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ORIGINAL ARTICLE

Molecular characterization of the S1 gene in GI-17 and GI-13 type isolates of avian infectious bronchitis virus (IBV) in Costa Rica, from 2016 to 2019

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Abstract Avian infectious bronchitis is one of the most important respiratory diseases affecting poultry production worldwide. The etiological agent of this disease is the avian infectious bronchitis virus (IBV). We analyzed 14 isolates of IBV obtained from poultry farms in Costa Rica, from 2016 through 2019. We sequenced the S1 region of the genome and the sequences obtained were submitted to GenBank. Phylogenetic analyses showed that the isolates obtained during 2016–2017 belong to the GI-17 lineage and are related to the Georgia 13-type Ga-13/14255/14 and CK/CR/1160/16 variants, with a 96.90–100% nucleotide sequence identity and a 92.25–100% amino acid sequence identity. The main differences were detected in the RBD and HVR-3 regions, where a series of mutations eliminate an N-glycosylation site in 10 out of 11 isolates. The isolates obtained during 2018–2019 belong to the GI-13 lineage and are closely related to the 4/91 vaccine variant, with over 98% sequence identity at the nucleotide and amino acids levels. Variations were detected in the RBD and HVR regions, with a possible N-glycosylation site detected in isolate CK/CR/0632/19. These results indicate that a GA13-like pathogenic variant circulated during the 2016–2017 period and that the 4/91 variant was detected after the introduction of the vaccine. The variations shown in both the GA13-like and 4/91 isolates examined, reveal the need for continuous surveillance of IBV in Costa Rica, to detect new variants that may be introduced to the country or develop during outbreaks. This information is highly relevant for vaccination planning and disease management programs.

Keywords 4/91-like · Georgia 13 · GA13-like · HVR · IBV · Infectious bronchitis · N-glycosylation

Abbreviations

4/91-CR	4/91 Variant from Costa Rica
BHI	Brain Heart Infusion Broth
GI-13	Genotype 1 lineage 13
GI-17	Genotype 1 lineage 17
GA13	Georgia 13 variant
GA13-CR	Georgia 13 variant from Costa Rica
HVR	Hypervariable region
HVR-1	Hypervariable region 1
HVR-2	Hypervariable region 2
HVR-3	Hypervariable region 3
IBV	Avian infectious bronchitis virus
LANASEVE	Laboratorio Nacional de Servicios Veterinarios
RBD	Receptor-binding domain
SENASA	Servicio Nacional de Salud Animal (National Service of Animal Health)
SPF	Specific pathogen free
SPR	Subtree-Pruning-Regrafting

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Introduction

Infectious bronchitis (IB) is a viral disease that affects layer and broiler chickens (*Gallus gallus domesticus*) and is distributed worldwide [1, 11, 12, 15, 19]. This is an acute and highly contagious disease that affects the respiratory tract and sometimes enteric and urogenital tissues, which results in important economic losses in production [1, 59]. IB is caused by avian infectious bronchitis virus (IBV) [10, 15], which belongs to the order *Nidovirales*, family *Coronaviridae*, genus *Gammacoronavirus* [10]. The IBV virions contain a 27.6 Kb single-stranded, positive-sense genome. The 5'-proximal region of the viral genome codes for two polyproteins (1a, 1ab), which contain the information for nonstructural proteins (NSPs) including the viral replicase. The rest of the genome contains genes that code for the spike (S), coat (E) membrane (M), and nucleoprotein (N) structural proteins, as well as several accessory proteins [1, 15, 17, 55]. The S glycoprotein is a class I fusion protein that suffers a posttranslational cut which results in two subunits: the hypervariable S1 subunit involved in host cell receptor binding and the S2 conserved subunit, which mediates fusion of the viral envelope with the endosomal membrane, through a pH-dependent process [6, 13, 45]. In the endoplasmic reticulum, the S polyprotein is glycosylated and forms trimers [45], which are later proteolyzed by furin in the Golgi complex to form the S1 and S2 subunits [6]. The S1 subunit contains a receptor-binding domain (RBD, residues 19–272) and three hypervariable regions: HVR-1 (residues 38–67), HVR-2 (residues 91–141) and HVR-3 (residues 274–387) [42, 46, 55, 65]. These regions are important due to their role in cell tropism and immune response by the avian hosts [37]. In vitro studies have indicated that as few as 3 amino acid changes in the RBD can generate a change in cell tropism, while the HVRs have been associated with the induction of neutralizing antibodies against the virus [9, 33, 37, 40, 42, 53]. Thus, sequence analyses of the RBD and HVRs in the S1 region, yield the most useful information for the study of genetic variation in IBV [9, 43, 49]. Analysis of the full S1 region is also useful, to include other antigenic regions that could modify viral serotypes [49].

The high diversity of viral types reported since 1931 [5, 19] has been attributed to mutations in the HVRs of the S1 region of the viral spike glycoprotein [19, 44, 55] as well as to recombination events among different viral types [24, 44]. Recombination is an important component of IBV evolution and is influenced by large numbers of chickens kept at high densities, as well as cocirculation and coinfection of different serotypes [11, 24]. More than 50 viral types have been described worldwide, based on the variability of the S1 region [16, 19, 44], which are currently classified in 6 genotypes and 32 lineages [15, 55].

Dynamic and constant surveillance aimed at the detection of circulating viral types should be one of the main strategies to control infectious bronchitis in poultry [27]. Furthermore, the diagnosis should include the sequence analysis of the full S1 region, to detect additional antigenic regions involved in serotype modification [9, 43, 49].

In 2016, egg consumption in Costa Rica showed an increase of 200 000 units compared to previous years, with 650 farms dedicated to this activity and thousands of families that raise poultry for small-scale production and self-consumption (William Cardoza, personal communication, May 17, 2016). IBV is highly involved in respiratory outbreaks that occur in poultry farms in Costa Rica [29]. Since 1990, IBV variants have been detected in Costa Rica, including Massachusetts-like and Arkansas-like variants [5, 29, 38], as well as genotype IBV-CR-53 [5, 38, 47]. In 2016, there was an infectious bronchitis outbreak that exhibited higher mortality after 35 days of age and the associated IBV isolate was classified as a Georgia 13 variant. In response to this outbreak, a new 4–91 vaccine strain (793B) was introduced in 2017 to be used in combination with a Massachusetts vaccine (Ma5), based on the protectotype concept [47]. The emergence of new variants hinders the control of infectious bronchitis in vaccination programs [38] and although the implementation of the Massachusetts and 4/91 protectotype was effective against heterologous variants, the possibility of reversion to virulence or recombination should be considered in disease management [3, 23]. The objective of this research was to determine the genetic variability of the IBV variants isolated during the 2016–2017 outbreak, as well as those variants obtained after the introduction of 4/91 vaccine (2018–2019).

Materials and methods

Sample collection

Samples were obtained from all 7 provinces in Costa Rica from farms with different bird populations: subsistence (< 1000), small (1000–15,000), medium (15,000–50,000), and large (> 50,000). Only the farms with more than 15 000 birds had a vaccination program, performed with live and inactivated vaccines that included H120 and/or Ma5 variants (previous to 2017) and 4/91 (after 2017). A symptom-directed sampling was performed in broilers (young birds) and layers (young and adult birds) that were identified as suspicious cases if they exhibited either respiratory symptomatology, tracheitis, airsacculitis, hemorrhagic bowel, or reduced egg production. Samples were

referred to the National Laboratory of Veterinary Services (LANASEVE) by official and private veterinarians. Pooled samples of 5 tracheal or cloacal swabs from each farm were transported in brain–heart infusion (BHI) broth. In some cases, pooled samples of organs (lungs, tracheae, or ceca) from three individuals with symptoms, were also obtained and stored at −20 °C before processing. All the samples used in this study tested negative for avian influenza and Newcastle disease virus.

RNA extraction

RNA was extracted from either 50 mg of macerated tissue, 200 µL of swab samples, or 200 µL of allantoic fluid (see Viral Propagation), using 1 mL TRIzol™ reagent (Invitrogen Cat. No: 15596026) according to manufacturer´s recommendations [51]. RNA was eluted with 200 µL of nuclease-free water.

Diagnosis of IBV directed to the nucleocapsid gene

Endpoint RT-PCR was performed to detect the nucleocapsid gene in the IBV genome using primers N784 and N1145 [52] (Table S1). Final reactions of one Step RT-PCR (Qiagen, GmbH, Hilden, Germany) of 12.5 µL (8.5 µL mixture + 4 µL sample) containing 0.9 µM of each primer were processed with the following conditions: one cycle of 30 min at 52 °C and 15 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 40 s at 52 °C, and 20 s at 72 °C, with a final cycle for a final extension at 72 °C for 5 min. A 2.5 µL aliquot of amplified products was analyzed by agarose gel electrophoresis in a 1.5% agarose gel, using Ma41 vaccine as the positive control.

Viral propagation

Samples that tested positive by RT-PCR were propagated by inoculation into 9–12 day embryonated, specific pathogen-free (SPF) eggs. A 5 mg sample was homogenized in 1.5 mL of BHI broth with antibiotics and inoculated into the chorioallantoic cavity of three eggs per sample. Embryos were analyzed at 7 days post-inoculation looking for signs of dwarfing, feather underdevelopment, hemorrhage, or finger/body curling [32, 39]. Allantoic fluids were collected for RNA extraction and RT-PCR as previously described, to confirm the presence of the virus. Two or three additional passages were done in negative allantoic fluids.

Sequencing of the S1 gene region

Allantoic fluids that tested positive for the presence of IBV were used to amplify the spike protein-coding S1 gene region, using primers NewS1OLIGO 5' and S1OLIGO3' [18] (Table S1). The RT-PCR reactions were performed using the Qiagen One-Step RT-PCR kit (Qiagen, GmbH, Hilden, Germany) with the protocol modifications described by Gallardo and collaborators (2010), using 4 mM $MgCl_2$ and a final reaction volume of 25 µL (22.5 µL reaction mixture + 2.5 µL sample). Amplification of the RT-PCR product was confirmed by electrophoresis in a 1% agarose gel and bands corresponding to the S1 amplified products (approx. 1600 bp) were purified using the QIAquick PCR purification kit (QIAGEN, Valencia, CA) and sent to Macrogen® South Korea, for sequencing by the Sanger method using the same primers used in RT-PCR. Samples with sequences not covering the entire RT-PCR fragment were resequenced using internal primers shown in Table S1 [1, 19]. The resulting sequences were assembled using Geneious® 11.1.5 (https://www.geneious.com).

Phylogenetic and recombination analysis

A phylogeny based on the S1 region of the spike protein gene was constructed using a total of 162 reference sequences obtained from GenBank (Table S2), under the classification proposed by Valastro et al. (2016). Sequence alignment was performed with the MAFFT algorithm, available in the Guidance2 server (http://guidance.tau.ac.il/) [48]. Sequence editing, translation, and levels of identity were performed using the Geneious® Prime software (https://www.geneious.com). In addition, loss or gain of glycosylation sites in the S1 region was predicted using the NetNGlyc server (http://www.cbs.dtu.dk/services/NetNGlyc).

Phylogenetic trees were inferred using a Bayesian approach with Mr.Bayes 3.2.6 program [25] in the CIPRES Science Gateway site (https://www.phylo.org) [41], for 10 000 000 generations. The best substitution analysis was deducted using PartitionFinder2, with the Greedy algorithm and based on the AICc criterion [34], in the CIPRES Science Gateway V.3 site [41]. The Generalised Time Reversible model (GTR) was selected, with Gamma distribution (Γ) and invariable sites (I) for each of the three codon positions in the S1 sequences. The Tracer V1.7.1 software (http://tree.bio.ed.ac.uk/software/tracer/) was used to corroborate convergence of the Bayesian analysis [15]. Phylogenetic tree robustness was evaluated by analyzing the S1 sequence data set by the Maximum Likelihood method, using PhyML in the ATGC Montpellier Bioinformatics platform (http://www.atgc-montpellier.fr/phyml/) [21]. A GTR + I + Γ4 substitution model and the SPR (Subtree-Pruning-Regrafting) search procedure for branch swapping were used, with an evaluation of node support through a Shimodaira-Hasegawa procedure [55]. Table S3 shows GenBank accession numbers for IBV-positive

samples analyzed in this work. Recombination events were detected using the RDP4 V.4.95 program [1, 66] and they were only considered in this work if they were detected by at least five out of the seven methods available in the program [66].

Results

Diagnosis of IBV directed to the nucleocapsid gene and viral propagation

Out of 146 samples examined, 98 samples tested positive for IBV and a total of 14 isolates were recovered which generated lesions on chicken embryos associated with IBV infection, such as feather underdevelopment, finger curling, blood traces, or embryo dwarfing and curling (Fig. S1). IBV presence was confirmed on these 14 isolates by RT-PCR directed to the nucleocapsid gene. The low recovery from samples could be due to manipulation procedures previous to laboratory analysis [30].

Sequence analysis of the S1 gene in IBV isolates from Costa Rica

Phylogenetic analysis using Maximum Likelihood and Bayesian inference methods indicated that the 14 IBV isolates from Costa Rica classified as genotype I, within lineages GI-17 or GI-13 with high support values for the GI-13 and GI-17 in both phylogenetic trees (Table S3, Figs. 1 and S2), 11 isolates grouped within lineage GI-17 and exhibited 86.42–100% nucleotide sequence identity and 82.13–100% amino acid sequence identity when compared to reference sequences (Table 1). These isolates form a cluster with GA-13 variants GA-13/14255/14 and Ck/CR1160/16 (with high support values), exhibiting high levels of nucleotide (96.90–100%) and amino acid (92.95–100%) sequence identities (Table 1). Moreover, 10 of these GA13-CR isolates show over 99% sequence identity with the Ck/CR/1160/16 isolate from Costa Rica, while the CK/CR/491/17 isolate is closest to the GA-13–14,255/14 variant (Figs. 1 and S2), showing 95.97% and 97.99 sequence identities at the nucleotide and amino acid levels, respectively (Table 1).

The RBD of GA13-CR isolates exhibits low sequence variation, with $\geq$ 95% sequence identity compared to the GA-13/14255/14 variant. The main differences observed in the RBD are in sites that are conserved among the GA13-CR isolates but not present in the GA-13/14255/14 sequence, with the HVR-2 showing the highest variation at the amino acid level (Fig. 2a; Table S4). Moreover, the HVR-3 exhibits $\geq$ 82.76% identity at the amino acid level between the GA-13 CR isolates and the GA-13/14255/14 sequence (Table S4), due to nucleotide insertions at positions r.1009_1010insA, r.1026_1028insUUA and r.1039_1044 insGUUUA (nucleotide positions relative to the GA-13/14255/14 genome sequence). These insertions result in a frameshift variation that diminishes the sequence homology and inserts three amino acid residues between positions 338 and 350 of the sequence alignment (Fig. 2b) and are therefore closely related to the CK/CR/1160/16 isolate from Costa Rica. These mutations are observed in all GA13-CR isolates, except for the CK/CR/491/17 isolate, which exhibits a 92.92% amino acid sequence identity with GA-13/14255/14 (Fig. 2; Table 1).

Comparison of the GA13-CR isolates with vaccine variants indicates a low amino acid sequence identity ($\leq$ 63.33%) in HVR-1, while the HVR-2 sequence identity is closest ($\geq$ 73,68%) to the 4/91 vaccine variant and the HVR-3 sequence identity is higher than 73,68% in all isolates, except for isolate CK/CR/491/17 (Table S4) which exhibits differences with the vaccine variants between amino acid residues 338 and 350 (Fig. 2). All isolates exhibited high amino acid sequence identities (over 98%) at the carboxyl-terminus.

The three remaining isolates (Fig. 1; Table 1) were closely related to isolates in the GI-13 lineage (over 99.13% and 97.96% identity at the nucleotide and amino acid levels, respectively), including the 4/91.V vaccine strain (AF093794) which exhibited high sequence identities with the 4/91-CR isolates (99.26–99.81% and 99.14–99.44% at the nucleotide and amino acid levels, respectively). The two isolates from 2018 that correspond to this lineage are similar to the 4/91 reference sequence (Table 1) and even though some differences were observed in HVR-1 and HVR-2 (Fig. S3a and S3b), the entire RBD shows over 98% amino acid sequence identity (Table S4). The 2019 isolate (CK/CR/0632/19) showed a lower (98.14%; Table 1) amino acid sequence identity with the other 4/91-CR isolates, with five amino acid substitutions (p.Gly55Lys, p.Asp64Tyr, Gly120Asp, Ser140Phe) in the HVR1 and HVR2, two amino acid substitutions (p.Ala88-Pro and p.Ala193Val) positioned in the RBD but outside the HVRs, and one substitution (p.Pro452Phe) positioned outside the RBD (Fig. S3b). As shown in Table 1 and S4, isolate CK/CR/0632/19 exhibited the lowest amino acid sequence identity when compared with vaccine variant 4/91. It is noteworthy that this was the only 4/91 isolate that was associated with clinical symptoms (respiratory, digestive, and egg quality issues). The recombination analysis for the S1 region showed no specific recombination events for the GA13-CR and 4/91-CR isolates.

A total of 16 possible N-glycosylation sites were detected in most of the GA13-CR sequences based on the presence of the N-Xaa-T/S motif, except for three of the isolates examined (Tables 2 and S5). Isolates CK/CR/185/

Fig. 1 Phylogenetic tree made using the nucleotide sequence of the S1 region of the IBV spike protein gene. The tree was inferred using the Bayesian method using the MrBayes program. IBV isolates used in this study are shown in bold. The CR sequences grouped within the GI-17 and GI-13 lineages

Table 1 Nucleotide and amino acid sequence identities in the S1 region of the spike protein gene of IBV isolates during the 2016–2019 period, compared with reference vaccine sequences

Strain		Nucleotide percentage of identity												
		1	2	3	4	5	6	7	8	9	10	11	12	13
GA-13/14255/14	1		**96.89**	**97.21**	**97.15**	**97.09**	**97.15**	**97.27**	**97.21**	**97.21**	**97.02**	**97.23**	**96.90**	**97.99**
Ck/CR/1160/16	2	**93.23**		**99.94**	**99.88**	**99.82**	**99.88**	**100.00**	**99.94**	**99.94**	**99.76**	**99.82**	**99.51**	**99.15**
CK/CR/1167/16	3	**93.67**	**100**		**99.82**	**99.76**	**99.82**	**99.94**	**99.88**	**99.88**	**99.70**	**99.82**	**99.45**	**99.09**
CK/CR/1298/16	4	**93.5**	**99.64**	**99.64**		**99.70**	**99.76**	**99.88**	**99.82**	**99.82**	**99.64**	**99.76**	**99.39**	**99.03**
CK/CR/1329/16	5	**93.5**	**99.82**	**99.82**	**99.45**		**99.94**	**99.82**	**99.76**	**99.76**	**99.82**	**99.70**	**99.57**	**98.97**
CK/CR/1344/16	6	**93.68**	**100**	**100**	**99.64**	**99.82**		**99.88**	**99.82**	**99.82**	**99.88**	**99.76**	**99.64**	**99.03**
CK/CR/0068/17	7	**93.68**	**100**	**100**	**99.64**	**99.82**	**100**		**99.94**	**99.94**	**99.76**	**99.88**	**99.51**	**99.15**
CK/CR/175/17	8	**93.5**	**99.82**	**99.82**	**99.45**	**99.64**	**99.82**	**99.82**		**99.88**	**99.70**	**99.82**	**99.45**	**99.09**
CK/CR/176/17	9	**93.5**	**99.82**	**99.82**	**99.45**	**99.64**	**99.82**	**99.82**	**99.64**		**99.82**	**99.82**	**99.45**	**99.09**
CK/CR/185/17	10	**93.31**	**99.64**	**99.64**	**99.27**	**99.45**	**99.64**	**99.64**	**99.45**	**99.82**		**99.64**	**99.51**	**98.91**
CK/CR/186/17	11	**93.55**	**99.46**	**99.64**	**99.27**	**99.45**	**99.64**	**99.64**	**99.45**	**99.45**	**99.27**		**99.39**	**99.03**
CK/CR/307/17	12	**92.95**	**99.09**	**99.09**	**98.72**	**98.91**	**99.09**	**99.09**	**98.91**	**98.91**	**98.72**	**98.72**		**98.66**
CK/CR/491/17	13	**95.97**	**96.93**	**96.93**	**96.56**	**96.75**	**96.93**	**96.93**	**96.75**	**96.75**	**96.56**	**96.56**	**96.02**	
4/91.V	14	72.08	75.55	75.55	75.37	75.37	75.55	75.55	75.55	75.37	75.18	75.37	75.74	72.81
CK/CR/327/18	15	71.72	75.18	75.18	75.00	75.00	75.18	75.18	75.18	75.37	75.18	75.00	75.37	72.45
CK/CR/1094/18	16	71.72	75.18	75.18	75.00	75.18	75.18	75.18	75.18	75.00	74.82	75.00	75.37	72.45
CK/CR/0632/19	17	71.53	75.00	75.00	74.82	74.82	75.00	75.00	75.18	74.82	74.63	74.82	75.18	72.26
Ma5.V	18	73.09	75.46	75.46	75.46	75.27	75.46	75.46	75.27	75.46	75.27	75.27	75.82	73.09
M41	19	71.63	75.00	75.00	75.05	74.86	75.05	75.05	74.86	75.05	74.82	74.51	75.54	72.66
H120.V	20	72.52	75.36	75.36	75.41	75.23	75.41	75.41	75.23	75.41	75.18	74.87	75.72	73.02
ARK99	21	75.04	77.60	77.45	77.50	77.31	77.50	77.50	77.31	77.68	77.45	77.12	77.45	75.59
AR/11/ER/33	22	76.32	78.17	78.17	78.17	78.17	78.17	78.17	77.98	77.98	77.80	77.98	78.35	75.59
CHL/12.185/Q1	23	72.92	75.76	75.76	75.57	75.57	75.76	75.76	75.57	75.76	75.57	75.57	75.76	73.30
YX10	24	69.2	72.73	72.51	72.56	72.56	72.56	72.56	72.56	72.56	72.33	72.22	72.51	70.38
GA08	25	77.74	80.70	80.70	80.51	80.51	80.70	80.70	80.51	80.88	80.70	80.51	80.51	78.10
DE/072/92	26	44.41	47.18	46.43	46.52	46.52	46.52	46.52	46.70	46.52	46.43	46.73	46.79	44.68
Strain		1	2	3	4	5	6	7	8	9	10	11	12	13
		Amino acid percentage of identity												

Strain		Nucleotide percentage of identity													
		14	15	16	17	18	19	20	21	22	23	24	25	26	
GA-13/14255/14	1	75.55	75.43	75.37	75.31	78.14	78.15	78.02	78.76	78.65	76.21	73.52	81.14	54.68	1
Ck/CR/1160/16	2	76.65	76.53	76.47	76.41	79.24	79.93	79.51	80.12	79.57	77.66	75.13	82.42	56.47	2
CK/CR/1167/16	3	76.65	76.53	76.47	76.41	79.24	79.75	79.38	79.94	79.57	77.66	75.00	82.36	56.00	3
CK/CR/1298/16	4	76.59	76.47	76.41	76.35	79.12	79.66	79.30	79.92	79.45	77.54	74.80	82.30	55.96	4
CK/CR/1329/16	5	76.47	76.35	76.35	76.23	79.12	79.61	79.25	79.81	79.45	77.54	74.88	82.24	55.98	5
CK/CR/1344/16	6	76.53	76.41	76.35	76.29	79.12	79.66	79.30	79.85	79.45	77.60	74.80	82.30	55.90	6
CK/CR/0068/17	7	76.65	76.53	76.47	76.41	79.24	79.78	79.42	79.98	79.57	77.66	74.92	82.42	56.02	7
CK/CR/175/17	8	76.59	76.47	76.41	76.47	79.18	79.72	79.36	79.92	79.51	77.60	74.92	82.36	56.08	8
CK/CR/176/17	9	76.59	76.59	76.41	76.35	79.24	79.78	79.42	80.04	79.51	77.73	74.92	82.49	56.02	9
CK/CR/185/17	10	76.41	76.41	76.23	76.16	79.06	79.56	79.20	79.82	79.33	77.60	74.76	82.30	55.88	10
CK/CR/186/17	11	76.59	76.47	76.41	76.35	79.12	79.63	79.21	79.88	79.45	77.60	74.81	82.42	56.09	11
CK/CR/307/17	12	76.53	76.41	76.35	76.29	79.24	79.75	79.38	79.64	79.39	77.41	74.70	82.24	55.94	12
CK/CR/491/17	13	75.80	75.67	75.61	75.55	78.38	78.90	78.53	79.21	78.72	76.84	74.15	81.63	55.23	13
4/91.V	14		**99.81**	**99.75**	**99.26**	78.12	78.32	78.26	77.33	77.33	78.53	78.36	76.93	56.07	14
CK/CR/327/18	15	**99.44**		**99.81**	**99.20**	78.06	78.26	78.20	77.27	77.14	78.53	78.24	76.93	56.13	15

Table 1 continued

Strain		Nucleotide percentage of identity													
		14	15	16	17	18	19	20	21	22	23	24	25	26	
CK/CR/1094/18	16	**99.44**	**99.44**		**99.13**	78.00	78.20	78.13	77.08	77.21	78.34	78.18	76.75	56.19	16
CK/CR/0632/19	17	**98.14**	**97.96**	**97.96**		77.87	78.20	78.01	77.21	77.08	78.27	78.18	76.93	56.01	17
Ma5.V	18	74.31	73.94	73.75	73.75		97.64	99.88	77.99	78.23	76.36	77.15	79.02	57.44	18
M41	19	73.43	73.06	72.88	73.25	95.90		97.58	78.87	78.31	77.00	77.05	79.26	58.06	19
H120.V	20	74.54	74.17	73.99	73.99	99.81	95.82		78.29	78.37	76.49	77.17	79.14	57.99	20
ARK99	21	75.37	75.18	74.82	75.18	75.78	75.36	75.67		77.57	76.81	76.06	83.31	57.73	21
AR/11/ER/33	22	76.47	75.92	76.29	75.92	76.70	76.19	76.92	76.29		77.64	75.54	79.26	57.68	22
CHL/12.185/Q1	23	77.16	76.97	76.78	76.97	73.66	73.47	73.85	76.91	76.34		76.47	77.13	56.65	23
YX10	24	78.45	78.08	78.08	77.90	76.81	75.59	76.31	74.46	76.78	76.57		76.30	57.30	24
GA08	25	77.16	77.16	76.80	76.98	77.25	77.25	77.43	82.32	80.48	77.86	74.91		56.67	25
DE/072/92	26	46.00	46.00	46.00	46.00	50.37	50.62	50.71	48.77	48.19	48.31	49.73	47.38		26
Strain		14	15	16	17	18	19	20	21	22	23	24	25	26	
		Amino acid percentage of identity													

Variants exhibiting over 90% sequence identity with the Costa Rican isolates, are shown in bold

17 and CK/CR/186/17 show substitutions at sequence position 271 (p.Thr273Ile and p.Thr272Pro, respectively) that affect the N-glycosylation motif. Isolate CK/CR/491/17 maintains most of the conserved N-glycosylation sites in the GA13-CR isolates and the GA13-like reference sequences (CK/CR/1160/16 and GA-13/14255/14). However, a new N-glycosylation was predicted at position 314 which is not present in any of the GA13-like variants analyzed in this study (Tables 2 and S5; Fig. 2, blue box). A total of 17 N-glycosylation sites were predicted in the 4/91-CR isolates (CK/CR/1094/18 and CK/CR/0632/19) and an additional site was predicted at position 57 for the CK/CR/327/18 isolate (Fig. S3, pink box).

Discussion

Infectious bronchitis is an important disease that affects the poultry industry in many different countries including Costa Rica, where it has been reported since 1990 [5, 29, 38]. After a high mortality outbreak occurred in Costa Rica during 2016 [47, 58], samples were collected to undertake an IBV diversity study. We analyzed a total of 146 samples from broilers and layers with suspicious symptomatology during a 2016–2019 period and 14 of these samples were recovered for viral propagation in embryonated eggs.

Samples recovered between 2016 and 2017 were identified as GA13-like variants and the complete genome sequence of one of these variants was reported in 2021 [58]. There have been subsequent informal commercial reports of GA13-like detection in field samples, but to the best of our knowledge, this is the first detailed molecular study of GA13-like variants from an outbreak. Currently, the GA13 variant appears to be relatively infrequent and limited to the southeast of the United States, although it is still an important threat to the poultry industry due to the lack of specific commercial vaccines for this variant [28].

The GA13-like isolates (GA13-CR) described in this study are closely related to the reference sequence (GA-13/14255/14), but they show several sequence variations (Figs. 1 and 2; Tables 1 and S4). One of the most significant sequence variations detected was at 338–350 (Fig. 2b, blue box), which causes a frameshift mutation and a predicted three amino acid insertion. All these sequence variations are probably associated with high mutation rates of the RNA-dependent RNA-polymerase, which may result in nucleotide substitutions, insertions, or deletions [20, 62]. Particular selective pressures in subpopulations of the virus at different locations [17, 23, 63] may contribute to the establishment of a particular viral variant. Variable selective pressures may include the movement of workers, animals, and even farm materials between locations, which may facilitate viral propagation [35, 56] and even population bottleneck events and subsequent genetic drift [54]. Once again, these factors must be considered in biosecurity measures for disease management in the field.

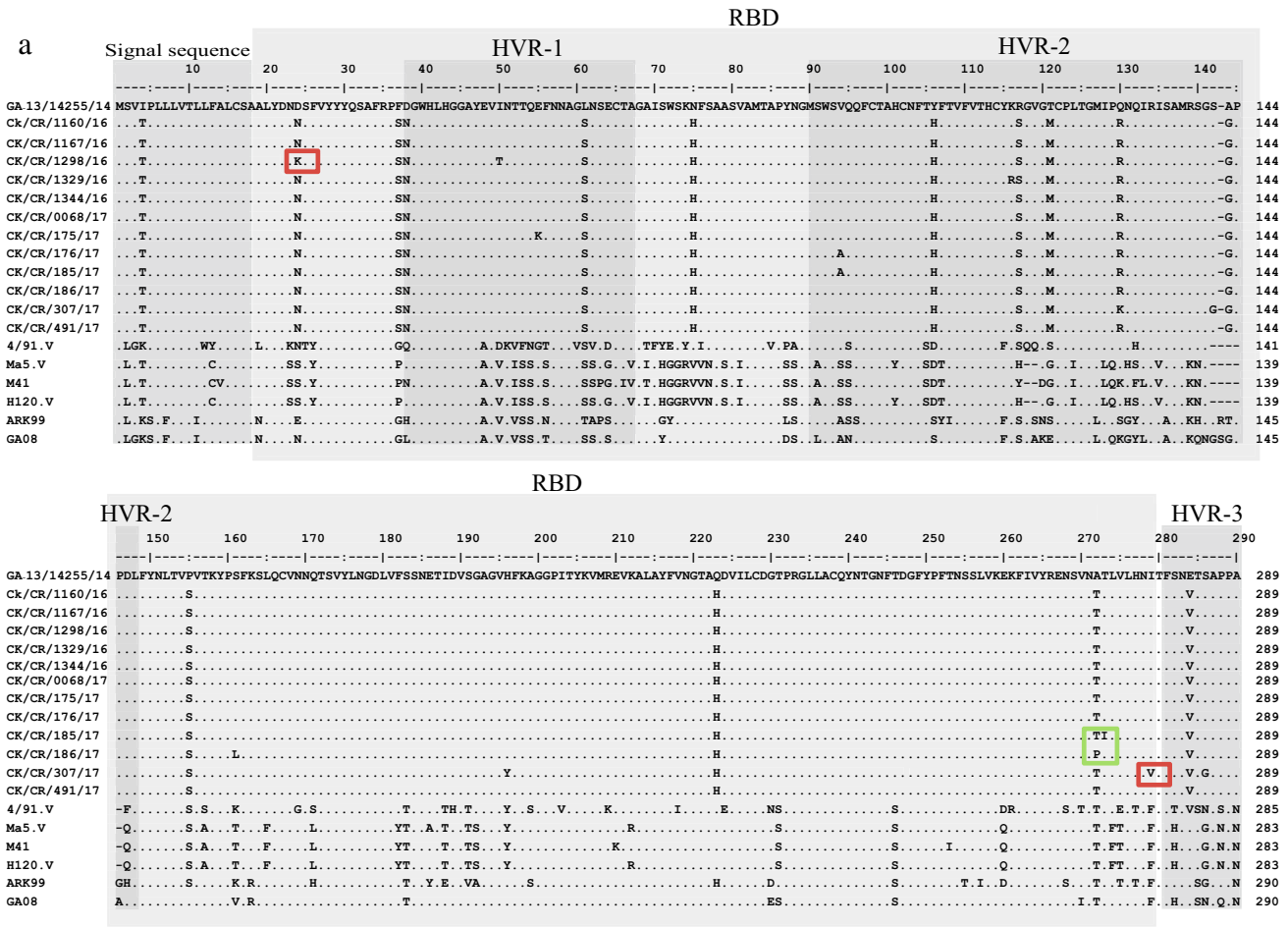

Fig. 2 Sequence alignment of amino acids from the S1 region of the IBV spike protein in the GA13-like isolates of CR. The deduced sequence for the S1 protein was aligned with reference IBV vaccine variant sequences. The dots indicate the residues that are identical with the GA-13/14255/14 variant at that position. The HVRs and the RBD were located according to the M41 variant [42, 46, 55, 65]. The red box shows a change in an N-glycosylation site of the sequence CK/CR/1298/16 and CK/CR/307/17. The green box shows the amino acid substitution that eliminates the N-glycosylation site in isolates CK/CR/185/17 and CK/CR186/17. The blue box indicates the region that differs between the GA-13/14255/14 and CK/CR/491/17 variants, compared to the other 11 GA13-like isolates of CR

The N-glycosylation site analysis shown in this study is novel, particularly regarding GA13-like isolates. Previous studies have predicted 30 to 35 N-glycosylation sites in the complete S1 and S2 regions of QX-like and Massachusetts-like variants [2], 10 to 14 sites in a 7937/B variant (closely related to the 4/91 variant) in the S1 region [57], and 10 sites in an M41 variant, six of which are essential for spike protein recognition by the host receptor [8, 45]. Our study indicates that N-glycosylation patterns are relatively conserved, given the observed sequence variation in the GA13-CR variants and the GA13 reference sequence (Table 2) and are distinct from the patterns detected in the vaccine strains. This type of analysis supports the study of diversity in IBV, together with recombination analysis and its relation to new serotypes, since mutations that modify N-glycosylation may also have an effect on the structure of the protein and lead to changes in antigenic patterns cellular tropism, and virulence [31, 37, 45, 61, 63].

In the case of the samples recovered during the 2018–2019 period, the three isolates obtained were closely related to the 4/91 vaccine variant, that was introduced to Costa Rica as part of a new vaccination program in May 2017, based on the protectotype concept [47]. Therefore, it was not surprising to detect the 4/91 variant during this period, since the vaccine had proven to be effective in controlling the outbreak in other countries where they used the 4/91 strains and they re-isolated the vaccine variant [36]. We consider that these variants are most probably re-isolations of the 4/91 vaccine used, due to the low sequence variation observed, primarily in the hypervariable regions 1 and 2 (Fig. S3, Table S4). Also, the number of glycosylation sites predicted here for the 4/91-CR isolates coincides with those reported elsewhere for other 4/91 vaccine

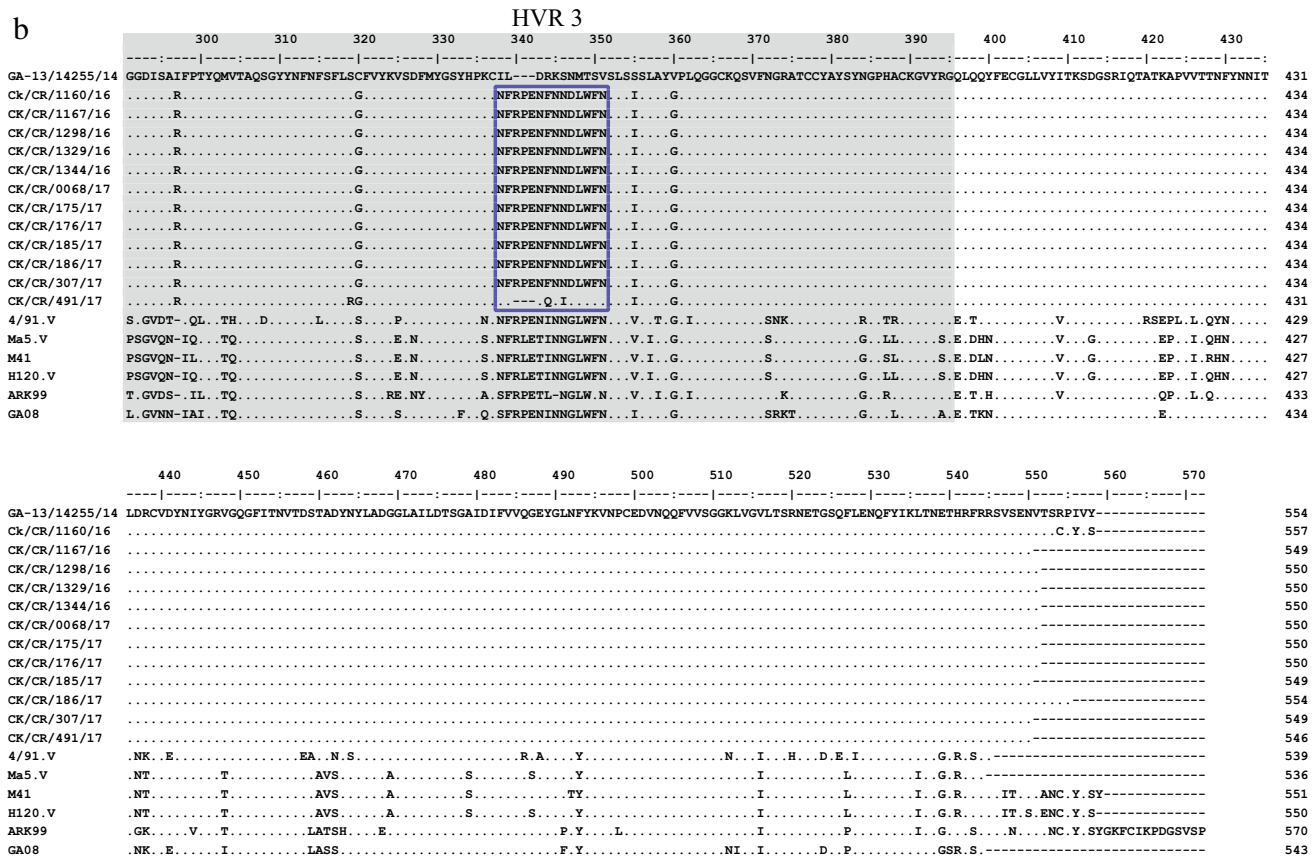

Fig. 2 continued

variants [57]. Finally, we detected an extra glycosylation site in one of the 4/91-CR isolates, which indicates that a single amino acid substitution may result in variation of glycosylation patterns of the spike protein.

It should be noted that a 4/91-CR isolate (CK/CR/0632/19) came from birds that exhibited symptomatology, but that does not necessarily mean that this is a vaccine revertant. Variants related to the 4/91 vaccine have been re-isolated in previous studies. Guzman et al., isolated a GI-13 homologous strain after a 4/91 vaccine introduction in birds with symptoms of infection [23], and another work reported the isolation of a 4/91 vaccine-derived strain, associated with mild symptomatology [50]. Moreover, postvaccination reactions vary in severity and are influenced by a series of factors, such as age of the bird, route of vaccination, vaccine concentration and degree of attenuation [60], mass spray vaccination [14, 35], environmental factors [60], and common secondary infections with microorganisms [7, 60]that may exacerbate the symptomatology of IBV infection [4, 26]. The introduction of new live vaccines has the potential to disperse new uncharacterized variants that may evolve in many different ways [22, 23] and the interactions occurring during viral co-infections in the field, may also have an impact on field strain replacement by vaccine variants [22].

Recombination is an important factor in the diversity of IBV variants. Recombination events have been reported between field variants and vaccine strains [64], and even the emergence of new variants with multiple recombination events [44]. Previous publications detected recombination events for GA13-CR [58] and 4/91-like variants [57], which reinforces the need for continuous surveillance that may detect novel IBV variants. However, no recombination events were detected in the analyses performed during this study.

In conclusion, the results shown here indicate that a GA13-like variant circulated in Costa Rica between 2016 and 2017. Isolates from this period exhibit differences in nucleotide and amino acid sequence as well as N-glycosylation sites, between themselves and compared to reference sequences of IBV variants. In 2018, re-isolations of 4/9-like variants were detected in Costa Rica and such variants should be monitored to detect possible virulent revertants, such as those identified in other studies [64]. This study aims to better understand the consequences of the introduction of vaccine variants of IBV and generate information that may aid in decision-making when

Table 2 Distribution of predicted N-glycosylation sites in IBV sequences from Costa Rica and reference vaccine variants

Position	Isolates/Variants[b]								
	GA-13/14255/14	Ck/CR/1160/16	GA13-CR	CK/CR/491/17[c]	4/91.V	4/91-CR	CK/CR/327/18[c]	M41	MA5.V
23	NDSF[23]	NNSF[23]	†NNSF[23]	NNSF[23]					
51	NNTQ[51]	NNTQ[51]	NNTQ[51]	NNTQ[51]				NISS[51]	NISS[51]
54					NGTN[54]	NGTN[54]	■NGTN[54]		
57							NNTV[57]		
75					NISA[75]	NISA[75]	NISA[75]		
77								NASS[77]	NASS[77]
103	NFTY[103]	NFTH[103]	NFTH[103]	NFTH[103]	NFSD[103]	NFSD[103]	NFSD[103]	NFSD[103]	NFSD[103]
151	NLTV[151]	NLTV[150]	NLTV[150]	NLTV[150]	NLTV[146]	NLTV[146]	NLTV[146]	NLTV[144]	NLTV[144]
170	NQTS[170]	NQTS[169]	NQTS[169]	NQTS[169]	NSTS[165]	NSTS[165]	NSTS[165]	NLTS[163]	NLTS[163]
185	NETI[185]	NETI[184]	NETI[184]	NETI[184]	NETT[180]	NETT[180]	NETT[180]	NETT[178]	NATT[178]
219	NGTA[219]	NGTA[218]	NGTA[218]	NGTA[218]	NGTA[214]	NGTA[214]	NGTA[214]	NGTA[212]	NGTA[212]
244	NFTD[244]	NFTD[243]	NFTD[243]	NFTD[243]	NFSD[239]	NFSD[239]	NFSD[239]	NFSD[237]	NFSD[237]
254	NSSL[254]	NSSL[253]	NSSL[253]	NSSL[253]	NSSL[249]	NSSL[249]	NSSL[249]	NSSL[247]	NSSL[247]
271	NATL[271]	NNTL[270]	*NNTL[270]	NNTL[270]	NNTL[266]	NNTL[266]	NNTL[266]	NNTF[264]	NNTF[264]
278	NITF[278]	NITF[277]	**NITF[277]	NITF[277]	NFTF[273]	NFTF[273]	NFTF[273]	NFTF[271]	NFTF[271]
283		NVTS[282]	NVTS[282]	NVTS[282]	NVSN[278]	NVSN[278]	NVSN[278]	NETG[276]	NETG[276]
314				NFSF[313]	NLSF[308]	NLSF[308]	NLSF[308]		
347	NMTS[343]			NMTS[343]					
433	NITL[433]	NITL[432]	NITL[432]	NITL[429]	NITL[427]	NITL[427]	NITL[427]	NITL[425]	NITL[425]
455	NVTD[455]	NVTD[454]	NVTD[454]	NVTD[451]	NVTE[449]	NVTE[449]	NVTE[449]	NVTD[447]	NVTD[447]
521					NETD[515]	NETD[515]	NETD[515]		
538	NETH[538]	NETH[537]	NETH[537]	NETH[534]	NGTR[532]	NGTR[532]	NGTR[532]	NGTR[530]	NGTR[530]
550	NVTS[550]	NVTS[549]	●NVTS[549]						

N-glycosylation sites were determined with the NetNGlyc server. The consensus N-Xaa-T/S tripeptide is underlined and the immediate following amino acid is show. Blanks indicate that N-glycosylation was not predicted at that alignment site

a-Position in alignment, based on deduced protein sequence, including the signal sequence

b-Superscripts indicate the position in each individual sequence

c-These isolates are shown separately because the exhibit distinct N-glycosylation sites

†-At this position, isolate CK/CR/1298/16 exhibits a NKSF sequence

*-No N-glycosylation site was predicted at this site for isolates CK/CR/185/17 and CK/CR/186/16

**-At this position, isolate CK/CR/307/17 exhibits a NVTF sequence

●-This N-glycosylation site was predicted only in the CK/CR/186/17 isolate

■-At this position, isolate CK/CR/0632/19 exhibits a NKT sequence

considering the introduction of vaccine variants not circulating in the country. Finally, full-genome sequencing of variants can also be implemented to aid in the detection of potential revertants in the field and relate this information to changes in genome sequence that affect pathogenicity and cell tropism. Such information would be very valuable in the selection of vaccination programs and the introduction of new vaccines.

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Data availability the S1 gene sequences obtained from the 14 isolates were uploaded in the genbank database under the accession

number MT230562, MT230563, MT230564, MT230565, MT230566, MT230567, MT230568, MT230569, MT230570, MT230571, MT230572, MT230573, MT230574, and MT230575.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval All procedures performed in animals were in accordace with the ethical standards of the "Comité Institucional de Cuido y Uso de Animales" (CICUA) of the University of Costa Rica.

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