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Variability Assessment of California Infectious Bronchitis Virus Variants

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SUMMARY. On the basis of the data from the California Animal Health and Food Safety Laboratory System, 1444 infectious bronchitis (IB) cases were diagnosed between 1997 and 2012. Epidemiologic analyses demonstrated two major IB virus (IBV) outbreak peaks, affecting mainly 35-to-49-day-old broiler chickens. California variant 1737 (CA1737) and California variant 1999 (Cal 99) IBV types were the most prevalent genotypes during the analyzed period. To further understand the increased prevalence of these genotypes, we assessed and compared the variability of the S1 gene hypervariable region of CA1737 and Cal 99 with the variability of IBV strains belonging to the Massachusetts 41 (M41) and Arkansas (Ark) types during serial passages in embryonated chicken eggs. On the basis of the S1 nonsynonymous changes, seven different subpopulations were detected in M41. However, the predominant population of the field strain M41 before passages continued to be predominant throughout the experiment. In contrast, Ark passaging resulted in the detection of 13 different subpopulations, and the field sequence became extinct after the first passage. In IBV Cal 99, eight different subpopulations were detected; one of these became predominant after the second passage. In CA1737, 10 different subpopulations were detected. The field strain major sequence was not detected after the first passage but reappeared after the second passage and remained at low levels throughout the experiment. Compared with M41 and Ark, Cal 99 and CA1737 showed intermediate variability.

RESUMEN. Evaluación de la variabilidad de los virus variantes de la bronquitis infecciosa de California.

De acuerdo con la base de los datos del Sistema de Laboratorios de Salud Animal y Seguridad Alimentaria del Estado de California, 1444 casos de bronquitis infecciosa aviar (IB) fueron diagnosticados entre los años 1997 y 2012. El análisis epidemiológico demostró dos picos principales de brotes del virus de la bronquitis infecciosa (IBV), que afectaron principalmente a pollos de engorde de 35 a 49 días de edad. Los tipos variantes California 1737 (CA1737) y California 1999 (Cal 99) fueron los genotipos más prevalentes durante el período analizado. Para comprender mejor el aumento en la prevalencia de estos genotipos, se evaluó y se comparó la variabilidad de la región hipervariable del gene S1 de los virus CA1737 y Cal 99 comparándola con la variabilidad de las cepas pertenecientes a los serotipos Massachusetts 41 (M41) y Arkansas (Ark) durante pasajes seriados en huevos embrionados de pollo. Con relación a los cambios no sinónimos del gene S1, se detectaron siete subpoblaciones diferentes del tipo M41. Sin embargo, la población predominante de la cepa M41 de campo antes de los pasajes continuó siendo la predominante durante todo el experimento. En contraste, el pasaje de los virus Arkansas resultó en la detección de 13 subpoblaciones diferentes y la secuencia de campo se extinguió después del primer pasaje. Con relación al tipo Cal 99, se detectaron ocho subpoblaciones diferentes; una de ellas se convirtió en la predominante después del segundo pasaje. Con relación al tipo CA1737, se detectaron diez subpoblaciones diferentes. La secuencia principal de la cepa de campo no se detectó después del primer pasaje, pero volvió a aparecer después del segundo pasaje y permaneció en niveles bajos durante todo el experimento. En comparación con el tipo M41 y Arkansas, los tipos Cal 99 y CA1737 mostraron variabilidad intermedia.

Key words: infectious bronchitis virus, variability, serial passages, subpopulations

Abbreviations: Ark = Arkansas; CA1737 = California variant 1737; CAHFS = California Animal Health and Food Safety Laboratory System; Cal 99 = California variant 1999; cDNA = complementary DNA; Conn = Connecticut; DPI = Delmarva poultry industry; EID_{50} = 50% embryo infective dose; IB = infectious bronchitis; IBV = infectious bronchitis virus; M41= Massachusetts 41; Mass = Massachusetts; RT-PCR = reverse transcriptase-PCR; S = spike protein; SNP = single nucleotide polymorphism; SPF = specific pathogen free

Infectious bronchitis (IB) is a worldwide endemic viral disease of chickens caused by IB virus (IBV), a gammacoronavirus. Dozens of serotypes and genotypes of IBV have been documented around the world and more are being recognized periodically. Coronavirus genetic diversity is generated by mutations or recombination events or both occurring during virus replication (13,14). Phenotypes favored by selections spread and successfully circumvent vaccination programs extensively applied in the poultry industry (19). Continuously changing environments, including, for example, seasonality and variation in dosage of standard IB vaccination programs provide ample opportunities for IBV variants to emerge during serial replication cycles in susceptible birds (11).

In the state of California, very little information is available on indigenous viruses isolated in the 1970s and 1980s. In the 1990s, most IBV viruses were classified as California variants (17). These IBV variants in the field successfully evade immune responses elicited by IBV vaccines (11) commonly used in California (1). In California, while commercial egg layer producers use Massachusetts (Mass), Connecticut (Conn), and Arkansas (Ark) serotype IBV vaccines during the rearing period and sometimes during production, broiler producers may use Mass and Conn, but many have been migrating away from field vaccination and even hatchery priming (C. Corsiglia, pers. comm.).

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IBV variants are commonly restricted to specific geographic areas (11), and sometimes their presence can be transient (1).

The S1 portion of the spike protein (S) of IBV is responsible for viral attachment to host cells and elicits neutralizing antibodies in chickens. S1 displays the most genetic and phenotypic variability among different IBV strains (4,6,13) and is therefore a good target to assess variability.

We conducted descriptive statistics on 1444 IBV diagnosed cases submitted to the California Animal Health and Food Safety Laboratory (CAHFS) between 1997 and 2012. In addition, we hypothesized that IBV variant strains showing high prevalence in California display increased population heterogeneity, as measured by single nucleotide polymorphisms (SNPs) on the S1 gene compared with the more stable Mass type, and instead, more similar to the highly variable Ark type. To test this hypothesis, two IBV types highly prevalent in California were subjected to selective pressure by serial passages in embryonated chicken eggs and compared with a Massachusetts 41 (M41) and an Ark-type virulent strain, and their S1 gene sequences were analyzed.

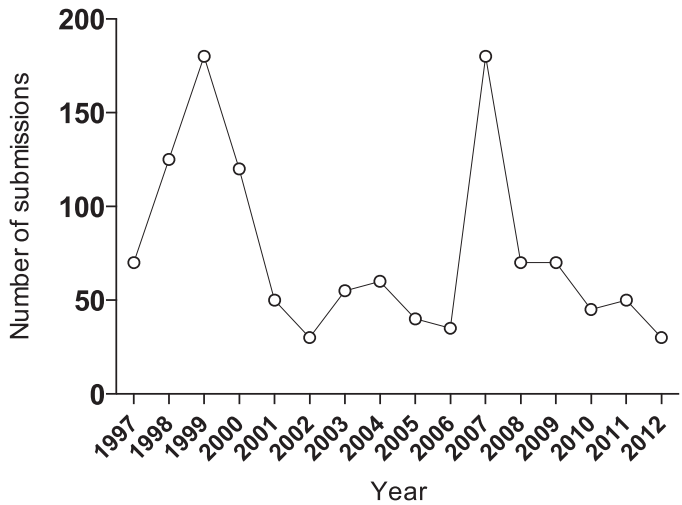

Fig. 1. IBV-diagnosed cases per year in California between 1997 and 2012 on the basis of data provided by the CAHFS. Total number of cases is 1444.

MATERIALS AND METHODS

IBV cases in California. Descriptive statistics were performed on a total of 1444 IBV diagnosed cases submitted to the CAHFS between 1997 and 2012. Data included IBV genotype, chicken type, and age at outbreak.

Viruses. A virulent M41 isolate (Mass-type virulent strain) and a virulent Ark-type previously described (7) were used. These field strains had been replicated once in embryonated specific-pathogen-free (SPF) eggs after their initial isolation. The results from our descriptive study (described in the following) determined that IBV California variant 1999 (Cal 99) and California variant 1737 (CA1737) were the most prevalent in the state. An IBV isolate belonging to each variant type (kindly provided by the CAHFS laboratory) were used in the current study. These isolates were received with one passage in embryonated eggs performed during their original isolation. All viruses were titrated in 10-day-old SPF embryonated eggs, as described (22).

Experimental design. Each virus was subjected to five serial passages in 10-day-old SPF chicken embryonated eggs (Sunrise Farms, Catskill, NY). Each passage was performed by inoculation via the allantoic route and incubated for 3 days. In the first passage, eggs were inoculated with 100 µl of each IBV strain containing M41 (Mass-type virulent strain) 10^8 50% embryo infective dose (EID_{50}); Ark-type (GenBank accession no.: JN861120) $10^{7.8}$ EID_{50}; CA1737 (GenBank accession no.: KU740249) 10^6EID_{50}; and Cal 99 (GenBank accession no.: KU740248) $10^{5.4}$ EID_{50}. Three-day postinoculation allantoic fluids from all eggs were harvested and stored individually. The inoculum for subsequent egg passages was prepared from a pool of the allantoic fluids of all eggs of each virus and diluted 1:2 in tryptose broth. Subsequent passages were performed by using 100 µl of pooled virus for a total of five passages.

Viral RNA amplification by reverse transcriptase-PCR (RT-PCR). Allantoic fluids (200 µl) were individually obtained for viral RNA extraction using the RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Complementary DNA (cDNA) was prepared from the extracted viral RNA by RT-PCR using a One Step RT-PCR Kit (Qiagen) and previously described primers, which allow amplification across IBV genotypes, S17F (TGA AAA CTG AAC AAA AGA CCG ACT TAG) and S18R (GGA TAG AAG CCA TCT GAA AAA TTG C) (8). After amplicon verification by agarose gel electrophoresis, the amplified cDNA was purified using the QIAquick PCR Purification Kit (Qiagen) and submitted for sequencing using the reverse primer. The sequence of the first 753 nucleotides of the S1 coding sequence was obtained. This sequence, containing the hypervariable region of the IBV S1 gene, has been repeatedly used to demonstrate IBV genetic diversity (7,8,9). Sequences were assembled and aligned by using the MacVector 12.7 software (MacVector Inc., Cary, NC). Sequence chromatograms were analyzed to identify SNPs and deduce amino acid changes.

Variability index. We developed a variability index on the basis of the genetic diversity and subsequent selection of viral gene sequences. Nucleotide and amino acid sequences of the hypervariable region of the S1 gene obtained from the different groups were investigated for repeatability on the basis of alignment to the S1 sequence of the original virus before egg passages. Variability was expressed as the ratio between the total number of distinct subpopulations detected in every group during passages and the number of selected subpopulations after the fifth passage. A distinct subpopulation is a subpopulation different in at least one nucleotide in the portion of the S1 gene analyzed compared with the reference sequence. Thus, a higher index value reflected higher variability, while a lower number reflected a more stable virus.

RESULTS

IBV descriptive statistics. Of 1444 IBV-related cases obtained between 1997 and 2012, approximately 90% (1300 cases) represented broilers and 10% layers (144 cases). Genotypic information was obtained from 786 IBV cases (54.4%) as follows: 151 (19.2%) cases were associated with Cal 99; 122 (15.5%) cases with CA1737; 115 (14.6%) cases with Conn; 32 (4.1%) cases with Mass, and 21 (2.7%) cases corresponded to Ark. The remaining cases involved genotypes not matching strains frequently found in the state.

Two major IBV outbreak peaks were detected in the analyzed period (Fig. 1). Analysis of the available data revealed limited genotypic data prior to 2001 and during 2007 (Fig. 2). These analyses indicated that CA1737 and Cal 99 were the two most prevalent genotypes during most recent years (Fig. 2). IBV CA1737 peaked in 2007, followed by two less severe peaks associated with Cal99 in 2009 and 2011 (Fig. 2). Most outbreaks occurred in broiler chickens ranging from 35 to 49 days of age.

Viral subpopulations. On the basis of nonsynonymous changes, the subpopulations detected for each IBV type prior to and after serial passages in embryonated eggs are shown in Tables 1–4. As seen in these tables, distinct subpopulations became detectable from all different viruses likely as a result of selective pressure while replicating in embryonated eggs.

IBV M41. As seen in Fig. 3, the IBV population predominant in the M41 virus isolate maintained predominance through five passages in embryonated eggs. On the basis of the amino acid changes, six

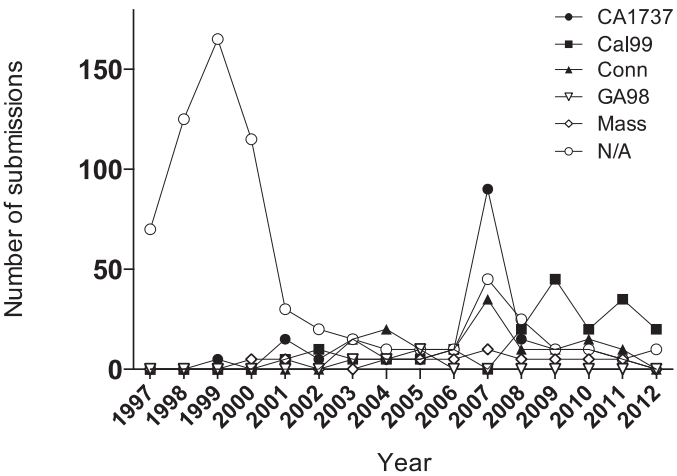

Fig. 2. IBV genotypes by year from 1997 to 2012 (N = 1444). CA1737: California variant 1737; Cal99: California 1999; Conn: Connecticut; GA98: Georgia 98; Mass: Massachusetts; N/A: not available.

additional distinct subpopulations emerged during IBV M41 embryo passages (Fig. 3a). Five other changes were synonymous and therefore not listed as subpopulations. Two of the amino acid changes detected may have structural consequences: Leu to Arg at position 126 and Val to Asp at position 218, changing from a nonpolar to an electrically charged amino acid.

IBV Ark. Rapid negative selection of the predominant population in this isolate became evident after the first embryo passage. Specifically, the S1 sequence of the major population prior to passages was no longer detectable thereafter. On the basis of the amino acid changes, 13 different subpopulations were detected during embryo passages (Fig. 3b). Five other changes were synonymous and therefore not listed as subpopulations. The most important amino acid changes involved polar to nonpolar changes (Thr to Pro at position 9), nonpolar to polar changes (Ile to Asp at position 135 and Pro to Ser at position 232), and electrically charged to polar (Arg to Ser at position 35 and Arg to Cys at position 134). The S1 gene sequence pattern of populations 7 and 13 showed predominance after the second and third passages, respectively (Fig. 3b). Finally, subpopulation 17 became predominant after passages four and five, ending with a frequency close to 90% of rescued sequences (Fig. 3b).

IBV Cal 99. Similar to IBV Ark, a rapid negative selection of the population predominant in the Cal 99 isolate was observed (Fig. 3c). After the second passage, a distinct subpopulation "5" became predominant. Interestingly, this subpopulation maintained predominance throughout the experiment (Fig. 3c). Additionally, seven different subpopulations emerged during embryo passages (Fig. 3c). Two other changes were synonymous and therefore not listed as subpopulations. Changes included nonpolar to polar amino acids (Ser to Phe at position 25), electrically charged to polar (Lys to Thr at position 95 and Arg to Gly at position 134), and polar to electrically charged (Gln to Arg at position 212).

IBV CA1737. A rapid negative selection of the population predominant in the field CA1737 was evident as soon as after the first passage. This subpopulation maintained low levels throughout the experiment. In addition, 10 distinct subpopulations became detectable, but none of them showed a strong predominance (Fig. 3d). Six other changes were synonymous and therefore not listed as subpopulations. Changes that could change the virus antigenicity included polar to electrically charged amino acids (Ser to Arg at position 91, Gln to Lys at position 207, and Gln to Arg at position 207) and polar to nonpolar amino acids (Gln to Pro at position 130, Ser to Phe at position 136, and Gln to Pro at position 207).

Variability index. The ratio calculated between the numbers of subpopulations based on nucleotide differences detected throughout the passages among each group versus the number of selected subpopulations at the end of the fifth passage showed differences between groups. Specifically, the calculated values were (M41) 4.0; (CA1737) 4.25; (Cal 99) 5.0; and (Ark) 9.5. Likewise, for subpopulations based on amino acid changes, the results show the same trend: (M41) 2.3; (CA1737) 2.5; (Cal 99) 3.5; and (Ark) 6.5.

Table 1. IBV M41 virus subpopulations, based on nucleotide changes resulting in amino acid changes, detected after five serial passages in embryonated chicken eggs.

Predominant population[A]	S1 sequence[B]						
Nucleotide	26	170	377	566	626	653	680
AA	9	57	126	189	209	218	227
M41 field strain	Val	Asn	Leu	Leu	Tyr	Val	Gly
4	**Ala**	Asn	Leu	Leu	Tyr	Val	Gly
6	**Ala**	Asn	**Arg**	Leu	Tyr	Val	Gly
8	**Ala**	Asn	**Arg**	Leu	Tyr	Val	**Val**
9	**Ala**	Asn	**Arg**	**Phe**	Tyr	Val	Gly
10	**Ala**	Asn	**Arg**	Leu	**Cys**	**Asp**	Gly
11	**Ala**	**Thr**	**Arg**	Leu	Tyr	Val	Gly

[A]Only subpopulations based on nonsynonymous nucleotide changes are listed.
[B]Bold indicates changed amino acids.

DISCUSSION

The analysis of IBV cases in California indicates a higher frequency of outbreaks affecting broiler chickens. This result is likely associated with tendencies by broiler companies directed toward discontinuing or reducing IBV vaccination programs. The results also indicate increased IBV associated problems in birds 35 to 49 days of age. Similar results have been reported for other states (20) and are probably linked to increased ammonia build up, waning of maternal immunity, and immunodeficiency (12,20).

The epidemiologic data indicated that IBV types CA1737 and Cal 99 have been the most prevalent genotypes in the period between 1997 and 2012. The Cal 99 variant was detected for the first time in early 1999 in 35-day-old broiler flocks in the San Joaquin Valley affected by respiratory signs and airsacculitis (16). In layers, Cal 99 has been associated with drops in egg production and nephritis (24). IBV CA1737 was first detected in 2004, affecting 6-wk-old replacement pullets. The virus was isolated from the respiratory tract, cecal tonsils, and kidneys (23). CA1737 represented 7%, 5.5%, 26%, 59%, and 36% of IBV cases in 2004, 2005, 2006, 2007, and 2008, respectively (23). In addition, this virus has been associated with nephritis in 4-to-6-wk-old pullets and respiratory distress in 32-to-46-day-old broilers (23).

Adaptation of IBV to embryo tissues after serial passages in embryonated eggs has been a well-known phenomenon since the early works performed by Bijlenga in 1960 (3). His finding that serial passages of the IBV H strain (e.g., H-120, where H stands for Huyben) would become attenuated for chickens, while maintaining immunogenicity, constituted one of the most relevant discoveries in IBV vaccine development history. In more recent years, Ammayappan *et al.* (2) demonstrated that the attenuation process was the result of genetic changes in the spike and replicase 1a genes. More recently, the importance of natural selection in IBV evolution was further elucidated by van Santen and Toro (21) and McKinley *et al.* (15) in IBV Ark-Delmarva poultry industry (DPI)–vaccinated chickens. The

Table 2. IBV Ark-type virus subpopulations, based on nucleotide changes resulting in amino acid changes, detected after five serial passages in embryonated chicken eggs.

Predominant population[A]	S1 sequence[B]											
Nucleotide	28	53	105	400	404	406	407	410	634	636	671	696
AA	9	18	35	134	135	136	136	137	212	212	224	232
Ark field strain	Thr	Ala	Arg	Arg	Ile	Ala	Ala	Ala	Gln	Ser	Asp	Pro
1	Thr	Ala	Arg	Arg	Ile	Ala	Ala	Ala	**Lys**	Ser	Asp	Pro
3	Thr	Ala	Arg	Arg	Ile	Ala	Ala	Ala	**Lys**	**Thr**	Asp	Pro
4	Thr	Ala	Arg	Arg	Ile	Ala	Ala	Ala	**Lys**	Ser	Asp	**Ser**
8	Thr	**Val**	Arg	Arg	Ile	Ala	Ala	Ala	**Lys**	**Thr**	Asp	Pro
9	Thr	Ala	Arg	Arg	Ile	Ala	**Val**	Ala	**Lys**	**Thr**	Asp	Pro
10	Thr	Ala	Arg	Arg	Ile	**Pro**	Ala	**Val**	**Lys**	**Thr**	Asp	Pro
11	Thr	Ala	Arg	Arg	Ile	Ala	Ala	**Val**	**Lys**	**Thr**	Asp	Pro
12	**Pro**	Ala	Arg	Arg	Ile	Ala	Ala	Ala	Gln	**Ser**	Asp	Pro
13	Thr	Ala	Arg	Arg	Ile	Ala	Ala	Ala	**Lys**	**Thr**	**Ala**	Pro
14	Thr	Ala	Arg	Arg	**Asp**	Ala	Ala	Ala	**Lys**	**Thr**	**Ala**	Pro
16	Thr	Ala	Arg	**Cys**	Ile	Ala	Ala	Ala	**Lys**	**Thr**	**Ala**	Pro
18	Thr	Ala	**Ser**	Arg	Ile	Ala	Ala	Ala	**Lys**	**Thr**	**Ala**	Pro

[A]Only subpopulations based on nonsynonymous nucleotide changes are listed.
[B]Bold indicates changed amino acids.

mechanisms of natural selection involved in IBV evolution have been reviewed recently (19). Moreover, applying selective pressure to IBV populations by serial passages in different biologic substrates has been shown to alter the original virus population not only regarding spike genes but also different nonstructural genes (10).

In the present investigation, selective pressure applied as a result of serial passages in chicken embryos was used to further understand the variability of IBV genotypes prevalent in California chickens. Alignment and comparison of S1 gene sequences of Cal99 and CA1737 isolates available in GenBank shows more than 99% similarity. Thus, the results obtained with the California isolates selected in this study should represent the behavior of other strains belonging to these genotypes. Five embryo passages determined 12 changes in nucleotide positions in the M41 strain and 7 amino acid changes. In IBV Ark, we detected 19 nucleotide position changes of which 13 led to amino acid changes. In Cal 99, 10 nucleotide changes were detected of which 7 constituted amino acid changes, while CA1737 showed variability in 17 nucleotide positions resulting in 10 amino acid changes. The nature of the amino acid changes detected in the current study (described previously) likely alters protein folding and antigenicity. Further studies confirming or disproving consistency of these changes are needed to understand their biologic importance. On the basis of the nucleotide changes, the variation index calculated for IBV M41 was 4.0, i.e., a relatively low value compared with all other viruses included in this study (see the following). In addition, the predominant population of the M41 strain prior to egg passages remained predominant throughout the experiment. This result is in agreement with our previous observations of M41 in which only one nonsynonymous mutation in the S1 gene was detected after infection in chickens (7). This results also agrees with results by Cavanagh *et al.* (5) who analyzed several Mass-type vaccine and field strains isolated over a period of 40 yr. Their results showed that S1 in Mass strains was strongly conserved. The same conservation was noted within the 3c open reading frame and the M gene (5). Thus, the present study corroborates the low variability of IBV M41 compared with other IBV strains.

In contrast, the calculated variability index for the Ark-type virus was 9.5, corroborating the enormous variability previously reported for this IBV genotype (8,9). Furthermore, we have examined changes occurring in the wild Ark virus in chickens vaccinated ocularly with attenuated Ark-DPI–derived vaccines or *in ovo*, with a replication defective recombinant adenovirus expressing a codon optimized IBV Ark S1 gene. Our findings showed five predominant populations in different vaccinated or challenged chickens, demonstrating the high variability of this viral strain. Interestingly, the populations emerging after serial passages in embryos differ from those found in vaccinated chickens (18). This finding further corroborates the

Table 3. IBV Cal99 virus subpopulations, based on nucleotide changes resulting in amino acid changes, detected after five serial passages in embryonated chicken eggs.

Predominant population[A]	S1 sequence[B]						
Nucleotide	12	74	284	400	635	637	638
AA	4	25	95	134	212	213	213
Cal 99 field strain	Leu	Ser	Lys	Arg	Gln	Leu	Leu
1	**Phe**	**Phe**	**Thr**	Arg	**Arg**	Leu	**Pro**
2	Leu	**Phe**	Lys	Arg	Gln	Leu	Leu
4	Leu	**Phe**	**Thr**	Arg	**Arg**	Leu	Leu
5	Leu	**Phe**	Lys	Arg	Gln	**Val**	Leu
7	Leu	**Phe**	**Thr**	Arg	Gln	**Val**	Leu
8	Leu	**Phe**	Lys	**Gly**	Gln	**Val**	Leu

[A]Only subpopulations based on nonsynonymous nucleotide changes are listed.
[B]Bold indicates changed amino acids.

Table 4. IBV CA1737 virus subpopulations, based on nucleotide changes resulting in amino acid changes, detected after five serial passages in embryonated chicken eggs.

Predominant population[A]	S1 sequence[B]						
Nucleotide	167	271	389	407	619	620	647
AA	56	91	130	136	207	207	216
CA1737 field strain	Ser	Ser	Gln	Ser	Gln	Gln	Tyr
1	Ser	**Arg**	Gln	Ser	Gln	**Arg**	Tyr
2	Ser	**Arg**	Gln	Ser	Gln	**Pro**	Tyr
3	Ser	**Arg**	Gln	Ser	Gln	**Pro**	**Asn**
5	Ser	**Arg**	Gln	**Phe**	Gln	Gln	Tyr
6	Ser	**Arg**	Gln	Ser	**Lys**	Gln	**Asn**
7	Ser	**Arg**	Gln	Ser	Gln	Gln	Tyr
8	Ser	**Arg**	Gln	Ser	Gln	Gln	**Asn**
10	**Tyr**	Ser	Gln	Ser	Gln	Gln	Tyr
15	Ser	**Arg**	**Pro**	Ser	Gln	Gln	Tyr

[A]Only subpopulations based on nonsynonymous nucleotide changes are listed.
[B]Bold indicates changed amino acids.

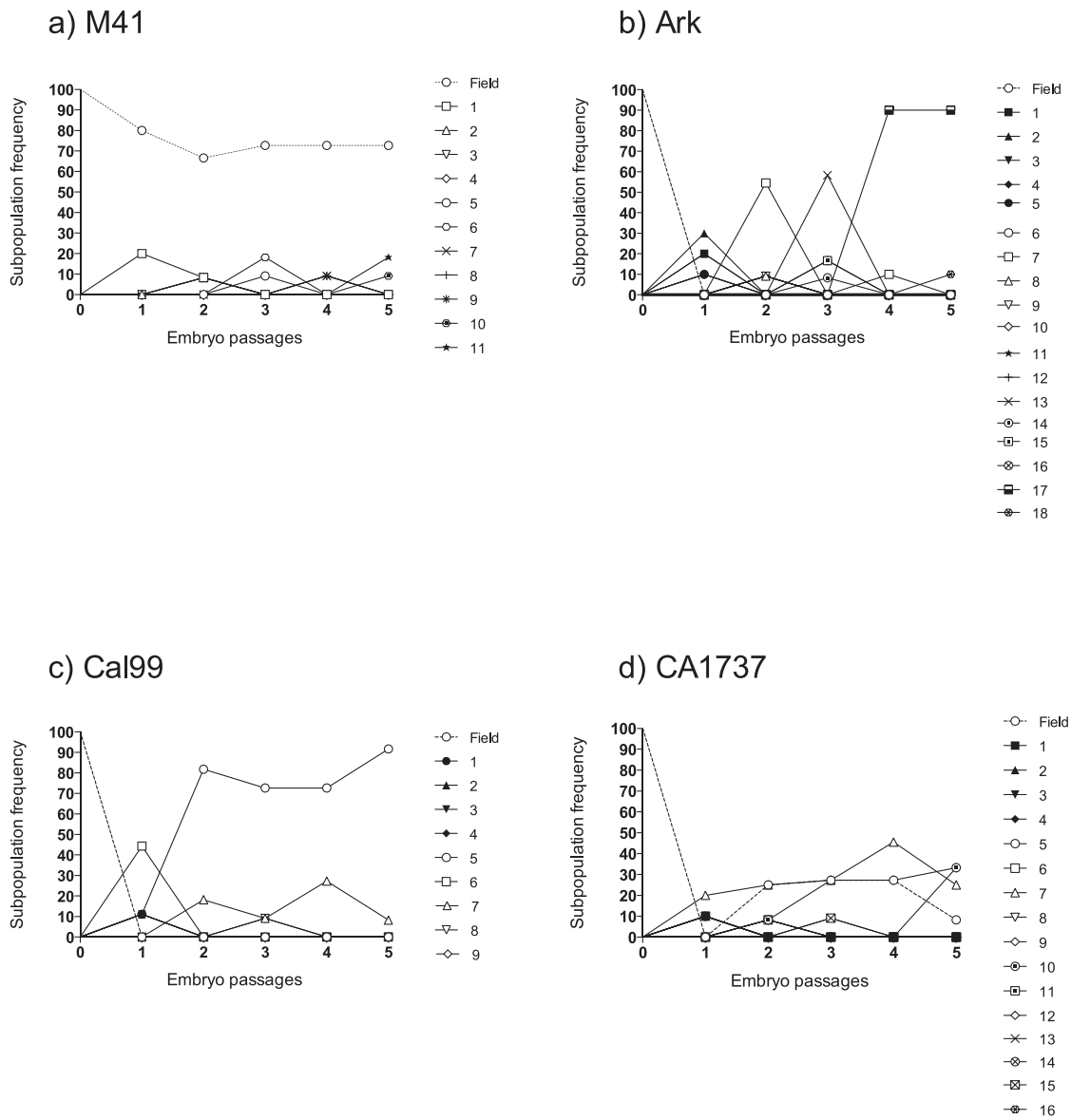

Fig. 3. Frequency of subpopulations (a) M41, (b) Ark type, (c) Cal99, and (d) CA1737 after five serial passages in embryonated chicken eggs.

enormous genetic diversity of IBV Ark viruses, allowing them to succeed in different environments. The calculated variability indices for both Cal99 (5.0) and CA1737 (4.25) were fairly similar. However, in the case of Cal99, a distinct population was successfully selected and became predominant, while in CA1737, four subpopulations intermittently dominated throughout the experiment. The level of population heterogeneity found in the present study may explain the persistence of these variants over the years in the California poultry industry. From these results, i.e., Cal 99 and CA1737 showing a moderate level variability compared with the most variable Ark-type viruses, we may speculate that production of effective type-specific attenuated vaccines would not pose the tremendous challenges of Ark-type strains.

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