Evidence of genetic diversity generated by recombination among avian coronavirus IBV

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Summary. Previously, we demonstrated that the DE072 strain of IBV is a recombinant which has an IBV strain D1466-like sequence in the S gene. Herein, we analyzed the remaining 3.8 kb $3'$ end of the genome, which includes Gene 3, Gene 4, Gene 5, Gene 6, and the $3'$ non-coding region of the DE072 and D1466 strains. Those two viruses had high nucleotide similarity in Gene 4. However, the other individual genes had a much different level of sequence similarity with the same gene of the other IBV strains. The genome of five IBV strains, of which the complete sequence of the $3'$ end of the genome has been determined, were divided at an intergenic (IG) consensus sequence (CTGAACAA or CTTAACAA) and compared phylogenetically. Phylogenetic trees of different topology indicated that the consensus IG sequences and the highly conserved sequence around this regions may serve as recombination 'hot spots'. Phylogenetic analysis of selected regions of the genome of the DE072 serotype field isolates further support those results and indicate that isolates within the same serotype may have different amounts of nucleotide sequence similarity with each other in individual genes other than the S gene. Presumably this occurs because the consensus IG sequence serves as the template switching site for the viral encoded polymerase.

Introduction

Infectious bronchitis virus causes a highly contagious upper-respiratory disease in chickens. The disease is characterized by increased ocular and nasal secretions, excess mucus in the trachea, decreased weight gain and feed efficiency in broilers, and declines in egg production and egg quality in layers. Although live attenuated vaccines are available, IBV continues to be a severe economic problem in commercial chickens because many different serotypes of the virus exist and do not cross protect [3].

Infectious bronchitis virus (IBV) is a coronavirus in the new order *Nidovirales* [4]. Members of the *Nidovirales* order have a single stranded positive sense RNA genome and produce a 3′ nested set of subgenomic mRNAs when they replicate [4]. Coronaviruses are divided into three antigenic groups based primarily on their structural proteins. Infectious bronchitis virus is the type strain of coronaviruses and is the only virus placed in antigenic group three. Characteristics of this group are a cleaved spike (S) glycoprotein, an N-glycosylated membrane (M) protein, and no hemagglutinin/esterase protein [19]. The genome of IBV is approximately 27 kilobases in length [1]. It is organized into six regions, each containing one or more open reading frames (ORF's), which are separated by intergenic sequences (IG) that contain the signal for transcription of subgenomic mRNAs [1, 17]. The viral RNA-dependent RNA polymerase is encoded in the 5′ two thirds of the viral genome by two overlapping open reading frames (ORF1a and ORF1b) [1]. The structural protein genes are located 3′ to the viral polymerase gene and are in order from 5′ to 3′, the S glycoprotein gene (gene 2), the small envelope (E) gene (gene3), the M glycoprotein gene (gene4), and the nucleocapsid (N) gene (gene6) [19, 20].

Evolution in IBV has been observed through the occurrence of variant viruses and analysis of known serotypes. More than twenty serotypes within IBV have been recognized worldwide and are thought to be generated by insertions, deletions, point mutations and RNA recombination [2, 3, 6, 14]. Evidence of natural recombination for several IBV strains has been reported [10, 15, 22]. However, because of the limited sequence information, recombination has only been described for a small part of the genome. So far, the complete sequence of the 3′ end of the genome (from the 3′ end of the polymerase gene to the poly A tail) of only three strains, Beaudette, KB8523 and CU-T2 have been determined [1, 11, 20].

The DE072 strain was first isolated in 1992 in the Delmarva peninsula region of the USA and initial characterization of this virus indicated this virus was serologically distinct from any other IBV serotypes in North America [7]. Previously, we demonstrated that the DE072 strain is a recombinant which has a D1466-like sequence in the S1 and S2 genes [18]. D1466 is an IBV vaccine strain of the D212 serotype from the Netherlands [7, 13, 14]. Herein, we describe the sequences of the remaining genes of the DE072 and D1466 strains with the exception of gene 1(the polymerase gene). We conducted phylogenetic analysis by dividing the genome in the IG sequence to elucidate possible role of this sequence in the homologous recombination in IBV. Further, we conducted sequence analysis of six isolates of the DE072 serotype in order to determine if recombination is frequently occurring in this region in field isolates of IBV.

Materials and methods

Viruses

Viruses used in this study are listed in Table 1. The viruses were propagated in 9-day-old embryonated specific-pathogen-free (SPF) chicken eggs (SELECT Laboratories, Gainesville,

Table 1. Viruses used in this study

Strain/isolates	Serotype	Origin	Source
DE072	DE072	Delmarva, USA	J. Gelb Jr.[a]
D1466	D212	Netherlands	Y. Weisman[b]
97-6370	DE072	Minnesota, USA	PDRC[c]
97-6386	DE072	Arizona, USA	PDRC
98-2831	DE072	Illinois, USA	PDRC
99-5381	DE072	Georgia, USA	PDRC
99-5425	DE072	Kansas, USA	PDRC
99-5658	DE072	Georgia, USA	PDRC

[a]University of Delaware, Newark, DE USA
[b]Kimron Veterinary Institute, Israel
[c]Poultry Diagnostic and Research Center, Athens, GA, USA

GA, USA). The D1466 strain of IBV was obtained as phenol-inactivated allantoic fluid using USDA import permit #42290.

Viral RNA extraction and RT-PCR

Viral RNA from IBV grown in embryonating eggs was extracted using the High Pure PCR Template Preparation Kit (Boehringer Mannheim, Indianapolis, IN, USA) according to the manufacturers recommendation. RNA from the phenol-inactivated allantoic fluid of D1466 was extracted with a modification in first several step of the High Pure PCR Template Preparation Kit. Briefly, 1.5 ml of the infectious allantoic fluid was placed into a microcentrifuge tube and centrifuged at 13,000 ×**g** for 5 min. The aqueous top layer, approximately 200 μl, was transferred to new tube. Binding buffer (200 μl) and 40 μl of proteinase K (18 mg/ml) was added and incubated for 10 min at 70 °C. Then 150 μl of chloroform/isoamyl alcohol (49:1) was added, vortexed gently for 5–10 sec and then placed on ice for 15 min. The mixture was centrifuged at 13,000 ×**g** for 10 min. The upper phase was transferred to a clean 1.5 ml tube and 100 μl of chloroform/isoamyl alcohol (49:1) was added. The mixture was vortexed gently for 5–10 sec. This was centrifuged for 2 min at 13,000 ×**g**, and the upper phase was transferred to a clean 1.5 ml tube. Remaining steps were followed sequentially as described by the manufacturer.

Gene 3, Gene 4, Gene 5, Gene 6, and a 421 bp hypervariable region (HVR) of the S1 gene were amplified separately using the Titan One Tube RT-PCR System (Boehringer Mannheim). Primer sets used to amplify Gene 3, Gene 4, and the HVR in S1 are listed in Table 2. The primers utilized for amplification of Gene 5 and Gene 6 have been reported [8, 23]. The reaction conditions for RT-PCR were previously described [16, 23].

Sequencing and analysis

PCR products were cut from 1% agarose gels and purified using the QIA quick Gel Extraction Kit (Qiagen, Santa Clarita, CA, USA). Purified PCR products were either sequenced directly or cloned into the TA cloning vector (Invitrogen, Carlsbed, CA, USA), and automated sequencing with the Prism DyeDeoxy terminator cycle sequencing kit (Perkin Elmer, Foster City, CA, USA) was conducted at the Molecular Genetics Instrumentation Facility, University of Georgia. Sequencing primers to various regions of the gene for DE072 and

Table 2. The oligonucleotide sequences of primers used in this study

	Primer	5′->3′ sequence	Position
Gene3	Gene 3 U	catgactggttgttgtggttg	−141−−121
	Gene 3 L	cctttcttatttccgctttg	1222–1242
Gene 4	Gene 4 U	tctttcttttgtaggttattg	920–940
	Gene 4 L	gccatttcatcgtccgtattt	1677–1697
HVR in S1	Ag072 5′	agtacaggcctcctaatgg	95–113
	Ag072 3′	caccygctgcttcaacatc	535–553

The relative primer positions were calculated using the ATG start site of Gene 3 as 1 for primers gene 3 and 4, and ATG start site of S1 gene as 1 for primers HVR in S1

D1466 were designed using OLIGO version 4.0 software (National Bioscience, Plymouth, MN, USA) and are available upon request.

Assembly of sequencing contigs, translation of nucleotide sequence into protein sequence, and initial multiple sequence alignments were performed with the Clustal V method in MegAlign software versin 1.03 (DNAStar Inc., Madison, WI, USA). Phylogenetic trees for each gene were generated using the maximum parsimony method with 100 bootstrap replicates in a heuristic search using the PAUP 3.1 software program [21].

Nucleotide sequence accession numbers

The nucleotide sequences reported here have been deposited with the GenBank. The accession numbers are as follows: DE072 (Gene 3), AF202998; DE072 (Gene 4), AF202999; DE072 (Gene 5), AF203000; DE072 (Gene 6), AF203001; DE072 (3′ end non-coding region), AF203002; D1466 (Gene 3), AF203003; D1466 (Gene 4), AF203004; D1466 (Gene 5), AF203005; D1466 (Gene 6), AF203006; D1466 (3′ end non-coding region), AF203007; 98-2831 (HVR in S1), AF206254; 99-5831 (HVR in S1), AF206255; 99-5425 (HVR in S1), AF206256; 99-5658 (HVR in S1), AF206257; 97-6370 (HVR in S1), AF206258; 97-6386 (HVR in S1), AF206259; 98-2831 (Gene 3), AF206260; 99-5381 (Gene 3), AF206261; 99-5425 (Gene 3), AF206262; 99-5658 (Gene 3), AF206263; 97-6370 (Gene 3), AF206264; 97-6386 (Gene 3), AF206265; 98-2831 (Gene 4), AF206266; 99-5381 (Gene 4), AF206267; 99-5425 (Gene 4), AF206268; 99-5658 (Gene 4), AF206269; 97-6370 (Gene 4), AF206270; 97-6386 (Gene 4), AF206266.

The complete sequence of the 3′ end of the genome of three strains, Beaudette, KB8523 and CU-T2 and Gene 6 of Holl52 strain have been previously reported [1, 11, 20, 23].

Results

Sequence analysis of DE072 and D1466

A total of 3839 nucleotide and 3861 nucleotide were found, respectively, in a region beginning from the 5′ end of gene 3 to the 3′ end of DE072 and D1466 genome. The intergenic sequence CTGAACAA or CTTAACAA was found immediately upstream of the start site for each gene of both strains. The sequences were identical to those found in the corresponding genomic areas of the Beaudette, KB8523, and CU-T2 strains (Fig. 1).

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                   Gene 3                                    3a
DE072      CTGAACAATA CAGACCTAAA AAGTCTGTTT AATGATTCAA AGACCCACAT CTTTT--CTA ATAGTATTAA TTTTTCTTGG    80
D1466      .......... .......... .......... ......C... ..T.....G. .C..C--T.. .......... ....G...T.
Beaudette  .......... .......... .......... ......C... ..T.....G. .C..C--... .......... ...C....T.
KB8523     .......... .......... .......... ......A... .AC...... .....TTT. .....G.... ..C....T.
Cu-t2      .......... .......... .......... ........C .CC..A.T.. ....C--T.. .....G.... C.C.....T.
Ho1152


DE072      GTGTAAACTT GTACTAAGTT GTTTTAAAGA GTGTGTTATA GCACTCCAGC AATTAATACA AGTTTTACTC CAAATTATTA   160
D1466      .......... .......... .......G... ..T.A..... ..G..T..A. ..C...C... .......... .........CG
Beaudette  .......... .......... .......G... ..T.A..... ..G....A. ..C....... .......... .........C.
KB8523     .......... ..C.T..... .......... ...C...... ..G..T.... .......... ....C..... ..........
Cu-t2      .......... ......T... .......... .......... ...T...G. .TTT..... ..G....... .....C....
Ho1152

                                                     ***3b
DE072      GTAATAACTT ACAGTCTAGA CTTCTGCTCT TGCACAGTCT AGACTAATGT TAGATTTTGA AGCAATTATT GAAACTGGTC   240
D1466      A..G...T.. .......... ..GACC..T. G......... .......... ..A.C..A.. .......... .........G
Beaudette  A..G...... .......... ..GACC..T. G......... .......... ..A.C..A.. ..T...... .........G
KB8523     A..G..G... .......... .......T. G......... .......... .......... .......... ..........
Cu-t2      .......... .......... .......T. G......... .......... .......... .......... ..........
Ho1152


DE072      AGCAAATAAT TCAGCAAATC AGTTTCGATT TACAGCACAT TTCAAGTGTG CTAAGCACTG AATTATTTGA CCCCTTTGAA   320
D1466      .T...G.G.. ...AA..... ......A... .......T.. .........A T...A...A. ..G....... .........C
Beaudette  .....G.G.. ...AA..... ......A... .......T.. .........A T...A...A. ..G....... T........C
KB8523     .....G.... ...AT.C... ......A.C. .......T.. .........A T...AT..A. .G........ ..........
Cu-t2      .......... .......... .......... .......... .......... .......... .......... ..........
Ho1152

                                                             3c              **
DE072      GTTTGTGTTT ACAGAGGAGG TAATTATTGG GAAGTAGAGT CAGCTGACGA GTTTTCAGGT GATGACGAAT ATATTGAATA   400
D1466      TA....TA.. .......... .....T.... ...A...... .......A.. T.G....T... T.........
Beaudette  TA....TA.. .......... .....T.... ...A...... .......A.. T.G....... .....T.... T.........
KB8523     .......... .......... .........T ..GT...... ..T....... T......... .......... T........
Cu-t2      ..C....... .......... .......... ..GT...... .......... .......... .......... T......G..
Ho1152

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DE072      AATCGCTAGA GGATAACGGA AGTTTCCTAA CAGCAGTTTA CATATTTGTT GCATTTGTAG CACTTTACCT ATTAGGTAGA   480
D1466      .G........ ...G..T... .....T.... ....GC.... .........A .G...T... T....T.. TC........
Beaudette  .G........ ...G..T... .....T.... ....GC.... ....A...A .G...T... ......T.. TC........
KB8523     .......... ...G...... .....T.... .......... .......... .G........ .......... ..........
Cu-t2      .......... ...G...... .......... ...CG..... .......... .G........ .......... .C........
Ho1152


DE072      GCACTCCAAG CATTTGTACA AGCTGCCGAC GCTTGGTGTT TATTTTGGTA TACATGGGTA GTAGTTCCTG GAGCCAAGGG   560
D1466      .....T.... .......... G.....T..T .....T.... .......... .......T.. ..A....A. ...TT.....
Beaudette  .....T.... .......... G.....T..T .....T.... .......... .......... ..A...A. ....T.....
KB8523     .......... .......... ...A..A..T .....T.... .G....... C......... .......... .....T.....
Cu-t2      .......... .......... .......... .....TC... .......... .......... A......... ..........
Ho1152

                                    Gene 4
DE072      TACAGCCTTT GGATATAATC ATACATATGG TAAAAAACTT AACAAACCGG AATTAGAAGC AGTTATTGTT AACGAGTTTC   640
D1466      .......... .T...C..GT .......... ..G....... ..TT... .......... ....G..... ..........
Beaudette  .......... .T...C..GT .......... ..G....... .....T.... .......... .......... ..........
KB8523     ......T... .TG...... .......... .......... .......... .......... ....G..... ..........
Cu-t2      .......... .T......T .......... ....C..... .......... .T......A. G......... ..........
Ho1152

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DE072      CTAAGAACGG TTGGAATAAT AAAAATCCAG TAAATTTTCA AGATGTCCAA CGAAACAAAT TGTACTCTTG ACTTTGAACA   720
D1466      .......... .......... .......... C......... .......... .......... .......... ..........
Beaudette  .......... .......... .......... C......... .....C.... ...G...... .......... ..........
KB8523     .......... .......... .......... C......... .......... .......... .......... ..........
Cu-t2      .C..A..... ......AC.- ---------- ---------- --...AATG. TAC..TG... ..C..A...A .TAC.C.G..
Ho1152


DE072      GT---CAGTT GAGCTTTTTA AAGAGTATAA TTTATTTATA ACTGCATTCT TGTTGTTCTT AACCATAATA CTTCAGTATG   800
D1466      ..---..... .......... .......... .......... .......... .......... .......... ..........
Beaudette  ..---..... .C........ .......... .......... .......... .......... .......... ..........
KB8523     ..---..... .......... .......... .......... .......... .......... .......... ....A.......
Cu-t2      .CAAG...C. .......... ....A..C.. .G.T...G.. ..G..T...C .T.....TC. T.GT...C.. ..........
Ho1152


DE072      GTTATGCAAC AAGAAGTAAG TTTATTTATA TACTTAAAAT GATAGTGTTA TGGTGCTTTT GGCCCCTTAA CATTGCAGTA   880
D1466      .......... .......... .......... .......... .......... .......... .......... ..........
Beaudette  .C........ .......... G......... C...G..... .......... .......... .......... ..........
KB8523     .......... .......... ....T..G. .......... .......... .......... .......... ..........
Cu-t2      .G..C..... T......CG. .......... ....G..... ....T.... .....T.... .......... ..........
Ho1152
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Fig. 1 (*continued*)

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DE072      GGTGTAATTT CATGTATATA CCCACCAAAC ACAGGAGG-- -TCTTGTCGC AGCGATAATA CTTACTGTGT TTGCGTGTCT  960
D1466      .......... .......... .......... ........-- -.......A. .......... .......... ..........
Beaudette  .......... ......C... .......... ........-- -.......... .......... .....A.... ..........
KB8523     .......... .......... T........T ........-- -.......... .......... .......... ....T.....
Cu-t2      .......... ........T .......... ........GT C.T....... .......... .....A.... G..G......
Hol152

DE072      T---TCTTTT GTAGGTTATT GGATCCAGAG TATTAGACTC TTTAAGCGGT GTAGATCTTG GTGGTCATTT AACCCAGAAT 1040
D1466      .----..... .......... .......... .......... .......... .......... .......... ..........
Beaudette  -GTC.--... .......... .......... .......... .......... .....G..A. .......... ..T.......
KB8523     .----..... .......... ....T..... .TG....... .....AA... .....G.... ......T... .....C....
Cu-t2      .GTC.T...C T.G....... .......... ...C...... .........G ....G..A. .G..G.CA.. ..A..CCG..
Hol152

DE072      CTAACGCCGT AGGTTCAATA CTCCTAACTA ATGGTCAACA ATGTAATTTT GCTATAGAGA GTGTGCCGAC GGTGCTTTCT 1120
D1466      .......... .......... .......... .......... .......... .......... .........T ..........
Beaudette  ....T..... .......... .......... .......... .......... .......... ......A.T .........
KB8523     ....T..... .......... .....T..A. .......... .......... .......... ......T.T .......G..
Cu-t2      ..T.T....G ....C..... .......... ....C..... .......... .......... ....C..AGT .........C
Hol152

DE072      CCAATTATAA AGAATGGTTT TCTTTATTGT GAGGGCCAGT GGCTTGCTAA GTGTGAACCA GACCACTTGC CTAAAGATAT 1200
D1466      ..T....... .......G. .......... .....T.... A......... .......... .......... .......C..
Beaudette  .......... .......G. .......... .....T.... .......... .......... .......... .......C..
KB8523     .......... .......G. C......... .....T.... .......... .......... .......... .......C..
Cu-t2      .......... .......G. .......... .......... .......... .......... .......... ..........
Hol152

DE072      ATTTGTTTGT ACACCGGATA GACGTAATAT CTACCGTATG GTGCAGAAAT ATACTGGTGA CCAAAGCGGA AATAAGAAAC 1280
D1466      ......A..C .....A.... .......... ...T...... .......... .C.T...... .......... .........A
Beaudette  .......... .......... .......... .......... .......... .......... .......... .........A
KB8523     .........C ...G...... ....C..C.. .......... .......... .C........ .......... .........A
Cu-t2      .......... .......... .......... .......... .......... .......... .......... ..........
Hol152

DE072      GTTTTGCTAC GTTTGTCTAT GCAAAGCAGT CAGTAGATAC TGGCGAGCTA GAAAGTGTAG CAACAGGAGG GAGTAGCCTT 1360
D1466      .G........ A......... .......... ....C..... .......... .......... .......T.. A.........
Beaudette  .G........ .......... .......... .......... .......... .......... .......... A....T...
KB8523     .G........ A......... .....A.... .G.....C.. .......... .......... .......... A....T...
Cu-t2      .......... .......... .......... .......... .......... .......... .......... ......T...
Hol152
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DE072      TACACATAAA TGTGTGTGTG TAGAGAGTAT TTAAAATTAT TCTTCAATAG TGCCTCTATT TTAAGAGCGC GGAAGAGTAT 1440
D1466      .......... .......... .......... .......... .......... .......... .......... ..........
Beaudette  .......... .......... .......... .......... ....T..... C......G.. .......... AT........
KB8523     .......... .......... .......... .......... ....T.G... .......G.. .......T.. AT........
Cu-t2      .......... .......... .......... .......... ....T..... .......... .......... AT..T.....
Hol152

DE072      TTGTTTTGAG GATATTAATA TAAATCCTCT TTGTTTTGTA CTCTCTTTAC AAGAGTTATT ATTTAAGCAA CAGTTTTTCC 1520
D1466      .......... .......... .......... .......... .......... .......... .......... ..........
Beaudette  ..A....... ....C..... .......... ........A.. .....C..T. .....C.... .--------- ----------
KB8523     .AT....... .......... .......... ......CA.. ........T. .......... ......AA.. ..........
Cu-t2      .AT....... .......... .......-. C......A.. ......T. .....C.... ......AA.. ..........
Hol152

DE072      TTTCCTTTGT TTGGAAGAAA GTTGTTGTTA ATGGTGTAGA ATTCCAAGTA GAAAATGGAA AAGTCCACT- ACGAAGGAAA 1600
D1466      .......... .......... .......... .......... .......... .......... .........- ..........
Beaudette  ---------- ---------- ---------- .C.....TAC C..T...... ..T....... .....T...- .........C
KB8523     AC..T..... GCCA..A.CT .......... ........AC C..T...... ..T....... .....T...- ..........
Cu-t2      AC..T..... GCCA..A.CT .......... ........AC C..T...... ..T....... .....T...T .........C
Hol152

DE072      CCCCATTTTC CAAAAAGGTT GTTGTAGGTT GGGGTCTCAT TATAAGAAGG ATTAAATGGA TTAAACCACC TACACTACTT 1680
D1466      .......... T......... .......... .T....C... .......... .......... .......... ..........
Beaudette  A..AG..... .......... .........A. .T....CA.. ........A. .A..-----. ..G....... ...TAC....
KB8523     A..AA.C... .......... ......T.. .T....CA.. ........A. .....G..TAG ....GT.... A..TAC....
Cu-t2      A..AG..... .......... .......AA. .T....CA.. ........A. .....G.ATA. ....GT.... ...TAC....
Hol152

                                                  Gene 5           5a
DE072      ACTTGTAATA AGGGCGTTTG GACTTACAAG CGCTTAACAA ATACGGACGA TGAAATGGCT GACTAGTTTT GGAAGAGCAG 1760
D1466      .......... .......... .......... ....A..... .......... .......... .......... ..........
Beaudette  .T..T..TA. GA..T....T ACT.A....A AA........ .......... .......... .......... ..........
KB8523     .T..T..TA. GA......T ATT......A .......... .......... .......... .......... ..C.........
Cu-t2      .T..T..TA. GA......T ATT......A .......... .......... .......... .....T.... ..........
Hol152
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Fig. 1 (*continued*)

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DE072      TTATTTCATG TTATAAAGCC TTACTATTGA CTCAATTAAG AGTGTTAGAT AGGTTAATTC TAGATCACGG ACCAAAACGC 1840
D1466      .......T.. .......... C.......A. ....G..... ...A...... .......... .......T.. .......... ..........
Beaudette  .......T.. .......T.. C.......A. .....C.T.. .......... .........T .......... ..T.CT....
KB8523     .......... .......... C.......A. .......... .......... .......... .......... ..........
Cu-t2      .......... .......... C.......A. .......... .......... .......... .......... ..........
Hol152

DE072      GTTTTAACGT GTAGTAGGCG AGTGCTTTTG TTTCAGTTAG ATTTAGTTTA TAGGTTGGCG TTTACGCCCA CCCAATCGCT 1920
D1466      ..C....... ..G....... .C..A.... .........A .......... .........A .A........ ..........
Beaudette  .......... .......... C.......A G....A.... .......... .......... .A........ ..........
KB8523     .......... ..G....A.. .......C.A G.G..A.... .C........ .......... .A........ .T........
Cu-t2      .......... .......... .......... .......... ........C .C........ .......... ..........
Hol152

                      5b***
DE072      GGTATGAATA ATAGTAAAGA TAATCCTTTT CGCGGAGCAA TAGCAAGAAA AGCGCGAATT TATCTGAGAG AAGGATTATA 2000
D1466      .......... .......... .......... .......... .......... .......... .......... .......G.
Beaudette  ..C....... .......... .......... .......... .......... .......T... .......... .......G.
KB8523     .......... .......... .......... .......... .......... .......... ...T.... .......G.
Cu-t2      .......... .......... .......... .......... .......... .......... ...T.... .......G.
Hol152

                    Gene 6
DE072      TTGTGTTTAC TTTCTTAACA AAGCAGGACA AGCAGAGCCT TGTCCCGCGT GTACCTCTCT AGTATTCCAA GGGAAAACTT 2080
D1466      G......... .......... .......... .......... .......... .......C.. .......... .......C.
Beaudette  .......... .......... .......... .......... .......... .......... .......... ..........
KB8523     .......... .......... .......... .......... .......... .......... .......... ..........
Cu-t2      .......... .......... .......... .......... .......... .......... .......... ..........
Hol152

DE072      GTGAGGAACA CATAAATAAT AATAATCTTT TGTCATGGCA AGCGGTAAGG CAACTGGGAA AACAGACGCC CCAGCGCCAG 2160
D1466      .......... ..C.G..... .......... ........G .........A .......... G......... ..........
Beaudette  .......... ....C..... .......... .......... .......A. ..G....A. .......... ..........
KB8523     .......... ....C..... ..C....... .......... ..T....... ......A.. G......... ..........
Cu-t2      .......... .......... .......... .......... ..T...... ......A.. G......... ..........
Hol152      .....G ........A .......... G......... ..........

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DE072      TCATCAAACT AGGAGGACCA AAGCCACCTA AAGTTGGTTC CTCTGGAAAT GCATCGTGGT TTCAAGCAAT AAAAGCCAAG 2240
D1466      .......... ......G... ..A....... .......... T......... .....T.... .......C... ..........
Beaudette  ....T..... .......... ..A....... ....C..... T......... .....T.... .......... ..........
KB8523     .......... .......... .......... .......... T......... .....T.... .......... ..........
Cu-t2      .......... .......... ..A....... .......... T......... .....T.... .......... ..........
Hol152      .......... ......G... ..A....... .......... T......... .....T.... .......C... ..........

DE072      AAGCTAAATT CACCTCAACC TAAGTTTGAA GGTAGTGGTG TTCCTGATAA TGAAAATCTT AAAACTAGCC AGCAACACGG 2320
D1466      ...T...... ......CT.. .......... .....C..C. .......... .......... ....TTA.... .......T..
Beaudette  ...T.....A ......CG.. C......... .....C.... .......... C.....CA.. ..GC.A.... .......T..
KB8523     .......... ......C..T .......... .......... .......... .......... ......A.... .......T..
Cu-t2      .......... ......C... ...T...... .....C.... .......... .......... ......A.... .......T..
Hol152      ...T...... ......CT.. .......... .....C..C. .......... .......... ....TTA.... .......T..

DE072      ATACTGGAGA CGCCAACTTA GGTTTAAGCC AAGTAAAGGC GGAAGAAAAC CAGTCCCGGA TGCTTGGTAC TTCTATTATA 2400
D1466      G......... ..T...GCC. ....AC..... .G........ .........T .......... .......... .......C.
Beaudette  .......... ......GCC. .......... .G.C.....T .......... .......... .......... ..T..C....
KB8523     G......... ......GC.. .......... .......... .......... .......... .......T .......... 
Cu-t2      .......... T.....GC.. .......... .G........ .......... .......... .....C.... ..........
Hol152      G......... ..T...GCC. ....AC..... .G........ .........T .......... .......... .......C.

DE072      CTGGAACAGG ACCAGCCGCT GATCTGAATT GGGGTGATAG CCAAGATGGT ATAGTGTGGG TTGCTGCAAA GGGTGCTGAT 2480
D1466      .......... .......... ..C....... .......T... .......... .......T... ..........
Beaudette  .......... ...T...... ..C.....C. ........C T.......... .......T.. ..........
KB8523     .......... .......... ..C....... .......... .......... .......... ..........
Cu-t2      .......... .......... ..C....... .......... ..G....... .....C.... ..........
Hol152      .......... .......... ..C....... .......... .......... .......... ..........

DE072      GTTAAATCTA GATCGAACCA GGGTACAAGG GACCCTGACA AGTTTGACCA ATATCCACTA CGATTCTCTG ATGGAGGACC 2560
D1466      AC........ ....T..... .......... ..T....T. .......... ...C..G... ........A. ..........
Beaudette  AC........ ....C..T.. .........A ..T.....T .......... ...C...... .......G. ....C....
KB8523     AC.....A.. ....T..T.. .........A .....C..T. .......... .......A. .C........
Cu-t2      AC.....C.. ....T..... .......... .......... .......... .......G. ..........
Hol152      AC........ ....T..... .......... ..T.....T .......... ...C..G... ........A. ..........

DE072      TGATGGTAAT TTCCGTTGGG ACTTCATTCC TCTGAATCGC GGTAGGAGTG GAAGATCAAC AGCAGCTTCA TCAGCAGCAT 2640
D1466      .......... .......... .......... AA.A.....T .......... .......... ...G...... ..........
Beaudette  .......... .......... .......... CC....C..T .......... .......... .......... .........G
KB8523     .......... .......... ....T..... .......T .......... ...AG..... ......C... ..........
Cu-t2      .......... .......... .......... AA.A.....T .......... ........T. ...G...... ..........
Hol152      .......... .......... .......... AA.A.....T .......... .......... ...G...... ..........
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Fig. 1 (*continued*)

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DE072      CTAGTAGAGC ACCGTCGCGT GACGGCTCGC GTGGTCGTAG AAGTGGTTCT GAAGGTGATC TTATTGCTCG TGCAGCAAAG 2720
D1466      .......... .......... ..T....... .......A... ...C..AG.. ....A..... ....A..... ..........
Beaudette  .......... ...A..A... ..A..T.... .......... .....A.... .G..A...C. .......... ..........
KB8523     .......... G..A..A... ..A..T.... .......... .......G.. ....A..... .......... ..........
Cu-t2      .......... .......... ..T....... .......A... ...C..AG.. ....A..... ....A..... ..........
Hol152     .......... .......... ..A..T.... .......A.. ......C..A ....A..... .......A.. ..........


DE072      ATAATCCAGG ATCAGCAGAA GAGGGGTTCT CGCATTACTA AGGCTAAGGC TGATGAAATG GCTCATCGCC GGTATTGCAA 2800
D1466      ..C..T.... .......... ..A....... .......... ...A...... C......... .......... .......T..
Beaudette  .......... .......... A.A..C.... .......C.. ....A..... A......... .......... ..........
KB8523     ..T..T.... ....A..... ..A...CG.. ........A. .......... .......... ..C....... ..........
Cu-t2      .C..T.... .......... ..A....... .......... .A...... C......... .......... .......T..
Hol152     ..C..T.... .......... .A........ .......... ...A...... C......... .......... .......T..


DE072      GCGCACTATT CCACCTGGTT ATAAGGTTGA TCAAGTGTTT GGTCCCCGTA CTAAAGGTAA GGAGGGAAAT TTTGGTGATG 2880
D1466      ...T.....C .......... .......... .......A... .......... .....T..... .......... ..........
Beaudette  .........C ......AA.. ...G...... .......... .......... .......... .......G... ..........
KB8523     .......... .......... .......... .......... ......T... .......... .......... ..........
Cu-t2      ...T.....C .......... .....A.... .......A... .......... .......... .......... ..........
Hol152     ...T.....C ......A... .......... .......A... .......... .......... .......... ..........


DE072      ACAAGATGAA TGAGGAAGGT ATTAAGGATG GGCGTGTTAC GGCAATGCTC AACCTAGTCC CTAGCAGCCA TGCTTGTCTT 2960
D1466      .......... ......G... .......... ....C..... A......... .......... .......... ..........
Beaudette  .......... .......... .......... .......... A......... .......... .......... ..........
KB8523     .......... .......... .......... .......... A......... .......... .......T.. ......C...
Cu-t2      .......... ......G... .......... ....C..... A......... .......... .......... ..........
Hol152     .......... ......G... .......... ....C..... A......... .......... .......... ..........


DE072      TTTGGAAGTA GAGTGACGCC CAAACTTCAA CCAGATGGGC TGCACTTGAA AATTGAATTT ACTACTGTGG TTTCACGTGA 3040
D1466      .......... .......... .......... .......... .......G.. .T........ .......... ..........
Beaudette  .......... ......A.. .......... .T........ .T......G .T........ .......... .CC..T....
KB8523     .......... .......... ......C... .......... .......... .T........ .......... .........A.
Cu-t2      .......... .......... .......... .......... .......G.. .T........ .......... ..........
Hol152     .......... .......... .......... .......... .......G .T........ .......... ..........


DE072      TGATCCGCAG TTTGATAATT ATGTGAAAAT TTGTGATCAG TGTGTTGATG GTGTAGGGAC ACGTCCAAAA GATGATGAAC 3120
D1466      .......... .......... .......... .......... .....C.... .......... G......... ..C.....
Beaudette  ...C...... .......... .......... .......... .....C.... .......A.. G......... .....C....
KB8523     .......... .......... .......... .......... .......... .......A.. G.....T... ..C.....
Cu-t2      .......... .......... .......... .......... .....C.... .......... .......... ..C.....
Hol152     .......... .......... .......... .......... .....C.... .......... G......... ..........


DE072      CGAGACCAAA GTCACGCTCA AGTTCAAGAC CTGCTACAAG AACAAGTTCT CCGGCGTTAA GACAACAGCC TCAAAAGAAG 3200
D1466      .......... ......C.. .A........ .......... .......... ..A...CC.. .......... ..........
Beaudette  .A.A...... .......... .......... .......... .GG..A.... ..A...CC.. .........G C.C......
KB8523     ...A...... .......... .......... .......... .......... .....CC.. ......C..G C.C......
Cu-t2      .A........ .......... ......C.. .A........ .......... ..T...CC.. .......... ..........
Hol152     .......... ......C.. .A........ .......... .......... ..A...CC.. .........G ..........


DE072      GAGAAAAAGC CAAAGAAGCA GGATGATGAA GTAGATAAAG CATTGACCTC AGATGAGGAG AGGAACAATG CACAGCTGGA 3280
D1466      .....G...T .......... .......... .........G. .......... .......... .......... ..........
Beaudette  .......... T......... .......... .C........ .......... .......... .......... ..........
KB8523     .........A .T........ .......... ..G....... .......... .......... .......... ..........
Cu-t2      .......... .......... .......... .......... .......... .......... .......... ......A..
Hol152     .....G...T .......... .......... .......G. .......... .......... .......... ..........

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DE072      ATTTGATGAT GAACCCAAGG TGATTAACTG GGGTGATTCA GCTTTAGGTG AGAATGAACT TTGA------ ---------G 3360
D1466      .......... .......... .......... ...G..G... ..AC.T..A. .......GT. G.A.AGCTAG ATTTCCAACT
Beaudette  ....T..... ..G....... .A........ ...G..G... ...C....A. .......... ....------ ----------
KB8523     G......... .....T..A. .......T.. ...G...... ..C.... ..AC.T.... .A........ ....------ ---------T
Cu-t2      .......... .......... .......T.. ...G...... .......... .......... ....------ ----------.
Hol152     ....G..... .......... .......... ...G...... ..AC.T..A. .......GT. G.A.AGCTAG ATTTCCAACT


DE072      TAACATAATG GACCTGC--- TGC-ATTTTG TGGTAC-AT- --TTTGTTAA -ACACTATTT CTGTGCC-TT CCTATCAATC 3340
D1466      ......C... ...G...GTA ...TG....T CCC...T..A GAC..T...G C.T.T..... T.-...TA.. TG...GGT.T
Beaudette  .......... .......T--- ---TG...CC ......-..- --.......- .......... T-.. ......T..... ........T
KB8523     .......... ...T...--- ...-.C...C .CTGG.---- -A...C..TG TGTTT.G... T...AG-A.. T..CA....T
Cu-t2      .....A.... .......--- A..-...T --......-.- --......CG -CT....... T.....T-.. ..........
Hol152     ......C... ...G...GTA ...TG....T CCC...T..A GAC..C...G C.T.T..... T.-...TA.. TG...GGT.T


DE072      ATTACAGGCA TTGATTGTGG TTATGTGCAA TATTTAAGCT TCTTTTG-GT TGCTTT-TTG CTTGTTGTAT TGTTGCTGTG 3520
D1466      ........TG AA......AT G...T..TTG ..CACTC.TA .G..C.ATA. .ATG..-..C TG.AG.-.G. .A..AG...T
Beaudette  .......... ........A ......T... .C........ ......-..- ......-... ..A....... ..........
KB8523     ....TT..-. A........A .......G ...C.T..T- .GG...A-A. .ATC..G... .AGT....G. .C..T.....
Cu-t2      .--....... .........A .C.......A .C........ .......-. .......-.. .......... ..........
Hol152     ........TG AA......AT G...T..TTG ..CACTC.TA .G..C.ATA. .ATG..-..C TG.AG.-TG. .A..AG...T
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Fig. 1 (*continued*)

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DE072      CTTTTTATTA TTGTGATTCT CATTAGTTTG TTTTATCGTA GAAGTTCAAT AGTAAGAGTT AAGGAAGATA GGCATGTAGC 3600
D1466      G..C..G..C ..ACTC.A.. GT.CTC...T C.....TT.. ..GTA..... ---....A.C .......... ........T
Beaudette  .........G .......... .......... C......... ...A...... .......... .......... ..........
KB8523     ...A..G..T ..A..C.G.. TTA.TC.... ..A....... ......---. .T.G.A.... .......... ..........
Cu-t2      .......... .......... .......... .......... .......... .......... .......... ..........
Hol152     G..C..G..C ..ACTC.A.. GT.CTC...T C.....TT.. ..GTA..... ---....A.C .......... ........T


DE072      TTAGC-ACCT ACATGTCTAT CGCCAGGGAA ATGTCTAATC TGTCTACTTA GTAGCCTGGA AACGAACGGT AGACCCTTAG 3680
D1466      ..GATT.... .......... .......... .......... .......... .......... .......... ..........
Beaudette  ..GATT.... .......... .......... .......... .......... .......... .......... ..........
KB8523     ..GATT.... .......... .......... .......... .......... .......... ..T....... ..........
Cu-t2      .....-.... .......... .......... .......... .......... .......... .......... ..........
Hol152     ..GATT.... .......... .......... .......... .......... .......... .......... ..........


DE072      ATTTTAATTT AGTTTATTTT TTAGTTTAGT TTAAGTTAGT TTAGAGTAGG TATAAAGATG CCAGTGCCGA GGCCACGCGG 3760
D1466      .......... .....A... .......... .......... .......... ........A. .........G ..........
Beaudette  .......... .....A... .......... .......... .......... .......... .........G ..........
KB8523     .......... .....A... .......... .......... .......... .......... .........G ..........
Cu-t2      .......... .....A... .......... .......... .......... .......... .........G ..........
Hol152     .......... .....A... .......... .......... .......... ........A. .........G ..........


DE072      AGTACGATCG AGGGTACAGC ACTAGGACGC CCACTAGGGG AAGAGCTAAA TTTTAGTTTA AGTTAAGTTT AATTGGCTAA 3840
D1466      .......... .......... .......... .......... .......... .......... .......... ..........
Beaudette  .......... .......... .......... ...T...... .......... .......... .......... ..........
KB8523     .......... .......... .......... ........C. .......... .......... .......... ..........
Cu-t2      .......... .......... .......... .......... .......... .......... .......... ..........
Hol152     .......... .......... .......... ......A.. .......... .......... .......... ..........


DE072      GTATAGTTAA AATTTATAGG CTAGTATAGA GTTAGAGC 3878
D1466      .......... .......... .......... ........
Beaudette  .......... .......... .......... ........
KB8523     .......... .......... .......... ........
Cu-t2      .......... .......... .......... ........
Hol152     .......... .......... .......... ........
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Fig. 1. The nucleotide sequence alignment of gene 3, gene 4, gene 5, and gene 6, and 3′ non-coding region. Dots indicates nucleotide identical to that of DE072 strain. The conserved nucleotide sequences ctgaacaa or cttaacaa, which is located at the starting site of each gene, is in bold character. Heavy underlines indicate the putative start codons, asterisks above the sequence indicate the stop codons

Gene 3 of both strains contained three ORFs, 3a, 3b, and 3c. Gene 4 consisted of the M protein gene with a single ORF and a non-coding region between the 3′ end of the M protein gene and gene 5. Gene 5 contained two ORFs (5a and 5b). Gene 6 consisted of the N protein gene with a single ORF and a 3′ non-coding region. Downstream from the stop codon of the N gene, a 15 base insertion was found in the D1466 genome which also occurs in the genome of the Holl52 (Fig. 1).

Sequence comparison and phylogenetic analysis

The 3′-terminal 3.8 kb of the genome of five strains and gene 6 of the Holl52 strain were compared. The nucleotide sequence similarities among coding regions of gene 3, M, gene 5, and the N protein gene of DE072 and other strain were between 83.3–97.6%. Those of D1466 and other strains were between 78.7–98.2% identical. D1466 showed only 1.8% nucleotide difference with Holl52 in Gene 6. Gene 3c and gene 5b were relatively more conserved than the other genes (Table 3).

Genes were divided by IG sequences (CTGAACAA/CTTAACAA) and phylogenetic analysis was conducted. The DE072 strain clustered with the CU-T2

Table 3. Percentage nucleotide homologies between coding regions of gene 3, M protein gene, gene 5, and N protein gene of IBV strains

IBV	Percent homology with DE072							Percent homology with D1466						
	Gene 3			M	Gene 5		N	Gene 3			M	Gene 5		N
	3a	3b	3c	M	5a	5b	N	3a	3b	3c	M	5a	5b	N
D1466/DE072	83.3	83.6	87.8	96.9	92.4	95.2	92.4	83.3	83.6	87.8	96.9	92.4	95.2	92.4
Beaudette	85.6	83.6	88.4	96.5	90.9	96.8	91.1	95.4	98.5	97.6	95.6	91.4	94.4	90.1
KB8523	85.6	89.7	93.6	94.1	92.4	96.8	93.7	80.5	86.2	91.2	94.8	92.9	95.2	91.3
CU-T2	85.6	97.4	90.8	84.8	97.0	97.6	94.7	78.7	82.6	83.7	82.3	91.4	95.6	95.5
Holl52	N/A	N/A	N/A	N/A	N/A	N/A	92.6	N/A	N/A	N/A	N/A	N/A	N/A	98.2

N/A Not available
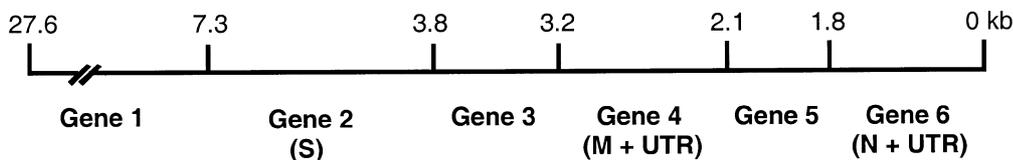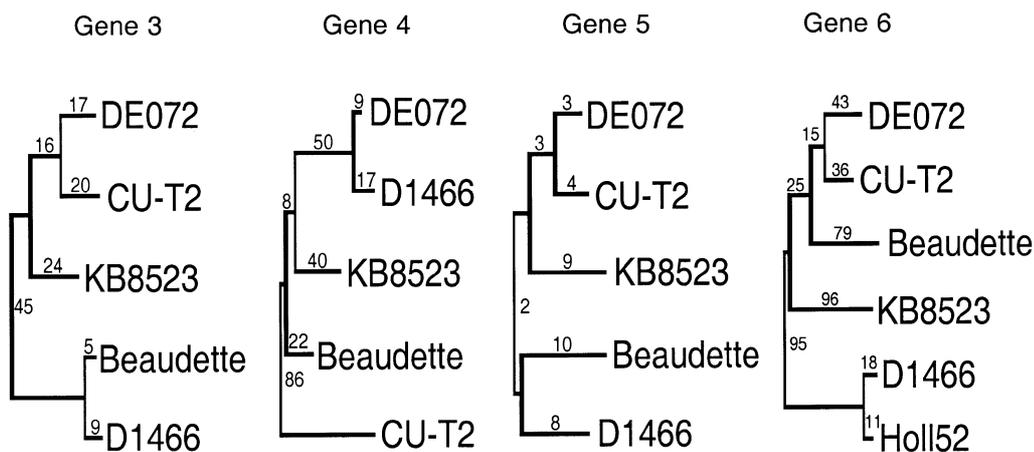
Fig. 2. Phylogenetic analysis of DE072 and D1466 with other IBV strains in Genes 3, 4, 5, and 6. **A** The linear structure of IBV genomic RNA. Genes are divided by intergenic (IG) sequences which is a stretch of consensus sequences (CTGAACAA or CTTAACAA). **B** Phylogenetic analysis using parsimony for five IBV genes based on nucleotide sequence. All trees were constructed by general bootstrap analysis using 100 replicates and midpoint rooted. Branch lengths are provided in each tree

strain in all genes except gene 4, where it clustered with D1466 and separated far from CU-T2. On the other hand, D1466 clustered with Beaudette in gene 3 and gene 5, and clustered with Holl52 in gene 6 (Fig. 2). KB8523, which is only the nephropathogenic strain, was solely placed in all genes compared.

Phylogenetic analysis of field isolates of DE072 serotype

In order to demonstrate the genetic heterogeneity of the same serotype isolates of IBV, we conducted phylogenetic analysis using six DE072 serotype field isolates. Phylogenetic analysis of the hypervariable region (HVR) in S1, clustered all the DE072 serotype isolates in one group with the prototype strain of the DE072
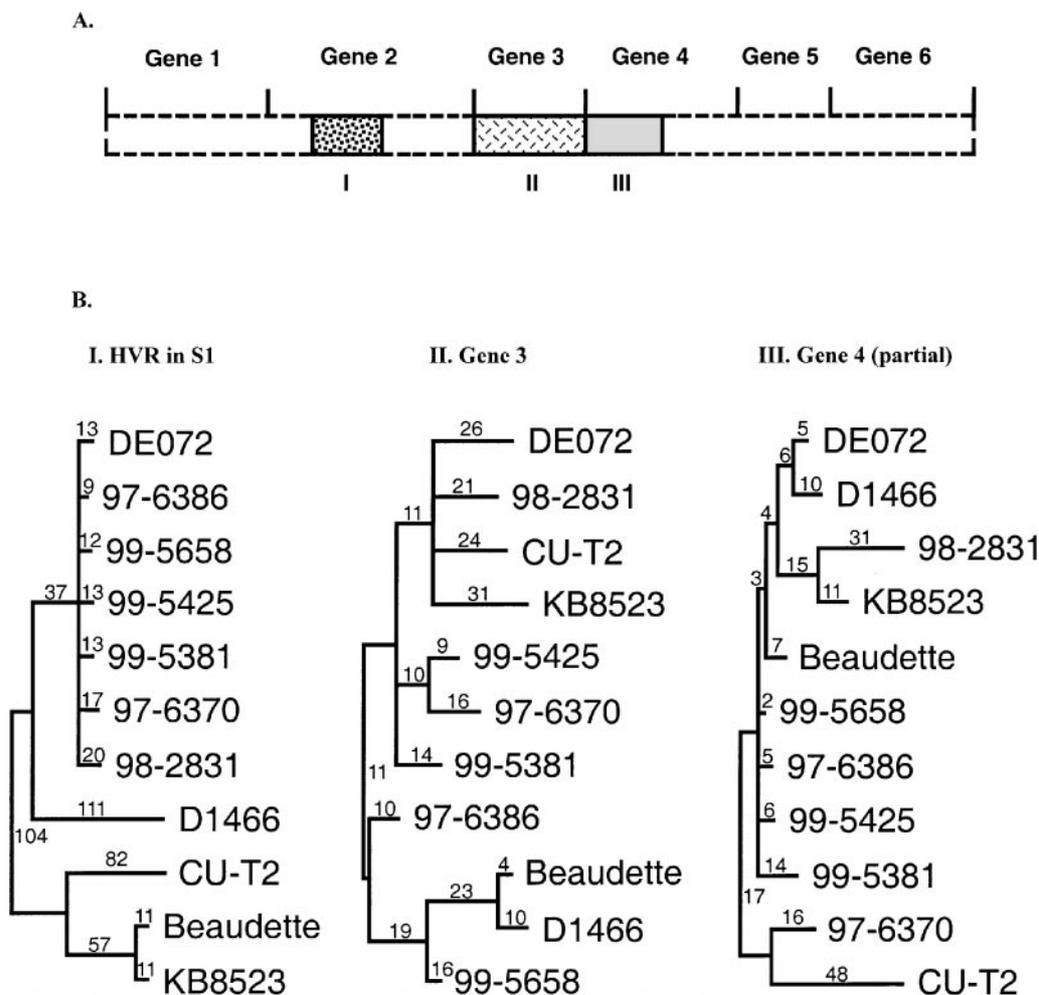
Fig. 3. Phylogenetic analysis of field isolates of DE072 serotype IBV. **A** Schematic representation of the genome of IBV. I–III indicate the regions used to construct the phylogenetic tree. **B** Phylogenetic trees for the regions I–III as indicated in **A**. *I* Trees of HVR in S1, which is 421 bp; *II* trees of gene 3 sequences; *III* trees of partial gene 4 sequences upstream residue 670 bp

serotype of IBV. This group was far from other serotypes of IBV strains in tree length. However, phylogenetic tree of gene 3 and gene 4 showed differences in tree topology among six isolates. In Gene 3, only one isolate, 98-2831, clustered with DE072. In gene 4, no isolates clustered with DE072 and formed groups randomly with other serotypes of IBV (Fig. 3).

Discussion

DE072 is a recent isolate made in 1992 [6]. In a previous study of the S gene, we demonstrated that this virus was closely related to D1466 which is an IBV vaccine strain of the D212 serotype from the Netherlands [7, 13, 18]. Analysis of gene 4 also reveals a high sequence relatedness between DE072 and D1466 (Table 3). However, in the other genes analyzed in this study, DE072 shares high sequence similarity with the CU-T2 strain which has also been reported to be a recombinant between Arkansas and Massachusetts strains [10]. Considering the fact that both strains were isolated in the northeastern USA, it is possible that they had undergone similar selection pressure. On the other hand, D1466 shows high similarity with Beaudette and Holl52 strains in genes other than the S gene. The percent similarity in the N gene and a 15 base insertion in the 3′ non-coding region suggests that both D1466 and Holl52 are closely related (Table 3, Fig. 1). The Holl52 strain has been extensively used as a live vaccine in Europe [5]. This finding provides more convincing evidene that vaccine strains are contributing to the emergence of variants in the field. Based on these results, we suggest that DE072 and D1466 had the same origin, but diverged a long time ago and evolved independently in different geographical locations.

Since recombination in coronaviruses is thought to occur by a template switching mechanism [8, 19], we speculate that IG sequences may serve as 'hot spots' for homologous recombination. So far, recombinations suggested in IBV have been used on a small part of the genome [10, 15, 22]. Examining only a small part of the genome may result in misleading conclusions because of point mutations or conserved regions of the gene. We conducted phylogenetic analysis by dividing 3.8 kb of the 3′ end of the genome among five IBV strains at the IG sequences. Phylogenetic trees of this sequence data had very different topology (Fig. 2), which indicates that recombination had occurred. It has been reported that RNA recombination in IBV can occur randomly in non-localized sites in vitro [12]. However, considering the selection pressure in vivo recombination in the IG sequences should be advantageous to virus in two aspects. First, since crossovers occur at the site of consensus IG sequences, there would be no shift in the codon reading frame. Second, since whole genes are substituted, there would be no drastic change in the conformation of proteins encoded by individual genes. Further, cross-overs at each of the five IG sequences would generate tremendous genetic diversity. This amount of diversity may contribute to persistence and to the continuing emergence of new variants of IBV despite vaccination efforts.

Finally, we conducted sequence analysis of 6 isolates of the DE072 serotype to demonstrate how random recombination occurs within the same serotype.

Phylogenetic analysis of the HVR in S1 shows that these 6 isolates cluster together because they are the same serotype. However, these 6 isolates had a much different level of nucleotide sequence similarity with each other in gene 3 and gene 4, and clustered randomly with other serotypes of IBV (Fig. 3). Based on this result, it is clear that isolates of the same serotype can differ substantially in individual genes. Thus, every field isolate of IBV could be unique in each gene sequence because of recombination.

Acknowledgements

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