transkription-Polymerase-Kettenreaktionen (RT-PCR) eingesetzt, bei denen die Oligonukleotide auf Sequenzen beruhten, die von Hühnerbronchitisvirus (IBV), einem Coronavirus des Haushuhns, stammten. Die RT-PCRs bestätigten, dass Turkey/UK/412/00 ein Coronavirus war und zeigten außerdem, dass es die gleiche partielle Gen-Anordnung (S-E-M-5-N-3′ UTR) hatte wie IBV. Diese Gen-Reihenfolge ist anders als die von irgendeinem der bekannten Säuger-Coronaviren, die kein Gen haben, das dem Gen 5 von IBV entspricht. Das Gen 5 des Putenvirus hat zwei offene Leserahmen (ORFs), 5a und 5b, wie beim IBV und den in Nordamerika aus Puten isolierten Coronaviren. Das Turkey/UK/412/00 ähnelte IBV, aber nicht Säuger-Coronaviren, auch insofern, als es in dem Protein E kodierenden Gen (Gen 3) drei ORFs hat. Die prozentualen Unterschiede zwischen den Nukleotid-Sequenzen der Gene 3 und 5 und dem 3′ UTR von Turkey/UK/412/00 waren beim Vergleich mit denen von IBVs ähnlich wie die Unterschiede, die festgestellt wurden, als verschiedene IBV-Stämme miteinander verglichen wurden. Es wurden keine Sequenzen nachgewiesen, die allein auf die Putenisolate beschränkt waren. Diese Resultate zeigen zum ersten Mal, dass ein Coronavirus außerhalb von Nordamerika mit einer Erkrankung bei Puten verbunden war, und dass es wie IBV ein Coronavirus der Gruppe 3 ist.

RESUMEN

Detección de un coronavirus, genéticamente relacionado con el virus de bronquitis infecciosa de los pollos, en pavos jóvenes en Europa

Se analizaron los contenidos intestinales de pavos jóvenes de 13 días de edad en Gran Bretaña que mostraban crecimiento lento, desigualdad en el lote y cojera, con un 4% de mortalidad. Al examen postmortem, la lesión más importante fue un contenido cecal e intestinal fluido. El examen histológico de los tejidos no presentó ningún tipo de alteración remarcable, salvo dilatación de las criptas y un epitelio aplanado que se observó en algunas secciones. La observación de tinciones negativas de contenidos cecales al microscopio electrónico reveló la presencia de partículas virales, que en tamaño y morfología tenían la apariencia de un coronavirus. Se extrajo el ARN (turkey/UK/412/00) y fue utilizado en la técnica de transcriptasa reversa-reacción en cadena de la polimerasa (RT-PCR) con oligonucleótidos basados en secuenciasderivadas de un virus de bronquitis infecciosa (IBV), un coronavirus de aves domésticas. La técnica de RT-PCR confirmó que el turkey/UK/412/00 era un coronavirus y, además, demostró que tenía el mismo orden de genes (S-E-M-5-N-3′ UTR) de IBV. Este orden de genes es diferente de cualquier otro coronavirus de mamíferos, que no tiene un gen análogo al gen 5 de IBV. El gen 5 del virus de pavo tenía dos secuencias de lectura abierta (ORFs), 5a y 5b, como el IBV y los coronavirus aislados de pavos en América del Norte. El turkey/UK/412/00 también se parecía al IBV, pero no a otros coronavirus de mamíferos, al presentar 3 ORFs en el gen que codificaba para la proteína E (gen 3). El porcentaje de diferencias entre la secuencia de nucleótidos de los genes 3 y 5 y el 3′ UTR del turkey/UK/412/00 cuando se comparaba con los de los IBVs era similar a las diferencias observadas cuando las diferentes cepas de IBV se comparaban entre ellas. No se identificaron secuencias únicas en estos virus aislados de pavo. Estos resultados demostraron que, por primera vez, un coronavirus estaba asociado con enfermedad en pavos fuera de América del Norte y que éste es un coronavirus del Grupo 3, como el IBV.