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Sequence analysis of infectious bronchitis virus isolates from the 1960s in the United States

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Abstract To better understand the molecular epidemiology of infectious bronchitis virus (IBV) in the United States following the introduction of commercial IBV vaccines, we sequenced the S1 and N structural protein genes of thirteen IBV field isolates collected in the 1960s. Analysis of the S1 sequence showed that seven isolates were of the Massachusetts (Mass) genotype, five were SE17, and one was of the Connecticut (Conn) genotype, suggesting that these three IBVs were circulating in commercial poultry raised in different regions in the United States during the 1960s. The S1 genes of Mass-type isolates had high levels of sequence variation, representing 81.3-81.9 % nucleotide (nt) and 77.3-78.7 % amino acid (aa) identity when compared to those of the SE17-type isolates. In contrast, the N genes from the same isolates were less variable (>92 % nt and >93 % aa identity) when compared to those of the SE17-type isolates. Phylogenetic analysis based on the S1 gene indicated that one isolate (L748) was more closely related to the Mass type. In contrast, phylogenetic analysis based on the N gene showed that L748 was more closely related to the SE17 type, indicating that there had been exchange of S1 genetic materials between Mass- and SE17-like viruses. In addition, the Mass-type isolates had high levels of sequence identity in the S1 gene compared with widely used modified live vaccines (Mass41, Ma5 and H120) and modern field strains from the USA and other countries, suggesting a common ancestor.

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Introduction

Avian infectious bronchitis virus (IBV) is a ubiquitous, highly contagious respiratory pathogen of chickens that inflicts serious economic losses to the commercial poultry industry worldwide [3, 4]. IBV, along with turkey coronavirus, belongs to the genus *Gammacoronavirus* in the subfamily *Coronavirinae*, family *Coronaviridae,* order *Nidovirales* [2, 5]. IBV has a single-stranded, positive-sense RNA genome of approximately 27.6 kilobases. The genes (open reading frames [ORFs]) encoding IBV structural proteins are located downstream of the viral replicase genes (ORFs 1a and 1b) and in order from 5′ to 3′ are as follows: S (spike), E (envelope), M (membrane) and N (nucleocapsid). The S glycoprotein is posttranslationally cleaved into a transmembrane domain (S2) and an outer domain (S1), which expresses the serotype-specific epitopes of the virus [4]. The N phosphoprotein, which forms the capsid of the virion, is involved in RNA replication and carries group-specific antigenic determinants [10]. Both S1 and N proteins play critical roles in the induction of immune responses against IBV [4, 10].

IBV was first isolated in the early 1930s in the United States [19]. The Massachusetts (Mass)-type viruses were believed to be the only serotype found in the USA until the mid-1950s, when a second IBV serotype Connecticut (Conn) was reported [12]. Immediately thereafter, the use of commercially produced Mass- and Conn-type modified live virus (MLV) vaccines began [4]. A number of new IBV serotypes, antigenic variants and field strains were isolated in the subsequent years in the USA, and many IBV serotypes are currently known to exist worldwide [4, 21]. MLV vaccines containing strains of IBV from different serotypes are routinely used to protect commercial chickens against infectious bronchitis (IB). MLV vaccine strains are selected to represent the antigenic spectrum of likely challenge viruses by incorporating the serotypes of viruses most commonly circulating in a particular country or region. In the United States, most IBV isolates causing IB belong to the Mass, Conn, and Arkansas (Ark) serotypes [4]. Disease outbreaks in vaccinated chickens may result when the flock is infected with IBVs that are antigenically unrelated to the vaccines used to immunize them. New variants of IBV can emerge from either wild-type or vaccine viruses by acquiring point mutations and/or genomic recombinations [7]. One of our previous studies indicated that antigenic and genetic diversity existed during the 1940 s [11], well before the introduction of IBV vaccines. However, many IBV strains from the USA isolated following the introduction of commercial MLV vaccines have never been characterized by sequencing, and it is essential to sequence these isolates to understand the evolution of IBV geographically and to implement effective vaccination strategies to control modern IBV strains that are in circulation. The main objective of this study was to examine a number of archived IBV isolates from the 1960s in order to determine (1) which genotypes of IBV were prevalent in the United States during that time and (2) whether genetic information obtained from these isolates would shed light on the evolution of the currently circulating serotypes.

Origin of archived viruses

During the 1960s, clinical signs of bronchitis with upper respiratory distress were frequently observed in broiler flocks, even in IBV-vaccinated birds, but rarely in layer flocks [6]. Through the cooperation of numerous hatcheries and laboratories, a number of field isolates of IBV were recovered in the mid-1960s from the respiratory tracts of naturally-infected chickens raised in different regions of the United States (Winterfield and Hitchner, unpublished data). Following isolation at Cornell University, the viruses were passed a minimum number of times (no more than three) in embryonated chicken eggs, and allantoic fluids from the embryos were lyophilized and stored at 4 °C. For the present study, we used thirteen representative IBV field isolates from all regions of the US, generously provided by Dr. Benjamin Lucio-Martinez, College of Veterinary Medicine, Cornell University, Ithaca, NY (Table 1). The lyophilized samples were reconstituted in 1 ml of diethylpyrocarbonate (DEPC)-treated water and used directly for RNA extraction without further passage in chicken embryos.

IBV RNA was extracted from 200 µl of each sample using TRizol LS Reagent (Gibco BRL, Grand Island, NY) according to the manufacturer's instructions and resuspended in 8 µl of RNase-free water. The reconstituted RNA from each isolate was used as the template for RT-PCR amplification of the S1 and N protein genes. Briefly, the RT reaction was performed using a Superscript III first-strand synthesis kit (Invitrogen, Inc., Carlsbad, CA) with random hexamer primers according to the manufacturer's instructions. The RNA template was removed by digestion with 2 U of *E. coli* RNase H for 30 min at 37 °C. Amplification of cDNA with gene-specific primers (NewS1Oligo5′ and Degenerate3′ [15] for S1, and NFor and NRev [23] for N genes) was performed using an Advantage 2 Polymerase Mix kit containing high-fidelity DNA polymerase (Clontech Laboratories, Inc., Mountain View, CA). The amplicons (1,610 bp [S1] and 1,230 bp [N]) were analyzed by gel electrophoresis using 0.5 % agarose with ethidium bromide (0.5 µg/ml). With the exception of isolate L969, all other isolates resulted in 1,230-bp N gene RT-PCR products, and therefore, only twelve N gene sequences were included in the analysis. RT-PCR products were gel-purified using a QIAquick Gel Extraction Kit (QIAGEN Inc., Valencia, CA), and directly sequenced using a BigDye Terminator Cycle Sequencing Kit (Perkin-Elmer, Branchburg, NJ) and an Applied Biosystems model ABI 377 automated DNA sequencing system. A combination of flanking and internal primers was used to sequence both strands of DNA in their entirety.

Assembly of contiguous sequences and translation of nucleotide sequences into amino acid sequences were performed with Vector NTI Advance 11 software (Invitrogen Inc., Carlsbad, CA). The BlastN program (http://www.ncbi.nlm.nih.gov/BLAST/) was used to search GenBank for homologous IBV S1 and N gene sequences. Selected sequences from GenBank were included in the alignment. After aligning the S1 and N sequences, the Geneious program (Biomatters Ltd, Auckland, New Zealand) was used to construct phylogenetic trees by the neighbor-joining method. Subsequently, bootstrap analysis was performed with 1,000 replicates to determine the best-fitting tree for each gene. Pairwise BLAST searches were also performed when there were no significant hits in the BlastN search. All of the S1 and N gene sequences reported

Table 1 Infectious bronchitis virus isolates from the 1960s examined in this study

Virus ID	Date of isolation[a]	Source/origin	Number of embryo passages	GenBank acces. no. of S1 gene	GenBank acces. no. of N gene
L74	1/20/65	Forrest Farms, Hattiesburg, MS	P1	JQ964060	JQ964073
L165	3/10/65	Farm Service Co-op, Fayetteville, AR	P2	JQ964061	JQ964074
L423	7/10/65	Valley Feed Co. Dardale, AR	P2	JQ964062	JQ964075
L455	7/29/65	Dr. B. G. Maxfield, Cullman, AL	P2	JQ964063	JQ964076
L554	10/27/65	Magee Coop, Magee, MS	P2	JQ964064	JQ964077
L569	11/18/65	Dr. James Poole, Albertville, Ala	P3	JQ964065	JQ964078
L613	12/3/65	Dr. Ostendorf and Zehr, Milford, IN	P2	JQ964066	JQ964079
L718	1/13/66	B & L Feed Supply, Morgaton, NC	P2	JQ964067	JQ964080
L748	1/26/66	H. H. Haralson Jr., Forrest, MS	P2	JQ964068	JQ964081
L806	3/2/66	Research Central Soya, Decatur, AL	P2	JQ964069	JQ964082
L905	4/27/66	Leret Mills, Chattanooga, TN	P2	JQ964070	JQ964083
L919	5/6/66	Hilbun Farms, So, MS	P2	JQ964071	JQ964084
L969	6/3/66	Dept. of Agriculture, Honolulu, HI	P3	JQ964072	ND

[a] Isolated by Drs. Winterfield and Hitchner

ND not done

herein have been deposited in the NCBI GenBank database, and the accession numbers are listed in Table 1. The S1 and N gene sequences of other IBV strains were also obtained from GenBank.

Sequence properties

The 1,611-bp S1 gene sequences (nt 20,368-21,978, numbered according to the Beaudette strain, GenBank accession number DQ001338) of thirteen 1960s isolates were compared with those of 47 other IBV strains (Supplementary Table 1) from the USA and other countries. A phylogenetic tree based on the nt sequences of the complete S1 genes showed that the 1960s IBV isolates were divided into three distinct lineages, Mass, SE17 and Conn genotypes (Fig. 1), which suggests that these three IBVs were circulating in commercial poultry raised in different regions of the USA in the 1960s. Seven IBV field isolates (L74, L423, L455, L569, L748, L806, L969) were clustered with Mass-type reference strains (Beaudette, Mass41), vaccine strains (Mass/Bvial1, Mass/Cvial1, Ma5, H120) and field strains from the USA and other countries. These viruses had 97.6-99.9 % nt (96.3-99.8 % aa) sequence identity when compared to prototype Mass41. Five IBV isolates (L163, L613, L718, L905, L919) were clustered with SE17 virus, and they had 98.7-99.4 % nt (97.9-98.7 % aa) sequence identity when compared to prototype SE17. Interestingly, only one IBV isolate (L554) clustered with the Conn-type reference virus (Conn46), the vaccine strain (Conn/Cvial1), and field strains from the USA and other countries. This virus had 99.8 % nt (99.3 % aa) sequence identity to the prototype Conn46 strain. Less-frequent isolation of the Conn-type virus was probably due to the high level of Conn-type immunity from widespread use of the vaccine in young flocks [6]. The S1 sequences of IBVs closely related to the Mass-type isolates were 1,611 bp (537 aa) in length. The S1 sequences of all IBVs grouped with SE17 were 1,632 bp (544 aa) in length due to an additional 21-nt (7-aa) insertion at positions 358-363 and 427-441. The S1 sequence of the Conn-type isolate L554 was 1543 bp long (513 aa), the same as the Conn46 virus. The Mass-type isolates were genetically distantly related to other 1960s viruses and had only 81.3-81.9 % nt (77.3-78.7 % aa) sequence identity when compared to SE17-type isolates and 94.5-95.2 % nt (89-89.9 % aa) sequence identity when compared to Conn-type L554 isolate. The S1 gene of the 1960s isolates were also distantly related to those of the Gray, JMK, Ark99, N-M24 and N-M39 strains, showing 79.8-85.9 % nt (76.5-84.7 % aa) sequence identity. The deduced aa sequence of the S1 proteins had multiple substitutions that were distributed throughout the S1 protein (data not shown). The Mass-type isolates had 1-18 aa differences when compared to Mass reference (Beaudette, Mass41) or vaccine (Ma5, H120) strains. Isolate L748 had a maximum 18 differences, but only two (S24R, L386R) differed from the Mass41 sequence. The SE17-type isolates had 1-5 differences compared to prototype SE17 virus, and the Conn-type L554 had three substitutions compared to Conn46 virus. The majority of the differences in Mass, SE17 and Conn-type viruses were concentrated between aa positions 51 and 77, and 115 and 151, which correspond to the two hypervariable regions (HVRs) [13] that are known to carry serotype-specific antigenic determinants. The cleavage recognition sites

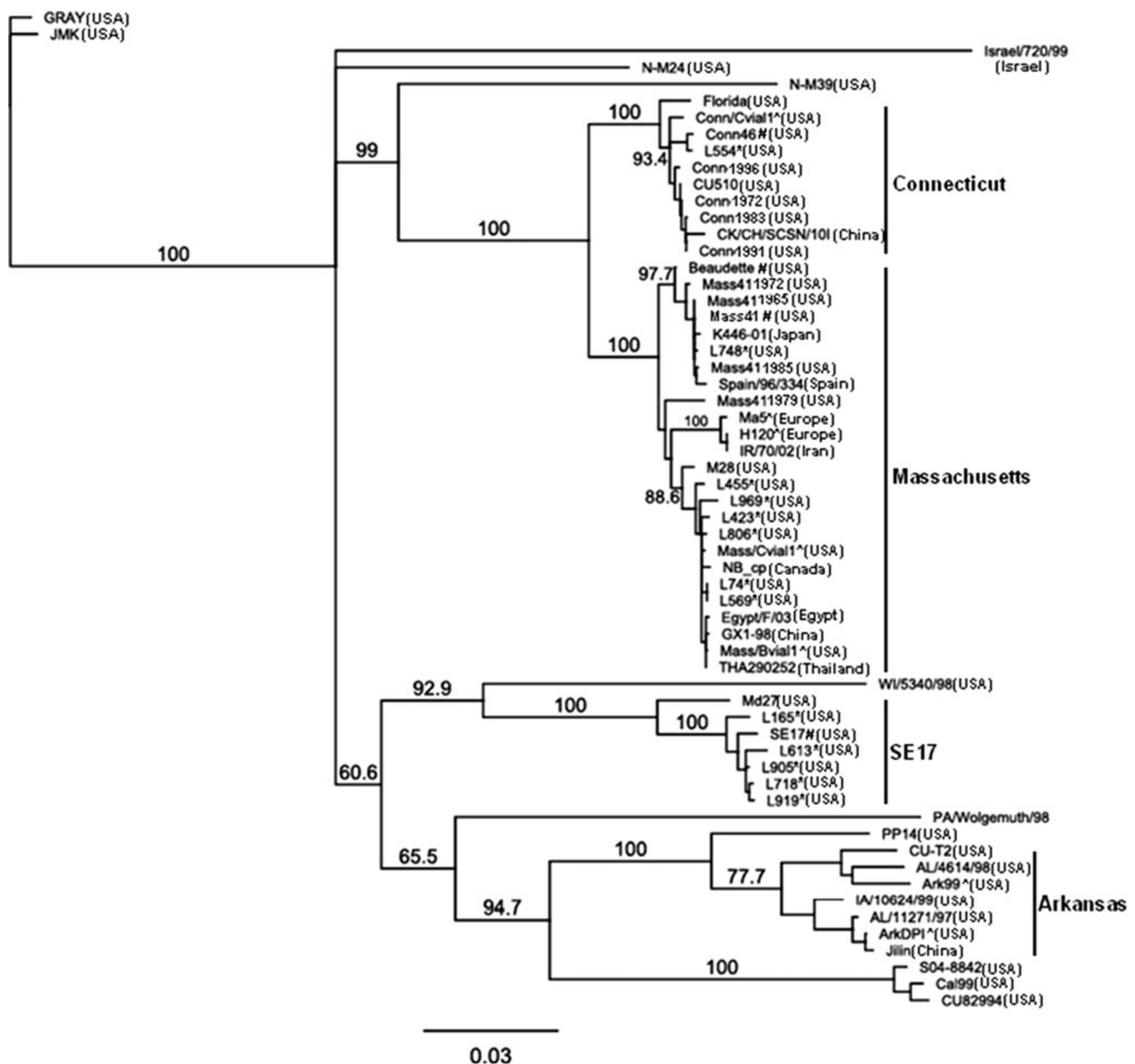

Fig. 1 Phylogenetic tree created from the nucleotide sequences of the S1 glycoprotein genes of infectious bronchitis virus (IBV) isolates. The neighbor-joining tree was constructed from the pairwise nucleotide differences for the S1 genes, which represented the relatedness between 13 isolates from the 1960s and 47 heterologous IBV strains from the United States and other countries. The 1960s isolates (*) clustered with Massachusetts, Connecticut and SE17-type reference (#) or vaccine (^) strains of IBV. The length of each pair of branches represents the distance between the sequence pairs. The scale at the bottom indicates the number of substitution events

between the S1 and S2 subunits of the 1960s isolates were compared. All Mass-type isolates had an RRFRR sequence, as in other Mass viruses, and the SE17 group had an HRSRR sequence like the Gray and Ark99 strains (data not shown).

The S1 genes of the Mass isolates were found to be genetically closely related to those of widely used MLV vaccine strains and modern field isolates from the USA and other countries (Fig. 1), suggesting a possible common ancestry and emergence of virus strains that are essentially similar to the MLV vaccines that are used. Previous studies have indicated that some strains of IBV isolated in different countries may have almost identical S1 genes [16, 17], reflecting some of the common features of IBV evolutionary direction. The spreading of a virus from one region or country to another could be due to its inadvertent introduction by the trading of birds or by the use of MLV vaccines. It is known that the Mass strain of IBV is widely used as a MLV vaccine throughout the world [4].

Unlike the S1 genes, the N gene sequences of the 1960s isolates were highly conserved, having >92 % identity at

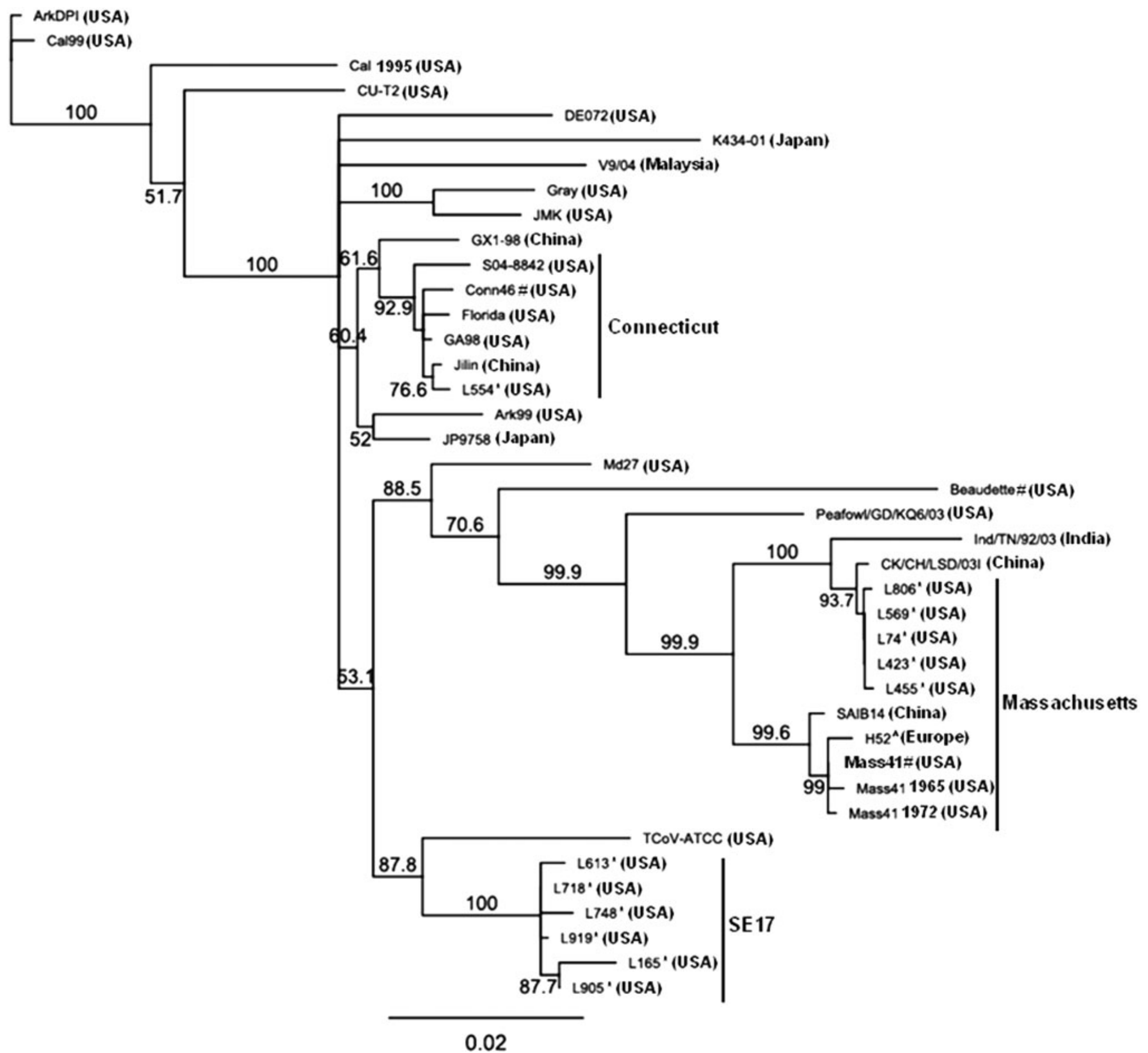

Fig. 2 Phylogenetic tree created from the nucleotide sequences of the nucleocapsid (N) genes of infectious bronchitis virus (IBV). The neighbor-joining tree was constructed from the pairwise nucleotide differences for N genes, which represented the relatedness between 12 isolates from the 1960s and 28 heterologous strains of IBV from the United States and other countries. The 1960s isolates (*) clustered with Massachusetts and Connecticut-type IBV reference (#) or vaccine (^) strains, and a turkey Coronavirus (TCoV-ATCC; in absence of the N gene sequence of the prototype SE17 virus). The length of each pair of branches represents the distance between the sequence pairs. The scale at the bottom indicates the number of substitution events

the nt (>93 % at the aa) level as compared to the N genes of the other 28 IBV strains (Supplementary Table 2). The phylogenetic tree based on the N gene nt sequences showed that the 1960s IBVs were also divided into three clades, representing the Mass, SE17 (unfortunately, the N gene sequence of prototype SE17 was not available in GenBank, and we did not have access to the isolate) and Conn genotypes (Fig. 2). Of them, five (L74, L423, L455, L569, L806) were closely related to the Mass strains (Mass41, H52), showing 98.2 % nt (98.5 % aa) sequence identity with Mass41, and they were even more closely related (>99 %) to isolates CK/CH/LSD/031 from China and Ind/TN/92/03 from India; six (L165, L613, L718, L748, L905, L919) IBV isolates, those clustered with SE17 (except for L748 which clustered with Mass) based on S1 phylogeny, were closely related to turkey coronavirus (TCoV) in the N gene, showing 96.5 % nt (96.8 % aa) sequence identity, and they had a similar (≥96 %) genetic relationship to the Ark99, Conn, and Gray strains of IBV. The isolate L554 clustered with Conn-type reference (Conn46) and field

strains, showing ≥99% sequence identity. The N gene of all the 1960s isolates was 1,230 bp (409 aa) in length and differed from one another by zero to 6.8 % nt (5.4 % aa) (data not shown). Our data is consistent with previous reports describing recombination between IBV and turkey coronaviruses [9].

Genetic variation was observed in the S1 and N genes of the isolate L748 in this study. Phylogenetic analysis of the S1 gene showed that the Mass-type isolates, including L748, clustered together (Fig. 1), as would be expected of viruses of the same serotype. Interestingly, the L748 isolate had much different levels of sequence similarity to each other in the N gene, and it clustered randomly with other SE17-type isolates (Fig. 2). The shift in sequence homology in the S1 and N genes indicated that exchange of genetic material took place subsequent to coinfection with a Mass-like virus and an SE17-like virus for L748, probably by homologous recombination. A similar recombination event has been observed in some Japanese isolates [20], where the S1 protein was found to be closely related to Australian isolates [18] and the N protein was very similar to those of North American viruses [14]. In addition, two US isolates (md27 and PP14) exhibiting 97 % and 95 % identity, respectively, to SE17 in the S1 genes are known to be derived from SE17 by recombination [1, 22]. SE17 was first reported by Hopkins [8] as a new serotype of IBV isolated in 1967 from a closed research flock of chickens with classic signs of IB. Herein, we report five SE17 isolates from samples collected in 1965 (L165, L613) and 1966 (L718, L905, L919) from commercial chickens raised in different regions of the USA. Unlike SE17, the Conn46 and Mass (Mass41, Ma5, H52, H120) strains have been used simultaneously as MLV vaccines in commercial poultry in the USA [4]. The use of SE17 or its derivatives as an MLV vaccine is not known. Because the chickens from which the 1960s isolates were isolated most likely had been exposed to live Mass vaccines [6], the vaccine might be implicated as a source for the Mass-like sequences in the SE17 S1 gene. The SE17 source of genetic material was probably a strain naturally infecting these affected flocks, since they had not been exposed to SE17 vaccine. Although this study cannot prove or disprove that recombination has occurred in L748, we can point to the evolutionary trends in the S1 gene as compared to the evolutionary trends in the N gene. This study demonstrates that the S1 gene of IBV tends to evolve more rapidly by mutation and genetic recombination when compared to the more conserved N gene [11]. This suggests that these two genes of IBV are under very different selective pressures and that the exchange of genetic material may have occurred between vaccine strains and field isolates.

Based on these findings, we conclude that widespread vaccination with MLV vaccines against previously known dominant serotypes (e.g., Mass41, Ark99), in association with the high recombination and mutation abilities of IBV, have led to the emergence and broad dissemination of the present-day novel serotypes. New strategies to control IBV infection effectively in chickens should be considered with the use of either inactivated or subunit vaccines.

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