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A novel variant of avian infectious bronchitis virus resulting from recombination among three different strains

W. Jia[1], K. Karaca[1,*], C. R. Parrish[2], and S. A. Naqi[1]

[1] Department of Avian and Aquatic Animal Medicine,
[2] The James A. Baker Institute for Animal Health, College of Veterinary Medicine,
Cornell University, Ithaca, New York, U.S.A.

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Summary. An antigenic variant of avian infectious bronchitis virus (IBV), a coronavirus, was isolated and characterized. This strain, CU–T2, possesses a number of unusual features, which have not been previously observed in IBV. The S1 glycoprotein of CU–T2 carries virus-neutralizing and serotype-specific epitopes of two IBV serotypes, Arkansas (Ark) and Massachusetts (Mass). Sequence analysis revealed that the virus, originally an Ark serotype, has acquired the Mass-specific epitope by mutation(s). This provides evidence that point mutations may lead to generation of IBV antigenic variants in the field. It was further observed that two independent recombination events involving three different IBV strains had occurred in the S2 glycoprotein gene and N protein gene of CU–T2, indicating that genomic RNA recombination in IBV may occur in multiple genes in nature. It was especially significant that a sequence of Holland 52 (a vaccine strain) had replaced half of the N gene of CU–T2. This proves that recombination among vaccine strains is contributing to the generation of IBV variants in the field. Based on these observations it is predicted that every IBV field isolate could have unique genetic nature. Therefore, several recently reported diagnostic and serotyping methods of IBV which are based on dot-blot hybridization, restriction fragment length polymorphism (RFLP), and polymerase chain reaction (PCR), may not reveal the true antigenic and/or genetic nature of IBV isolates, and may in fact yield misleading information.

Introduction

Infectious bronchitis virus (IBV), a member of the family *Coronaviridae*, causes a highly contagious respiratory and reproductive disease in chickens which

* Present address: Department of Veterinary Pathobiology, University of Minnesota, St. Paul, Minnesota, U.S.A.

results in heavy economic losses to the commercial poultry industry, worldwide [24].

IBV has a single-stranded, positive sense RNA genome. It is 27.6 kilobases long, encodes three major structural proteins, and is organized as 5′-POL-S-Gene3-M-Gene5-N-3′ (Fig. 1) [9, 35, 36]. The three structural proteins of IBV are the spike glycoprotein (S protein), the membrane glycoprotein (M protein) and the nucleocapsid protein (N protein). The S protein of IBV is cleaved post-translationally into N-terminal S1 and C-terminal S2 proteins [13]. The S1 protein carries antigenic epitopes which induce virus neutralizing (VN) antibody, and also determine the virus serotype [14, 15]. The N protein may induce cellular immune responses against IBV [6].

IBV isolates have been grouped into serotypes based on VN test [16, 18]. Although more than twenty distinct IBV serotypes have been reported in the USA [17, 32], most isolates causing disease in the field belong to Massachusetts (Mass), Connecticut (Conn), or Arkansas (Ark) serotypes [24]. Live vaccines containing strains of those three serotypes have been routinely used in the field. However, more frequently than ever, outbreaks of the disease are being observed in vaccinated flocks. These vaccine failures, however, are not always due to infection by distinct serotypes, but could be caused by antigenic variants of IBV emerging from wild-type or vaccine viruses by point mutation(s) or genomic RNA-RNA recombination [12, 25].

Although point mutation(s) are believed to contribute to the generation of new antigenic variants of IBV, no direct evidence for that has been presented. RNA-RNA recombination has been shown to occur at a high frequency both in vivo and in vitro in mouse hepatitis virus (MHV), a coronavirus [3, 22, 23, 27–29, 31], yet, no isolation of recombinant MHV from natural disease in mice has been reported. In the case of IBV, one Japanese and three European isolates have been suggested to be possible recombinants [11, 25]. However, the origin of

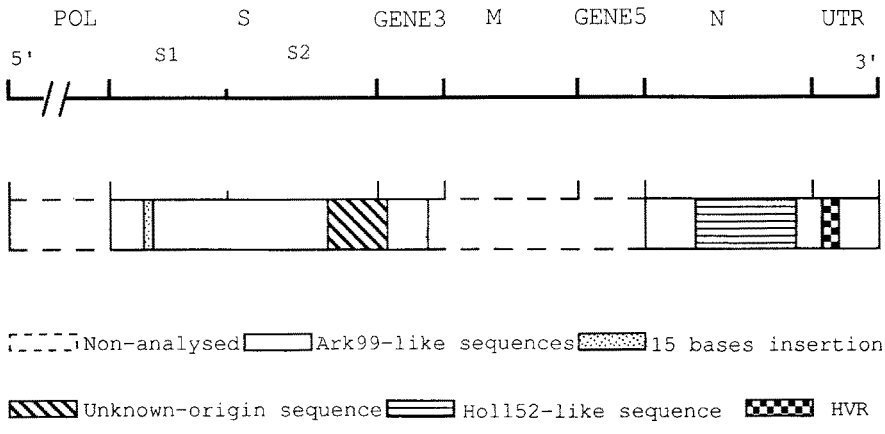


Fig. 1. Schematic characterization of the CU-T2 genome. *POL* RNA polymerase gene. *S* S protein gene; *S1* S1 protein gene; *S2* S2 protein gene. *N* N protein gene. *UTR* 3′ end untranslated region. *HVR* Hypervariable region

the recombination fragments in those isolates was unknown. More recently, two American field isolates were found to contain fragments of Mass-like sequences in the S1 gene, which were 94% to 95% homologous to IBV strain Mass41 [38]. All the recombinations suggested in IBV so far have been in the S gene, and none of those reports have addressed whether the recombinations had altered the antigenic characteristics of the viruses.

Here we describe an IBV serotype and recombination variant, CU–T2, which reacted with two monoclonal antibodies (MAbs) specific to the VN epitopes of IBV serotypes Mass and Ark, respectively. We present evidence that the CU–T2 was originally an Ark serotype which subsequently acquired a Mass-specific epitope on the S1 protein through mutation(s). We also show that CU–T2 had undergone two independent genomic RNA recombination events within the S2 and N protein gene (N gene), respectively, involving three different IBV strains. Furthermore, we provide evidence that CU–T2 had acquired a sequence of Holland 52 (Holl52), a vaccine strain of Mass serotype used in the field. Implications of such genetic and antigenic changes in diagnosis and serotyping of IBV are discussed.

Materials and methods

Viruses

Four previously characterized IBV strains, Ark99, Holl52, Conn46, and Mass41 [19, 20] were obtained from our own laboratory stocks. CU–T2 was isolated from an adult commercial chicken flock in New York State, which was experiencing reduced egg production and poor egg shell quality associated with IBV infection. Virus isolation from the flock was performed through the use of "sentinel" chickens. Briefly, ten specific-pathogen-free (SPF) chickens were placed among the affected birds in separate cages for ten days. Subsequently, the "sentinel" chickens were euthanized and the tracheas were collected for passage in SPF chicken embryos. CU–T2 strain was recovered from the allantoic fluid of inoculated embryos, and was subsequently propagated and plaque purified in chicken kidney cell (CKC) culture monolayers [16].

MAbs and enzyme-linked immunosorbent assay (ELISAs)

MAbs 1318, 1588, and 940 specific to S1-associated serotype-specific epitopes of IBV prototype Ark, Mass, and Conn, respectively, have been described elsewhere [21]. The protocols for both antigen-capture and competitive-binding ELISAs have also been reported previously [21,34]. The ELISAs were performed using whole virions, or purified S1 protein of CU-T2 according to the procedures used previously [21].

Preparation of viral genomic RNA

Viruses were propagated in chicken embryos and purified by sucrose gradient as described previously [37]. Viral genomic RNAs were extracted with phenol-chloroform and used for cDNA synthesis and RNA sequencing.

cDNA synthesis, cloning and sequencing

For cDNA synthesis, an Uni-ZAP II kit (Stratagene, La Jolla, CA) was used in conjunction with a synthetic primer complementary to a sequence located downstream of the S genes of

several Mass serotype strains [5] (5′-GAACTAGTCTCGAGGAAGGACGTGGGACTTTG-3′). The cDNA library was screened by hybridization with a 5′ end-radiolabeled probe prepared from a *Sty*I fragment derived from a cDNA clone (pBSM41M) of S gene of Mass41 obtained from Solvay Animal Health, Inc., Mendota Heights, MN. Phagemids containing IBV genes were prepared by in vivo excision in *Escherichia coli* (*E. coli*) using a protocol furnished by Stratagene (La Jolla, CA). DNA sequencing was carried out by the dideoxy method, using a Sequenase 2.0 DNA sequencing kit (United States Biological (USB), Cleveland, OH). cDNA clones pARK99-8p1, pBARK99-8p2, pBT2-t223, pBT2-t229, and pBT2-t237 were used for sequencing. Both strands of each cDNA clone were sequenced. Sequence data were obtained from cDNA clones, except for the first 1100 bases of the S gene, the last 200 bases of the N gene, the 3′ end non-coding region of CU-T2, and the partial Gene3 of Ark99 and Holl52, which were obtained from direct sequencing of IBV genomic RNA. Direct sequencing of IBV genomic RNA was also performed at least twice for those regions, using an RNA sequencing kit (USB, Cleveland, OH). Sequence alignments and analysis were performed using the Genetics Computer Group (GCG) program.

Nucleotide sequence accession numbers

The nucleotide sequences reported here have been deposited with the GenBank. The accession numbers are as follows: S protein gene of IBV Ark99, L10384; S protein gene of IBV CU–T2, U04739; N protein gene of IBV CU-T2, U04805; 3′ end non-coding region of IBV CU–T2, U04804.

Results

Demonstration of specific epitopes both for Ark and Mass serotypes on the S1 protein of CU–T2

Tables 1 and 2 summarize results of the three types of ELISAs performed with the Ark, Conn, and Mass serotype-specific MAbs. In both the indirect and antigen-capture ELISA, the Ark-specific and Mass-specific MAbs reacted with both the whole virions and the purified S1 protein of CU–T2 strain (Table 1).

Table 1. Reactivity of Ark- and Mass-specific MAbs with whole virions and S1 proteins of CU–T2

MAbs	Mab specificity	Indirect ELISAs against		Antigen-capture ELISAs against S1[a]
		virions	S1	
1318	Ark S1	1.75[b]	>2.90	Nd[c]
1588	Mass S1	0.26	1.19	0.75
940	Conn S1	Nd	0.13	0.10

[a] S1 antigen of CU–T2 was captured with MAb 1318 and reacted with biotinylated MAbs 1588 and 940

[b] Optical density (O.D.) at 490 nm. O.D. values $\geqslant 0.20$ were considered positive

[c] *Nd* Not done

Table 2. Results of two competitive-binding ELISAs with Ark- and Mass-specific MAbs

		Unlabeled competing MAbs		
Labeled MAbs	Mab specificity	1318 (Ark)	1588 (Mass)	940 (Conn)
1318	Ark	0.16/0.18[a]	1.60/2.20	1.40/2.69
1588	Mass	0.71/0.50	0.16/0.05	0.30/0.89

[a] ELISA optical density values at 490 nm from two independent assays. Values ≥ 0.20 were considered positive

In the competitive-binding ELISA, unlabeled Mass- and Ark-specific MAbs blocked reaction of the homologous labeled MAbs, but not with the heterologous MAbs.

Sequence analysis of S gene of Ark99 strain

In order to determine the genetic basis for the expression of the Ark- and Mass-specific epitopes on the S1 protein of CU–T2, the entire S gene of CU–T2 was sequenced. The entire S gene of Ark99 was also sequenced for comparison. All base positions indicated in this paper are calculated from the S gene start codon [4]. Amino acid positions indicated are of the S protein precursor, including the signal peptide.

The gene of Ark99 was found to be 3480 bases long, with a capacity to code 1159 amino acids. The S1 and S2 genes were 1629 and 1851 bases long, respectively, and were predicted to encode 543 and 616 amino acids, respectively. Comparison of the deduced amino acid sequences of the S proteins of Ark99 and Mass41 [5], revealed an 88.2% homology (1022/1159). However, the S1 protein showed only 78.1% homology (424/543), compared to 97.1% (598/616) for the S2 (data not shown). Amino acid variations in the S1 proteins of the two viruses were more than 7 times as frequent as for the S2 protein. Alignment of the S1 gene of Ark99 with that of Mass and several European IBV isolates revealed that fifteen bases (5′-TGGAAGTGCTACGCC-3′) between bases 419 and 435 of the Ark99 strain were unique to that virus (data not shown). A 95.3% homology was observed between the first 1100 bases of the S2 gene of Ark99 and Mass41, while the last 751 bases of the two genes were identical (Fig. 2).

Sequence analysis of S gene of CU–T2 strain

The nucleotide sequences of S1 genes of CU-T2 and Ark99 exhibited over 98% homology. The fifteen-base insertion in the S1 gene of Ark99 described above was also observed in the CU-T2 S1 gene, but its location was between bases

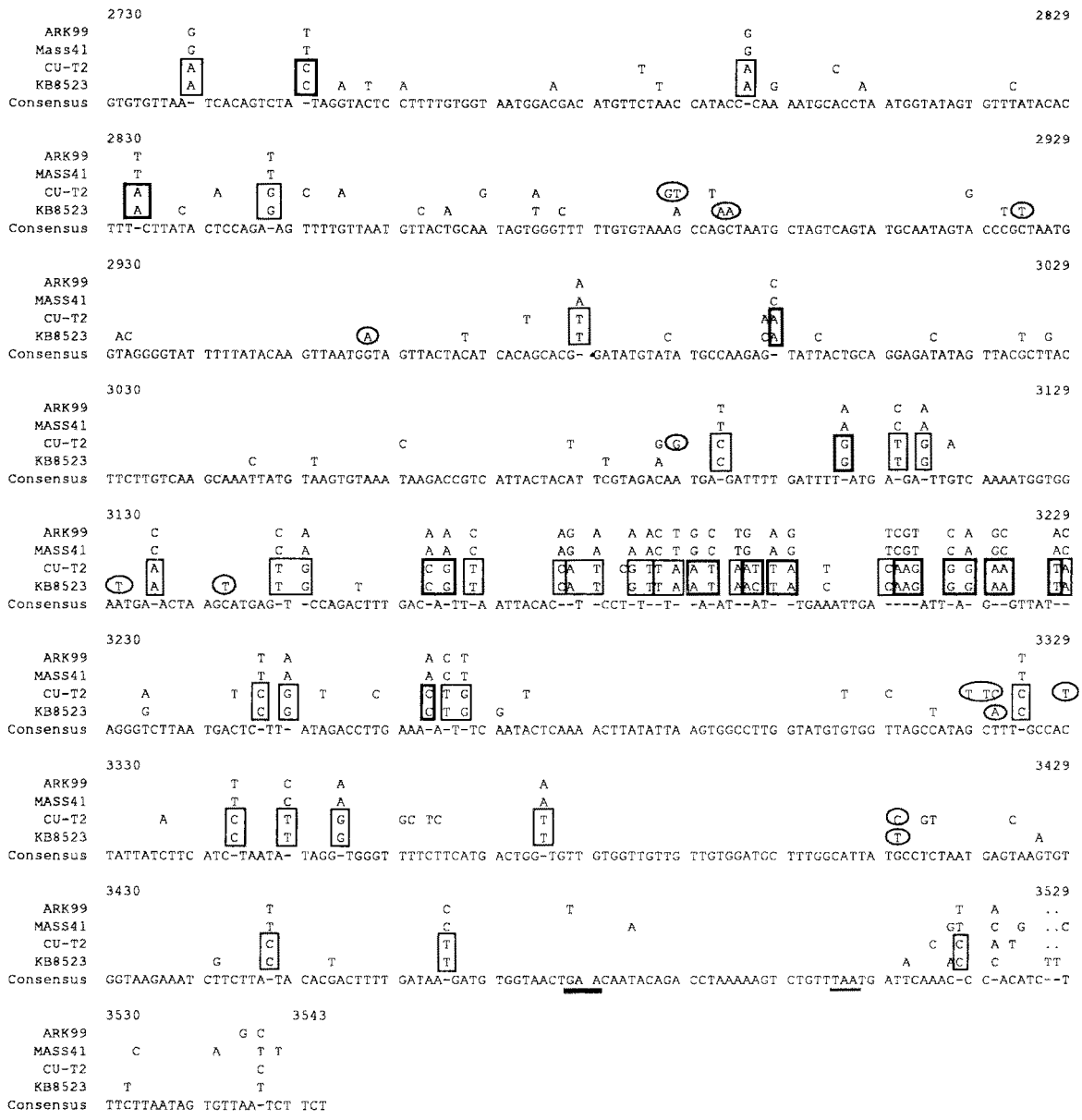


Fig. 2. Sequence alignment of S2 genes (only 3′ ends are shown) of Ark99, CU–T2, KB8523 and Mass41. The consensus nucleotide sequence of the strains is shown in the last line. In the other lines, the nucleotide differences among strains have been indicated. Identical nucleotides between CU–T2 and KB8523, but different from Ark99 and Mass41 in the crossover region, have been boxed. Nucleotide substitutions which are circled or are in the heavy-line boxes in the crossover region, produced non-synonymous codons. Nucleotide substitutions which are not circled or are in light-line boxes in the crossover region produced synonymous codons. Heavy underline indicates the stop codon of Ark99, and light underline indicates the stop codons of CU–T2, KB8523 and Mass41. The sequences of S2 genes of Mass41 and KB8523 have been previously reported [5, 37]

416 and 432, as compared to bases 419 and 435 for Ark99. The difference in the position of the insertion was due to a three-base deletion (TAA) between bases 356 and 360 of the S1 gene of CU–T2 compared to the S1 gene of Ark99. A single noncoding substitution was noted within the fifteen-base insertion of CU–T2 (T versus C at position 428).

A 96.7% homology was observed between the amino acid sequences of the S1 proteins of CU–T2 and Ark99. Seventeen amino acids were found to be different in the S1 protein of CU–T2 compared to those in Ark99, and those differences were distributed throughout the gene (data not shown).

A 96.6% homology was observed in the first 1100 bases of the S2 genes of Ark99 and CU–T2, but, the following 751 bases had only 87.6% homology (Fig. 2). Comparison with published sequences showed that the sequence of this 751-base region of CU–T2 was more similar to a fragment in the 3′ end of the S2 gene of a Japanese isolate KB8523 [37] than to those of all other strains. Although 76 nucleotide substitutions were found in that fragment of the CU–T2 and KB8523, 66 of those were synonymous substitutions (Fig. 2). The downstream crossover site of this fragment was about 12 to 13 bases downstream of the stop codon of the S2 gene of CU–T2. Through a substitution of T by G in the TAA stop codon, the S2 gene of CU-T2 was 27 bases longer than that of Ark 99, but of the same length as the S2 gene of KB8523 (Fig. 2), indicating that a fragment of approximately 751 bases was incorporated into the S2 gene of CU–T2 by recombination.

Sequence analysis of the Gene3, N gene and 3′ non-coding region of CU–T2 strain

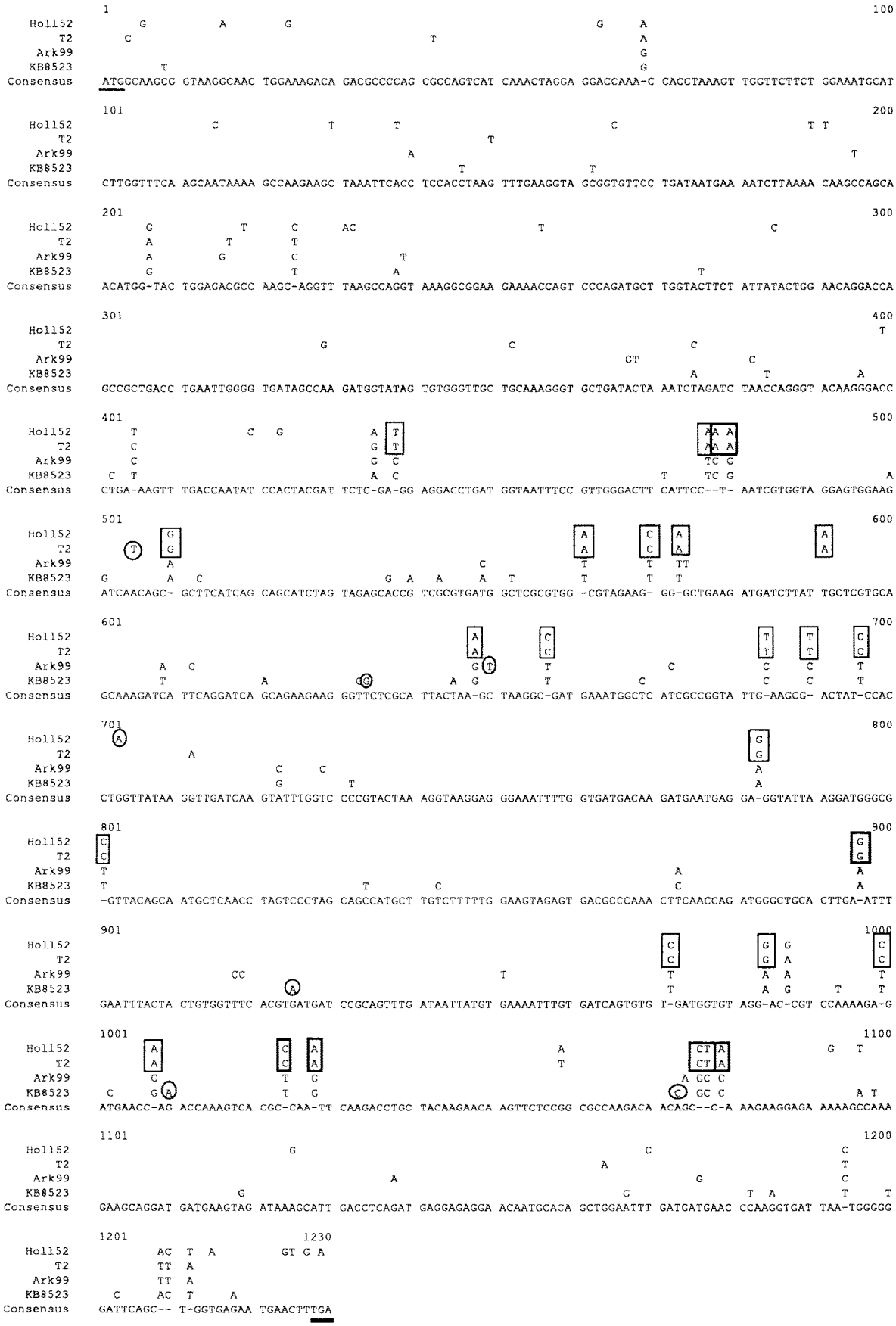
To further study the possible evolutionary origin of the CU-T2 strain, the first 268 bases of Gene3, the N gene and the 3′ non-coding region of CU–T2 were sequenced subsequently. The same regions of Gene3 of Ark99 and Holl52 were also sequenced for comparison. The first 268 bases of the Gene3 of CU–T2 matched more closely with those of Ark99 Gene3 (97.6% homology), than of Mass41 [7], KB8523 [37], or Holl52 (Table 3).

The N gene of CU–T2 was 1230 bases long, with a capacity to code 410 amino acids. Around 436 bases at the 5′ end and 150 bases at the 3′ end of

Table 3. Distances among Gene3 of different serotype of IBV[a]

	Holl52	Mass41	CU–T2	Ark99	KB8523
Holl52	1.000	0.973	0.865	0.873	0.906
Mass41		1.000	0.854	0.863	0.878
CU–T2			1.000	0.976	0.909
Ark99				1.000	0.928
KB8532					1.000

[a]Data was obtained from 5′ end 268 bases of the Gene3 without gaps


Holl52
T2
Ark99
KB8523
Consensus
ATGGCAAGCG GTAAGGCAAC TGGAAAGACA GACGCCCCAG CGCCAGTCAT CAAACTAGGA GGACCAAA-C CACCTAAAGT TGGTTCTTCT GGAAATGCAT
CTTGGTTTCA AGCAATAAAA GCCAAGAAGC TAAATTCACC TCCACCTAAG TTTGAAGGTA GCGGTGTTCC TGATAATGAA AATCTTAAAA CAAGCCAGCA
ACATGG-TAC TGGAGACGCC AAGC-AGGTT TAAGCCAGGT AAAGGCGGAA GAAAACCAGT CCCAGATGCT TGGTACTTCT ATTATACTGG AACAGGACCA
GCCGCTGACC TGAATTGGGG TGATAGCCAA GATGGTATAG TGTGGGTTGC TGCAAAGGGT GCTGATACTA AATCTAGATC TAACCAGGGT ACAAGGGACC
CTGA-AAGTT TGACCAATAT CCACTACGAT TCTC-GA-GG AGGACCTGAT GGTAATTTCC GTTGGGACTT CATTCC--T- AATCGTGGTA GGAGTGGAAG
ATCAACAGC- GCTTCATCAG CAGCATCTAG TAGAGCACCG TCGCGTGATG GCTCGCGTGG -CGTAGAAG- GG-GCTGAAG ATGATCTTAT TGCTCGTGCA
GCAAAGATCA TTCAGGATCA GCAGAAGAAG GGTTCTCGCA TTACTAA-GC TAAGGC-GAT GAAATGGCTC ATCGCCGGTA TTG-AAGCG- ACTAT-CCAC
CTGGTTATAA GGTTGATCAA GTATTTGGTC CCCGTACTAA AGGTAAGGAG GGAAATTTTG GTGATGACAA GATGAATGAG GA-GGTATTA AGGATGGGCG
-GTTACAGCA ATGCTCAACC TAGTCCCTAG CAGCCATGCT TGTCTTTTTG GAAGTAGAGT GACGCCCAAA CTTCAACCAG ATGGGCTGCA CTTGA-ATTT
GAATTTACTA CTGTGGTTTC ACGTGATGAT CCGCAGTTTG ATAATTATGT GAAAATTTGT GATCAGTGTG T-GATGGTGT AGG-AC-CGT CCAAAAGA-G
ATGAACC-AG ACCAAAGTCA CGC-CAA-TT CAAGACCTGC TACAAGAACA AGTTCTCCGG CGCCAAGACA ACAGC--C-A AAGAAGGAGA AAAAGCCAAA
GAAGCAGGAT GATGAAGTAG ATAAAGCATT GACCTCAGAT GAGGAGAGGA ACAATGCACA GCTGGAATTT GATGATGAAC CCAAGGTGAT TAA-TGGGGG
GATTCAGC-- T-GGTGAGAA TGAACTTTGA

CU–T2 N gene were very similar to the comparable regions of Ark99. However, two crossover sites were observed around bases 436 and 1090 (Fig. 3). Between those two sites only five bases were different (99.2% homology) between the CU–T2 and the Holl52 strains, and of those, only two were non-synonymous (Fig. 3). Within the same region, 42 bases were different between CU–T2 and ARK99 (93.6% homology), and eight of those changes were non-synonymous (Fig. 3). There were many more differences in that region between CU–T2 and other IBV strains, including Mass 41, Gray, KB8523 and Beaudette (data not shown).

The 3′ end untranslated region (UTR) of the IBV genome was previously shown to contain an approximately 190-base hypervariable region (HVR) [39] (Fig. 1). That region of the CU–T2 sequence had a high homology with Ark 99 (96.4%), but lesser homology with KB8523 (89.3%), Holl52 (77.3%) and Mass41 (52.4%) (data not shown).

Discussion

The results show that CU–T2 is a serotype and recombination variant which originated from an Ark-like IBV. This is based on both the sequence data and MAb studies of the virus. The nucleotide sequence homologies between the S1 gene, most of S2 gene, Gene3 and the 3′ UTR of CU–T2 and Ark99 all attest to the origin of CU–T2 from an Ark-like virus. The fifteen-base insert observed in the S1 genes of both Ark99 and CU–T2, which was absent in the Mass serotype strains, further strengthens this conclusion (Fig. 1).

The seventeen amino acids in the S1 protein of CU–T2 which were different from those in Ark99 were distributed throughout the gene, indicating that these amino acid changes were not due to recombination. Therefore, it may be concluded that CU–T2 acquired the Mass-specific serotype epitope on the S1 protein as a result of point mutation(s). The results of competitive-binding ELISAs indicated that the Ark- and Mass-specific epitopes on CU–T2 are independent. These facts provide direct evidence that point mutation(s) can result in the generation of antigenic variants during a natural infection.

The results show that an approximately 751 base-long fragment of unknown origin was incorporated into the 3′ end of CU–T2 S2 gene by recombination. Interestingly, this recombinant fragment was similar to one previously observed

Fig. 3. Sequence alignment of N genes of CU–T2, Ark99, KB8523 and Holl52. The consensus nucleotide sequence of the strains is shown in the last line. In the other lines, the nucleotide differences among the strains have been indicated. Identical nucleotides between CU–T2 and Holl52, but different from others in the crossover region, have been boxed. Nucleotide substitutions which are circled or are in heavy-line boxes in the crossover region produced non-synonymous codons. Nucleotide substitutions which are not circled or are in light-line boxes in the crossover region produced synonymous codons. Heavy underline indicates the stop codons, and light underline indicates the start codons. The sequences of N genes, except those of CU–T2, have been previously reported [8, 37, 39]

in KB8523, a possible recombinant strain of IBV [25]. Recombination fragments in other two European isolates (6/82 and D207) were also found within the same regions [25], although they were of shorter length than those of CU–T2 and KB8523 strains. In an in vitro transfection experiment it was observed that RNA recombination in MHV occurred preferentially at certain selected sites (hot spots) [1]. In IBV, a secondary structure most likely exists at the 5′ end of Gene3 [30]. We found that the downstream crossover sites of both CU–T2 and KB8523 were located around that secondary structure (data not shown). The clustering of recombinations in IBV suggests that a recombination 'hot spot' most likely exists around the 3′ end of the S2 gene and the 5′ end of the Gene3, although the clustering may be a result of selection [2].

An additional recombination which resulted in the replacement of half (52.4%) of the N gene was also observed, indicating that genomic RNA recombination of IBV may involve more than two strains and may occur in multiple genes during natural infection. More interestingly, that recombination fragment in the CU–T2 is almost identical to a sequence (99.2% homology) in the N gene of Holl52 strain. Since both Ark99 and Holl52 strains have been used simultaneously as live vaccines in the USA [24], this finding provides convincing evidence that recombination among vaccine strains is contributing to the emergence of variants in the field. The significance of recombination in the N gene must also be viewed in the light of a recent report that the N protein may induce cellular immune responses against IBV [6].

The sequence of S1 portion of Ark99 S gene obtained from a cloned PCR product has been published more recently [38]. When the S1 portion of the entire S gene sequence of Ark99 obtained in this study was compared with that sequence, nine bases were found to be different, which would result in four amino acid changes in the S1 protein at positions 143, 335, 348 and 356.

Our data suggest that both inter-strain recombinations and mutation(s) are contributing to the generation of IBV variants in the field. We have shown here that genomic recombination between IBV strains may lead to replacements of large RNA fragments in multiple genes. The replacement, however, may not result in change of antigenic nature of IBV. In contrast, mutation(s) on the S1 protein alone may generate new VN epitope(s). The data imply that every field isolate of IBV could be unique in RNA sequence. Therefore, recently reported diagnostic and serotyping methods of IBV, such as dot-blot hybridization [33], restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR) [26, 40], may not reflect the true antigenic and/or genetic nature of the virus. Indeed, an exclusive use of such assays may yield misleading information on the antigenic and/or genetic characteristics of the virus.

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Authors' address: Dr. S. A. Naqi, Department of Avian and Aquatic Animal Medicine, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, U.S.A.

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