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ANÁLISIS MOLECULAR DE VARIANTES DEL VIRUS DE LA BRONQUITIS INFECCIOSA AVIAR (IBV) OBTENIDAS TRAS LA INTRODUCCIÓN DE LA VACUNA ATENUADA 4/91 EN COSTA RICA DURANTE EL 2017

Tesis sometida a la consideración de la Comisión del Programa de Estudios de Posgrado en Biología para optar al grado y título de Maestría Académica en Genética y Biología Molecular

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RESUMEN

La bronquitis infecciosa aviar es una enfermedad viral altamente contagiosa que afecta los sistemas respiratorio, reproductivo, digestivo y renal de las gallinas (*Gallus gallus*) de todas las edades. Su agente etiológico es el virus de la bronquitis infecciosa aviar (IBV), el cual pertenece al género *Gammacoronavirus* de la familia Coronaviridae y del orden Nidovirales. El IBV, al igual que en la mayoría de los virus de ARN, presenta una alta tasa de mutaciones y eventos de recombinación, por lo que es un virus muy variable. Las vacunas atenuadas son unos de los principales métodos de control del IBV, su efectividad ha sido extensamente demostrada, sin embargo, también han sido relacionadas con riegos a largo plazo debido a eventos de recombinación y reversión de virulencia.

En Costa Rica se ha documentado la presencia del IBV desde 1990. Existen reportes de la presencia de las variantes Massachusetts, Arkansas, Pennsylvania y variantes locales como IBV-CR-53, además de un reciente brote en 2016-2017 asociado a la variante Georgia 13 del genotipo I, linaje 13 (GI-13). A partir de este último brote se introdujo la vacuna atenuada 4/91 (GI-17) en 2017, variante de la cual no existían reportes previos en su forma silvestre en el país. El objetivo de esta investigación fue realizar una caracterización molecular de las variantes del virus de la bronquitis infecciosa circulantes en el país seis años después de la introducción de la vacuna 4/91.

Se analizaron un total de 177 muestras de aves sintomáticas, de las cuales 43 resultaron positivas para el virus de la bronquitis infecciosa. Se obtuvieron siete secuencias completas de S1 y las cuales se agruparon dentro del linaje GI-13 mediante análisis filogenéticos. El análisis de secuencias mostró una alta similitud genética con la cepa vacunal 4/91, con identidades de secuencias de nucleótidos y aminoácidos superiores al 99,13% y 97,96%, respectivamente, a pesar de que estas muestras se tomaron de aves no vacunadas. El análisis de modificación postraduccional de la proteína S1 reveló sitios conservados de N-glicosilación y palmitoilación, mientras que se predijeron dos cambios de fosforilación de serina entre las secuencias obtenidas y la cepa vacunal. El análisis de presión selectiva identificó 10 sitios bajo selección positiva, ubicados principalmente dentro del dominio de unión al receptor y las regiones hipervariables de la subunidad S1. La presencia de variantes similares a la 4/91 en aves no vacunadas requiere atención, y su relación con la patología observada requiere más investigación. La vigilancia continua es esencial para detectar posibles mutantes que escapen a la vacuna y mitigar su impacto.

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LISTA DE ABREVIATURAS

BHI: Brain heart infusion broth (Infusión cerebro corazón)

BI: Bayesian Inference (Inferencia Bayesiana)

dN: Non-synonymous nucleotide substitutions (Sustituciones de nucleótidos no sinónimas)

dS: Synonymous nucleotide substitutions (Sustituciones de nucleótidos sinónimas)

E: Envelope protein (Proteína de envoltura)

FEL: Fixed Effects Likelihood (Verosimilitud de Efectos Fijos)

FUBAR: Fast Unconstrained Bayesian Approximation (Aproximación Bayesiana Rápida Sin Restricciones)

GA13-CR: Variante Georgia 13 de Costa Rica

GA13-like: Georgia 13 like strain (variante tipo Georgia 13)

GI-13: Genotype I linage 13 (Genotipo I linaje 13)

GI-17: Genotype I linage 17 (Genotipo I linaje 17)

HVRs: Hypervariable regions (Regiones hipervariables)

IBV: Infectious Bronchitis Virus (Virus de la Bronquitis Infecciosa Aviar)

M41: Massachusetts 41 variant (Variante Massachusessts 41)

M: Membrane protein (Proteína de membrana)

Ma5: Massachusetts variant (Variante Massachusetts)

MEME: Mixed Effects Model of Evolution (Modelo de Efectos Mixtos de Evolución)

ML: Maximum Likelihood (Máxima Verosimilitud)

N: Nucleocapsid (Nucleocápside)

Nsp: Nonstructural protein (Proteína no estructural)

PCR: Polymerase Chain Reaction (Reacción en cadena de la polimerasa)

RBD: Receptor Binding Domain (Dominio de unión al receptor)

RFLPs: Restriction fragment length polymorphisms (Análisis de polimorfismos de longitud de fragmentos de restricción)

RT-PCR: Reverse transcription PCR (PCR de transcripción inversa)

S: Spike protein (Proteína espicular)

SENASA: Servicio Nacional de Salud Animal

SLAC: Single-Likelihood Ancestor Counting (Conteo de Ancestros de Única Verosimilitud)

SPF: Embryonated specific pathogen-free eggs (huevos embrionados libres de patógenos específicos)

INTRODUCCIÓN

Historia de la bronquitis infecciosa aviar

La bronquitis infecciosa aviar es una enfermedad viral altamente contagiosa que afecta los sistemas respiratorio, reproductivo, digestivo y renal de las gallinas (*Gallus gallus*) de todas las edades (De Wit et al., 2011). Fue reportada por primera vez en la década de 1930 en Dakota del Norte, Estados Unidos (Schalk, 1932). Inicialmente, se creyó que la enfermedad estaba relacionada con una forma leve de laringotraqueítis infecciosa (Bushnell & Beandly, 1933), pero no fue sino hasta 1936, a través de estudios de inmunidad cruzada, que se demostró que la enfermedad era causada por un agente distinto (Beach & Schalm, 1936).

Desde su aparición, esta enfermedad ha representado un desafío significativo para la industria avícola a nivel mundial, por lo que se iniciaron esfuerzos para controlarla (De Wit et al., 2011). Gracias a estudios realizados en 1937 por Beaudette y Hudson, se logró aislar el virus en huevos embrionados (Beaudette & Hudson, 1937) y se determinó que a medida que se aumenta el número de pasajes en embriones, el virus se vuelve más letal para los embriones, pero menos patogénico para las gallinas (Delaplane & Stuart, 1941). Esto permitió que en 1940 se demostrara que era posible evitar los impactos económicos de las infecciones en períodos de postura, cuando se exponía a las aves en edades tempranas al virus atenuado mediante pasajes (Cook et al., 2012). La exposición al virus se realizaba en un pequeño grupo de aves, las cuales posteriormente se liberaban con las demás, para que de manera natural expusieran al resto de la parvada. Este método de inmunización se utilizó hasta principios de los años 1950, cuando se desarrolló y comercializó la primera vacuna atenuada con la variante M41 (Massachusetts 41) (Cook et al., 2012).

Sin embargo, en 1956 se evidenció la existencia de diferencias inmunológicas entre las variantes virales circulantes, las cuales podían evitar la protección cruzada de la vacuna M41 (Jungherr et al., 1957). Esto llevó al desarrollo de vacunas contra otras variantes y al concepto de "protectotipo" definido como la aplicación conjunta de variantes vacunales antigénicamente distintas, que aumenta la protección contra variantes heterólogas (Cook et al., 1999). También llevó al desarrollo de otros tipos de vacunas como las inactivas (Bhuiyan et al., 2021), y más recientemente a las vacunas de nueva generación, las cuales aún no se encuentran disponibles

comercialmente (Bande et al., 2015). En la actualidad, a pesar del avance y uso extensivo de vacunas y medidas de bioseguridad, la bronquitis infecciosa aviar sigue generando altos impactos económicos a nivel mundial (Bande et al., 2017; Colvero et al., 2015). La aparición de nuevas variantes sigue dificultando el control y prevención de la enfermedad (Abozeid, 2023).

Transmisión y características clínicas

La transmisión del virus de la bronquitis infecciosa aviar (IBV) por secreciones respiratorias o fecales que pueden ser transmitidas entre las aves por medio del aire o a través del transporte por objetos contaminados (Ramakrishnan & Kappala, 2019). Las células epiteliales ciliadas son los principales sitios de replicación de este virus (Cook et al., 2012), por lo que diversos tejidos del sistema respiratorio, digestivo, renal y reproductor pueden verse afectados en mayor o menor medida según el tropismo tisular de la variante viral que cause la infección (Cavanagh & Gelb, 2008).

Los signos clínicos respiratorios más comunes incluyen secreción nasal, ocular y traqueítis (Ramakrishnan & Kappala, 2019). En el sistema reproductor de las hembras se puede producir retraso en el desarrollo del oviducto, producción de quistes, necrosis y desprendimiento del epitelio de la superficie ovárica (Hassan et al., 2021). Estas lesiones llevan a una disminución en la producción y calidad de los huevos, los cuales pueden presentar cáscaras blandas o rugosas y producción de albúmina delgada y acuosa (Jackwood & De Wit, 2013). También se han visto lesiones en el sistema reproductor masculino, en donde el virus puede replicarse en los testículos, causar transtornos endocrinos y llevar a la apoptosis de células germinales, generando una disminución en la fertilidad (Boltz et al., 2004). Además, se han reportado variantes de IBV nefropatogénicas, las cuales pueden generar un aumento en el tamaño de los riñones, depósitos de urato, dilatación y necrosis en los túbulos renales (Najimudeen et al., 2022; Reddy et al., 2016). Otras variantes pueden infectar el tubo digestivo (esófago, proventrículo, intestinos, tonsilas cecales y cloaca), usualmente con escaso efecto clínico (Jackwood & De Wit, 2013).

Las variantes que pueden producir infección en sistemas distintos al respiratorio, se han relacionado con su capacidad de infectar monocitos (Hoerr, 2021). Estas células infectadas pueden desempeñar un papel crucial en la diseminación del virus a la circulación sanguínea y a los órganos

internos (Reddy et al., 2016). También se puede afectar la viabilidad de los macrófagos y dañar sus funciones fagocíticas, permitiendo una mayor replicación viral (Han et al., 2017).

Agente etiológico

El IBV pertenece al género *Gammacoronavirus* de la familia Coronaviridae y del orden Nidovirales (Carstens, 2010). Su virión tiene un diámetro de aproximadamente 120 nm, es pleomórfico y está cubierto por una envoltura fosfolipídica de la cual se proyectan estructuras espiculares triméricas de aproximadamente 20 nm de largo (Cavanagh & Gelb, 2008). Su genoma está compuesto por un ARN simple banda con sentido positivo, de aproximadamente 27.6 kb, con una caperuza en el extremo 5' y una cola de poliadeninas en el extremo 3' (Lai & Cavanagh, 1997).

El genoma de IBV sigue la estructura general de los coronavirus 5'-UTR-1a/ab-S-3a-3b-E-M-5a-5b-N-3'UTR, donde los "UTR" son regiones sin traducir de aproximadamente 500 nucleótidos (Lai & Cavanagh, 1997). Las primeras 20 kb del genoma contienen los genes 1a/1b, que producen la poliproteína 1a y 1b por medio de un desplazamiento en el marco de lectura ("frameshifting") y las cuales son escindidas por proteasas para producir al menos 15 proteínas no estructurales (nsp) individuales. Estas proteínas son responsables de formar el complejo de replicación y transcripción del virus (Cavanagh, 2007), pero también participan en procesos de corrección o "proofeading" (Denison et al., 2011), adición de la caperuza y modulación del sistema inmune (Peng et al., 2022). Adicionalmente, el genoma codifica para proteínas estructurales como la proteína espicular (S), de membrana (M), envoltura (E) y nucleocápside (N) (Cavanagh & Gelb, 2008). También contiene genes codificantes para proteínas accesorias como la 3a, 3b, 5a, y 5b, relacionadas con la patogenicidad del virus (Laconi et al., 2018). En algunas variantes también se ha descrito la presencia de genes para las proteínas accesorias 4b, 4c y 6b (Abozeid, 2023; Villalobos-Agüero et al., 2021).

Aislamiento y propagación del virus

Para realizar estudios de monitoreo e identificación de variantes virales, es necesario contar con protocolos de aislamiento viral. Para IBV se utilizan ampliamente los huevos embrionados libres de patógenos, los cuales proporcionan la diversidad de tipos de células necesaria para la replicación exitosa del virus y generan la cantidad de virus necesaria para diversos estudios (Banda

& Yan, 2022). Por medio de estos cultivos virales se hacen posibles estudios sobre producción de vacunas (Jordan, 2017) y sobre diferentes aspectos moleculares del virus (Cavanagh *et al.,* 2005).

Clasificación y diversidad del virus

Actualmente la clasificación del IBV se basa en la región S1 del gen de la proteína espicular. Esto se debe a que la heterogeneidad de nucleótidos es más prevalente en la subunidad S1 por la presencia de tres regiones hipervariables (HVR) (aa 38–67, 91–141 y 274–387) (Lai & Cavanagh, 1997) y al considerar esta región como el principal componente inmunogénico del virus (Tan et al., 2016). Valastro et al. (2016) propusieron la existencia de 6 genotipos y 32 linajes por análisis filogenéticos con secuencias completas de nucleótidos de la subunidad S1. Los linajes se definieron por distancias de 13% y 14% en las secuencias de nucleótidos y aminoácidos respectivamente, mientras que los genotipos por distancias de 30% en secuencias de nucleótidos y 31% de aminoácidos. Se propuso que los nombres se asignaran utilizando la abreviatura del genotipo al que pertenecen, seguido de un número consecutivo asignado según el orden temporal de la descripción de los linajes (Valastro et al., 2016). Con base en esta clasificación, se han definido nueve genotipos y 41 linajes (Abozeid, 2023; Ma et al., 2019; Marandino et al., 2023).

La diversidad de este virus, al igual que en la mayoría de los virus de ARN, se debe a la alta tasa de mutaciones y eventos de recombinación (Holmes, 2009). Las mutaciones se producen generalmente durante la replicación viral, debido a la falta de un mecanismo de corrección en la ARN polimerasa. A pesar de que la nsp14 contiene un dominio exoribonucleasa con función correctora, su eficacia no es tan alta como la actividad de corrección de errores de las ADN polimerasas (Eckerle et al., 2007). Los eventos de recombinación se dan cuando la ARN polimerasa cambia de una molécula de ARN a otra durante la replicación, mientras permanece unida a la cadena de ácidos nucleicos naciente, generando así una molécula de ARN con ascendencia mixta (Aaziz & Tepfer, 1999). Este proceso puede ocurrir de forma homóloga (entre genomas de la misma variante) y no homóloga (entre genomas de varientes disntintas) (Han et al., 2016; Lee et al., 2010). La probabilidad de que ocurran eventos de recombinación aumenta en granjas con altas poblaciones de aves y condiciones de hacinamiento (Cavanagh, 2007). La distribución de las variantes a nivel mundial se ha relacionado con el comercio internacional de aves (Abozeid, 2023), la introducción de variantes vacunales (Rohaim et al., 2019) y a la

distribución de aves migratorias (Domanska-Blicharz et al., 2014), siendo el último factor el menos estudiado.

Vacunas atenuadas y evolución de IBV

Las vacunas atenuadas has sido utilizadas ampliamente para el control del IBV. Se emplean comúnmente para inmunizar a los pollos de engorde en su primera semana de vida y para "preparar" a futuras ponedoras y reproductoras antes de la administración de una vacuna inactivada (Abozeid, 2023). Estas vacunas se fabrican mediante pasajes seriados en huevos embrionados libres de patógenos, lo que resulta en la producción de mutaciones y selección de subpoblaciones que genera un virus de menor virulencia (Cavanagh et al., 2005). Las vacunas atenuadas han demostrado ofrecer una excelente protección contra variantes homólogas, sin embargo, la protección contra variantes antigénicamente distintas es limitada (Abozeid, 2023). Para aumentar la protección contra variantes heterólogas se utiliza la combinación de dos variantes antigénicamente distintas, estrategia conocida como "protectotipo" (Cook et al., 1999) .

La efectividad de las vacunas atenuadas ha sido extensamente demostrada (Abozeid, 2023). Sin embargo, también han sido relacionadas con riegos a largo plazo debido a que estas pueden influir en la evolución del virus a través de eventos de recombinación, tanto con otras variantes vacunales como con variantes de campo (Han et al., 2016; Marandino et al., 2023). Además, se ha reportado selección de subpoblaciones vacunales en las aves vacunadas que pueden llevar a la producción de variantes con mayor virulencia (Van Santen & Toro, 2008). Adicionalmente, en casos donde la vacuna produce la erradicación de una variante, puede crear un nicho vacío que sea ocupado por nuevas variantes virales (Hanley, 2011). Esto demuestra la importancia que tiene la vigilancia constante del comportamiento del IBV y la caracterización de su dinámica epidemiológica, para identificar las variantes virales circulantes y controlar de manera efectiva la bronquitis infecciosa.

IBV en Costa Rica

En Costa Rica se cuenta con evidencia serológica y molecular de la presencia de bronquitis infecciosa aviar. El primer serotipo en ser aislado fue el Massachusetts, en 1990 (Jiménez et al., 2004). Desde entonces, se han identificado otros serotipos como Arkansas, Pennsylvania, dos

nuevas variantes (A y B) y cuatro aislamientos que no lograron ser identificadas por reacción en cadena de la polimerasa (PCR) y análisis de polimorfismos de longitud de fragmentos de restricción (RFLPs) (Jiménez et al., 2004). En 2004, Lindhal et al., reportaron la presencia de una variante única para el país, designada como IBV-CR-53. En este estudio se obtuvieron evidencias serológicas de la presencia de IBV en palomas (*Zenaida asiatica* y *Columba fasciata*), lo que sugiere que juegan un papel en la transmisión y persistencia de IBV en Costa Rica (Lindahl, 2004).

Más recientemente, entre mayo del 2016 y julio del 2017, ocurrió un brote de IBV en granjas del país, asociado a una variante estadounidense conocida como Georgia 13 (GA13) y que ocasionó sintomatología respiratoria y mortalidad principalmente en aves de menos de 35 días (Roman et al., 2018). Un estudio publicado en el 2021 describió la secuencia genómica completa de esta variante y determinó que comparte un 94.3% de la identidad de nucleótidos con la variante DMV/1639/GA9977/2019 (MK878536) de Georgia, EE. UU. Los análisis filogenéticos con la subunidad S1 la clasificaron dentro genotipo I, linaje 17 (GI-17). También se detectaron posibles eventos de recombinación en los genes S, E, M, 4b y 4c, con variantes tipo Massachusetts, Connecticut, Arkansas y MA5 (Villalobos-Agüero et al., 2021).

Posteriormente, en 2022 se publicó un estudio que analizó 14 aislamientos de IBV obtenidos de granjas avícolas en Costa Rica desde el 2016 hasta el 2019 (Villalobos-Agüero et al., 2022). Al secuenciar la región S1 y realizar estudios filogenéticos, se encontró que los aislamientos obtenidos durante 2016-2017 se clasifican como GI-17, relacionados con variantes GA13 y los cuales fueron nombrados variantes Georgia 13 de Costa Rica (GA13-CR). Los aislamientos obtenidos durante el 2018-2019 se clasifican como genotipo I, linaje 13 (GI-13) y están estrechamente relacionados con la variante vacunal 4/91. La vacuna atenuada 4/91 fue introducida en el 2017 en respuesta al brote reportado entre 2016 y 2017 IBV. Esta vacuna se implementó bajo el concepto de protectotipo, por lo que se trabajó en conjunto con la vacuna de Massachusetts Ma5 (Roman et al., 2018).

Vacuna 4/91

La variante 4/91 (también llamada 793B) fue identificada por primera vez en el Reino Unido en 1991, por brotes relacionados con mortalidad y miopatía muscular (Cook et al., 1996). Debido a la ineficacia de las vacunas utilizadas para proteger contra esta variante, se produjo la

vacuna atenuada 4/91 en 1992 (Cavanagh et al., 2005). Esta vacuna ha mostrado una alta efectividad contra variantes heterólogas al ser utilizada en conjunto con la vacuna atenuada Ma5, por lo que se ha comercializado por distintas partes del mundo (Bande et al., 2015). Sin embargo, la introducción de la vacuna 4/91 cuando su variante patógena homóloga no ha sido aislada previamente, sigue siendo controvertida por el efecto que esta podría tener sobre las poblaciones virales locales (Abozeid, 2023). También se han reportado algunos eventos de recombinación entre distintas variantes de IBV y la variante vacunal 4/91 (Hassan et al., 2019; Jiang et al., 2018; Rohaim et al., 2019).

JUSTIFICACIÓN

La bronquitis infecciosa aviar es una de las principales enfermedades diagnosticadas en la industria avícola mundial, por lo que es de gran importancia económica (Colvero, 2015). Los brotes de IBV son frecuentes incluso en aves vacunadas, debido a que existe poca protección cruzada entre serotipos (Cavanagh, 2007). En Costa Rica se han utilizado vacunas atenuadas del tipo Massachussets e inactivas del tipo Arkansas (Román *et al.* 2018). Sin embargo, en 2017 fue necesaria la introducción de la vacuna 4/91 para controlar el brote de GA13 que se generó en el 2016 (Román *et al.* 2018).

Según los resultados obtenidos en proyectos de investigación realizados previamente en la Universidad de Costa Rica (B6537-*Prevalencia de Bronquitis Infecciosa Aviar en Pequeños y Medianos Productores Avícolas en Costa Rica* y B9052-*Diagnóstico molecular y análisis de secuencias de dos virus de importancia económica en granjas avícolas de Costa Rica*) se han logrado detectar eventos de recombinación en el genoma completo de un aislamiento de GA13-CR y se han reportado cambios a nivel de nucleótidos y aminoácidos en la región S1 de la proteína espicular entre los distintos aislamientos. Adicionalmente, el ingreso de una nueva variante vacunal no reportada previamente en el país requiere de estudios de seguimiento que permitan evaluar su efectivad y comportamiento con otras posibles variantes circulantes. Obtener esta información es de gran utilidad para los productores, debido a que genera conocimiento básico que les permite tomar mejores decisiones con respecto a qué estrategias tomar para proteger de IBV a sus granjas.

Este proyecto pretende dar continuidad a los estudios de IBV anteriores, además de adaptar el protocolo de aislamiento viral en huevos embrionados libres de patógenos en las instalaciones de la Universidad de Costa Rica. La implementación de esta metodología permitirá desarrollar nuevas técnicas diagnósticas o modificaciones que mejoren la efectividad o sensibilidad de las pruebas diagnósticas en el futuro. Este estudio busca generar conocimiento que contribuya con el control del IBV en el país y con esto reducir sus posibles impactos económicos.

OBJETIVOS

Objetivo general: Caracterizar las variantes circulantes del virus de la bronquitis infecciosa aviar presentes en granjas de producción avícola en Costa Rica posterior a la introducción de la variante vacunal 4/91.

Objetivos específicos:

1. Detectar la presencia de IBV en muestras de granjas de producción avícola, mediante la extracción de ARN y amplificación de un fragmento del gen de la proteína de la nucleocápside.
2. Implementar una metodología de aislamiento viral de IBV en huevos embrionados libres de patógenos.
3. Analizar filogenéticamente las secuencias genómicas obtenidas a partir de las muestras colectadas para determinar la diversidad de la población viral que circula en el país, y la relación de sus secuencias genómicas.
4. Diseñar iniciadores específicos dirigidos a la región S1 de las variantes GA13-CR para su detección por medio de RT-PCR.
5. Identificar la relación entre cambios de nucleótidos encontrados en las secuencias obtenidas con posibles modificaciones postraduccionales.
6. Identificar regiones bajo selección positiva en las variantes obtenidas que puedan tener relación con evasión del sistema inmune.

ARTÍCULO

Objetivos abarcados:

- Detectar la presencia de IBV en muestras de granjas de producción avícola, mediante la extracción de ARN y amplificación de un fragmento del gen de la proteína de la nucleocápside.
- Implementar una metodología de aislamiento viral de IBV en huevos embrionados libres de patógenos.
- Analizar filogenéticamente las secuencias genómicas obtenidas a partir de las muestras colectadas para determinar la diversidad de la población viral que circula en el país, y la relación de sus secuencias genómicas.
- Identificar la relación entre cambios de nucleótidos encontrados en las secuencias obtenidas con posibles modificaciones postraduccionales.
- Identificar regiones bajo selección positiva en las variantes obtenidas que puedan tener relación con evasión del sistema inmune.

Título: Molecular analysis of 4/91-like variants of avian infectious bronchitis virus (IBV) obtained after the introduction of a 4/91 live-attenuated vaccine in Costa Rica during 2017.

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Molecular analysis of 4/91-like variants of avian infectious bronchitis virus (IBV) obtained after the introduction of a 4/91 live-attenuated vaccine in Costa Rica during 2017

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Abstract Avian infectious bronchitis virus (IBV) belongs to family *Coronaviridae*, genus *Gammacoronavirus* and is one of the most predominant causes of respiratory disease in poultry. Its high mutation rate constantly leads to the emergence of novel variants that complicate disease control. In 2016, a GA13-like IBV outbreak occurred in Costa Rica, prompting the introduction of the 4/91 live-attenuated vaccine. The objective of this research was to perform a molecular characterization of IBV variants circulating in the country six years after the introduction of the 4/91 vaccine. A total of 177 samples from symptomatic birds were analyzed, with 43 testing positive for IBV. Seven complete S1 sequences were obtained and clustered within the GI-13 lineage by phylogenetic analysis. Sequence analysis showed high genetic similarity to the 4/91 vaccine strain, with nucleotide and amino acid sequence identities over 99.13% and 97.96%, respectively, despite these samples being taken from unvaccinated birds. Post-translational modification analysis of the S1 protein revealed conserved N-glycosylation and palmitoylation sites, while two serine phosphorylation changes were predicted between the obtained sequences and the vaccine strain. Selective pressure analysis identified 10 sites under positive selection, mainly located within the receptor-binding domain and hypervariable regions of the S1 subunit. The presence of 4/91-like variants in unvaccinated birds needs attention, and its relation to observed pathology requires further research. Continuous surveillance is essential to monitor for potential vaccine escape mutants and mitigate their impact.

Keywords IBV · 4/91-like · Avian Coronavirus · Live-attenuated vaccine · Phosphorylation · Selective Pressure

Introduction

Infectious bronchitis virus (IBV) is an enveloped virus with a positive-sense single stranded RNA genome, classified in the order *Nidovirales,* family *Coronaviridae,* genus *Gammacoronavirus* (Zhao et al., 2023). IBV infects poultry of all ages, causing a highly contagious respiratory disease, with some variants leading to reproductive disorders and renal dysfunction (Cook et al., 2012). Its genome is approximately 27 kb long, organized as follows: 5′UTR-1a/1ab-S-3a-3b-E-M-5a-5b-N-3′UTR (Cavanagh, 2007). The large 1a/1ab gene constitutes two-thirds of the genome and encodes the nonstructural proteins that are responsible for RNA replication and transcription. The rest of the genome encodes the structural proteins: Spike (S), Envelope (E), Membrane (M) and nucleocapsid (N), as well as some accessory proteins (Bhuiyan et al., 2023).

The S protein is post-translationally glycosylated to form trimers and is later cleaved by proteolysis at the furin consensus site (RRFRR/HRRR) to form subunits S1 and S2 (Wickramasinghe et al., 2014). The S1 subunit binds to host cellular receptors while S2 mediates fusion of the viral membrane with the cellular membrane (Cavanagh, 2007). The S1 subunit contains the receptor-binding domain (RBD), spanning from residue 19 to 272, and three hypervariable regions (HVRs): HVR-1 (residue 38 to 67), HVR-2 (residue 91 to 141) and HVR-3 (residue 274 to 387) (Wickramasinghe et al., 2014). The S1 subunit also includes the primary epitopes recognized by the host immune system (Kant et al., 1992), and thus variance within the S1 subunit is a major reason behind the emergence of new variants and poor cross-protection between serotypes (Cavanagh et al., 1997).

Among the most important post-translational modifications of S protein are palmitoylation and glycosylation (Bhuiyan et al., 2023). The N-glycosylation of the S protein can impact receptor binding, contributing to antigenic shifting (Smati et al., 2002), and it is also associated with changes in virulence and cellular tropism (Bhuiyan et al., 2023). Palmitoylation participates in virus assembly, subcellular localization and transport and protein–protein interactions (Fung & Liu, 2018). Phosphorylation of proteins in IBV has been linked specially with M, and N proteins, however in more recent studies with SARS-CoV-2, it was shown to be present in the spike protein

too (Bouhaddou et al., 2020). Phosphorylation has been linked with viral assembly, replication and intracellular communication (Fung & Liu, 2018).

Genetic categorization of IBV is mostly performed through phylogenetic analysis using the complete sequence of the S1 gene (Valastro et al., 2016). To date, nine genotypes and 41 lineages have been classified based on the S1 gene (Abozeid, 2023; Ma et al., 2019; Marandino et al., 2023). The control of outbreaks and emergence of new strains largely depends on vaccination using live-attenuated and inactivated vaccines (Abozeid, 2023). IBV live vaccines are developed by serial passaging of the virulent field isolate in embryonated chicken eggs (Cook et al., 2012). These vaccines have proven effective in inducing a protective immune response, but their use is controversial because of the risk of recombinant events between the strains used as vaccines and virulent field strains (Bande et al., 2015). In rare occasions, live vaccines can also mutate and revert to virulence (Hanley, 2011). This highlights the importance of surveillance studies.

In Costa Rica IBV has been detected since 1990 (Jiménez et al., 2004). In 2016, there was an infectious bronchitis outbreak caused by a GA13-like variant in Costa Rica (Villalobos-Agüero et al., 2021). To control the outbreak, a 4/91 live-attenuated vaccine was introduced in 2017 and was used in combination with a Ma5 vaccine, based on the protectotype concept (Roman et al., 2018). Before the introduction of the vaccine, the 4/91 strain had not been reported in Costa Rica. Two years after the introduction of the vaccine Villalobos-Agüero et al., (2022) isolated 4/91 vaccine-like strains that exhibited high nucleotide similarity to the original 4/91 vaccine. The objective of this research was to perform a molecular characterization of IBV variants circulating in the country six years after the introduction of the 4/91 vaccine.

Materials and Methods

Sample collection

Samples were obtained from five provinces of Costa Rica (San José, Alajuela, Limón, Puntarenas, Cartago) with the majority (76%) collected from Alajuela, where bird production is concentrated in the country. The sizes of the farms sampled included subsistence (<1000 birds), small (1000-15,000 birds), medium (15,000-50,000 birds), and large (>50,000 birds). Broilers (young birds), layers (young and adult birds) and breeding (adult birds) were sampled when they exhibited respiratory symptoms, airsacculitis, mortality, slaughterhouse confiscation or reduced egg production. Samples were collected by certificated and private veterinarians, in pools of three

tracheal or cloacal swabs or three organs (trachea or cecal tonsils). Organ samples were transported in RNA*later* solution™ (Thermo Fisher Scientific Inc.), while swab samples were transported in viral medium using MicroTest™ M4RT (Thermo Fisher Scientific Inc.). Bird age and information about 4/91 vaccination and time of application were collected for all the samples. All the bird samples in this study tested negative in laboratory assays for Avian Influenza and Newcastle disease virus.

RNA Extraction and Diagnosis of IBV

RNA was extracted from 50 mg of macerated tissue, 200 µL of swab samples or 200 µL of allantoic fluid (see Viral Propagation), using 800 µL of TRIzol reagent (Invitrogen Cat. No: 15596026) according to Rio et al. (2010). RNA was eluted with 200 µL of nuclease-free water. Endpoint RT-PCR was performed to detect the IBV nucleocapsid gene using primers N784 and N115 (Sumi et al., 2012) (Table S1). Reactions were conducted with One Step RT-PCR (Qiagen, GmbH, Hilden, Germany) with a final volume of 12.5 µL (8.5 µL Master Mix + 4 µL sample). The Master Mix was prepared with the following work concentration: OneStep Buffer 1X, dNTPS 0.4 mM, Primers 0.9 µM, OneStep RT-PCR Enzyme 1X. The PCR conditions were: 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 volume of 3 µL of amplified product was analyzed by agarose gel electrophoresis in a 1.5% agarose gel to identify a band of 400 pb. A Ma5 IBV vaccine was used as positive control.

Viral Propagation and Sequencing of the S1 gene region

Positive samples were inoculated into 9-12 days embryonated specific pathogen-free (SPF) eggs provided by the National Animal Health Service of Costa Rica (SENASA). A total volume of 200 µL of swab samples was mixed with 1.5 mL of Brain Heart Infusion (BHI) broth and passed through 0.22 µm syringe filters before inoculation into the chorioallantoic cavity. Embryos were incubated at 37 °C for seven days and analyzed to detect IBV lesions such as finger/body curling, feather underdevelopment, hemorrhage and dwarfing (Ramakrishnan & Kappala, 2019). Allantoic fluids from embryos with lesions were collected for RNA extraction as previously described (see RNA Extraction and Diagnosis of IBV) and RT-PCR was performed to amplify the spike protein-coding S1 gene, using the primers NewS1OLIGO 5’ and S1OLIGO3’ (Gallardo et al., 2010)

(Table S1). RT-PCR reactions were performed with One Step RT-PCR (Qiagen, GmbH, Hilden, Germany) using a final reaction volume of 12.5 µL (7.5 µL Master Mix + 5 µL sample). Master Mix was prepared with the following work concentration: OneStep Buffer 1X, dNTPS 0.4 mM, Primers 0.6 µM, $MgCl_2$ 1.5 mM and OneStep RT-PCR Enzyme 1X. The PCR conditions were: one cycle of 30 min at 50 °C and 15 min at 95 °C; 10 cycles of: 30 s at 94 °C, 30 s at 59-50 °C and 2 min at 72 °C, followed by 30 cycles of: 30 s at 94 °C, 30 s at 47 °C and 2 min at 72 °C, with a final cycle for a final extension at 72 °C for 7 min. A volume of 3 µL of amplified product was analyzed by agarose gel electrophoresis in a 1% agarose gel to identify a band of 1600 pb. A Ma5 IBV vaccine was used as positive control. Products were purified using the QIAquick PCR purification kit (QIAGEN, Valencia, CA) and samples were sent to Macrogen® (South Korea) for Sanger sequencing, using the same primers used in RT-PCR. Sequence assembling, editing, translation and levels of identity were performed using Geneious 11.0.20.1 software (Kearse et al., 2012).

Phylogenetic analysis

A phylogeny based on the S1 subunit of the spike protein gene was constructed using a total of 112 reference sequences obtained from GenBank (Table S2), based on the classification proposed by Valastro et al., (2016). Sequence alignment was generated using MAFFT server https://mafft.cbrc.jp/alignment/server/, with default parameters (Katoh et al., 2018). GUIDANCE2 Server https://taux.evolseq.net/guidance/, was used to check the overall quality of the alignment and determine unreliable sequences (Sela et al., 2015). Sequence editing was done with Geneious 11.0.20.1 (Kearse et al., 2012). PARTITION FINDER v.3 (Lanfear et al., 2017) implemented in the CIPRES Science Gateway (http://www.phylo.org/sub_sections/portal/), with the Akaike Information Criterion (AIC), was used to determine the best-fit model of evolution, and General Time Reversible (GTR) with gamma distribution and invariant sites applied. Bayesian Inference (BI) and Maximum Likelihood (ML) phylogenetic analyses were applied to the dataset. The ML analysis was carried out in RAxML v.8.2.12 (Stamatakis, 2014) implemented in the CIPRES Science Gateway, with 1,000 non-parametric bootstrap iterations. To corroborate the robustness of the Phylogenetic tree, Bayesian Inference was generated with Mr.Bayes 3.2.3 (Huelsenbeck & Ronquist, 2001) in CIPRES Science Gateway, for 10 000 000 generations. Tracer v1.7.1

(http://tree.bio.ed.ac.uk/software/tracer) was used corroborate convergence of Bayesian analysis. The resulting trees were visualized using the iTOL v4 online tool (https://itol.embl.de/).

Prediction of Post-Translational Modifications

S1 glycoprotein gene sequences of 4/91-like isolates obtained in 2023 were subjected to post-translation modification analysis and compared with modifications present in S1 glycoprotein gene sequences of 4/91 vaccine, 4/91 pathogenic strain, and isolates obtained in Costa Rica during 2018-2019 (Villalobos-Agüero et al., 2022). The predicted N-glycosylation sites were predicted with the server *NetNGlyc-1.0* (Gupta & Brunak, 2002.) (https://services.healthtech.dtu.dk/services/NetNGlyc-1.0/). The potential phosphorylation sites were determined using NetPhos-3.1 (Blom et al.,1999) (*https://services.healthtech.dtu.dk/service.php?NetPhos-3.1*). Potential palmitoylation sites were identified using GPS-Palm 5.0 Software (https://gpspalm.biocuckoo.cn/)
(Ning et al., 2021).

Selective Pressure Analysis

A total of 50 sequences coding for the S1 subunit of the Spike protein classified as 4/91, 4/91-like or 793B were obtained from GenBank (Table S3). Sequence alignment was performed as previously described (see Phylogenetic analysis). To identify statistically supported sites for positive and negative selective pressure, we used Datamonkey Adaptive Evolution Server (https://www.datamonkey.org/). Gene and site-specific selection pressures for the S1 protein gene were measured as the ratio of non-synonymous (dN) to synonymous (dS) nucleotide substitutions per site, using the models: FEL (Fixed Effects Likelihood, FUBAR (Fast Unconstrained Bayesian Approximation), SLAC (Single-Likelihood Ancestor Counting) and MEME (Mixed Effects Model of Evolution). Positive selection is inferred when $dN/dS > 1$, purifying selection is inferred when $dN/dS < 1$ while neutrality is inferred when $dN/dS = 1$ (Zhang et al., 2005). A p-value < 0.1 for FEL, SLAC and MEME and a posterior probability > 0.90 for FUBAR were used as statistical support for amino acid sites found under selection, as previously reported (Lo Presti et al., 2020). Positively selected sites that were confirmed by at least two different methods were included in this study.

Homology modeling of S1 subunit and epitope prediction

A 538 aa sequence of S1 protein of 4/91 strain (PP398709) was used to generate a model structure on Swiss-Model (https://swissmodel.expasy.org/) (Waterhouse et al., 2018). The structure was modeled using the repository's best fit template (6cv0.1.A) of a cryoelectronic microscopy structure of the IBV spike protein. Structural predictions of epitopes were made using ElliPro antibody epitope prediction toll (http://tools.iedb.org/ellipro/) (Ponomarenko et al., 2008) from the PDB file of the S1 model generated using Swiss-Model. Epitope structure predictions were performed using default parameters (a minimum score value of 0.5 and maximum distance of 6Å). The model was edited with Chimera software to visualize epitopes that contains sites under positive selection (Pettersen et al., 2004).

Results

IBV diagnosis and obtention of S1 sequences

Out of 177 samples, 43 tested positive for IBV. Among these 43 positive samples, 33 were broilers aged between 20 to 41 days, none of which had received the 4/91 vaccine. The remaining 10 positive samples were from layers or breeders aged between 11 to 64 weeks, with only three having received the 4/91 vaccine a year prior. A total of seven isolates that tested positive by RT-PCR using S1 gene primers were recovered from allantoic fluid from embryos that exhibited lesions associated with IBV infection, such as finger curling, feather underdevelopment, blood traces, and embryo dwarfing and curling. The seven isolated samples originated from tracheal and cloacal swabs from unvaccinated broiler birds. More details about S1 sequences obtained from these samples are provided in Table S4. The low sample recovery might be related to manipulation procedures occurring before laboratory analysis (Forman & Valsamakis, 2011).

Sequence analysis of the S1 gene

Phylogenetic analysis using Maximum Likelihood and Bayesian inference methods clustered all obtained isolates with the Genotype I lineage 13 (GI-13) (Fig. 1), sharing close genetic relationships with strains identified as 4/91, 793/B, or CR88 (Cook et al., 1996). Comparison of the S1 gene sequence from the 4/91 vaccine (AF093793) showed a high degree of identity, with over 99.13% at the nucleotide level and 97.96% at the amino acid level. Furthermore, comparison

with a pathogenic 4/91 strain (AF093794) revealed over 99.32% nucleotide identity and 98.51% amino acid identity. When compared with 4/91-like isolates from Costa Rica obtained during 2018-2019 by Villabos-Agüero et al. (2022), our sequences exhibited over 99.06% and 97.03% of nucleotide and amino acid identity respectively (Table 1). Figure 2 shows amino acid differences between the field variants and the Ma5, H120 and 4/91 vaccines that have been applied in Costa Rica. In total, 18 amino acid residue changes were identified between the 2023 isolates and the vaccine sequence, with 11 occurring within HVRs. Notably, three substitutions (p.Ala95Ser, p.Ile508Val, and p.Pro530Leu) were present across all obtained sequences, while a substitution at residue 55 (p.Gly55Glu) was present in five out of the seven sequences. Changes at amino acid positions 55 and 95 reside within HVR-1 and HVR-2, respectively, whereas substitutions at positions 508 and 530 are located outside both HVRs and the RBD.

Prediction of Potential N-Glycosylation, Phosphorylation, and Palmitoylation Site

A total of 17 possible N-glycosilation sites were predicted based on the presence of the N-Xaa-T/S motif (Fig. S5). No differences were observed in glycosylation sites when compared with the 4/91 vaccine, 4/91 pathogenic strain, and 4/91-like isolates from 2018-2019. Phosphorylation sites were identified by the NetPhos 3.1 server, which predicts Serine, Threonine, or Tyrosine phosphorylation sites in the S1 glycoprotein. Table 2 details the conserved phosphorylation sites among the 4/91 vaccine, 4/91 pathogenic strain, 2023 isolates, and 2018-2019 isolates. Most predicted phosphorylation sites are conserved across these sequences, except for a Serine phosphorylation site at position 91, which is present only in the 4/91 vaccine and a sequence from 2018 (CK/CR/1094/18). Additionally, a Serine phosphorylation site at position 95 is present in all 2023 isolates and the 4/91 pathogenic strain but absent in the 4/91 vaccine and 2018-2019 isolates. Palmitoylation predictions revealed no differences between commercial vaccine and the isolates. All of the sequences predicted a single palmitoylation site at position 372 under a medium threshold (0.77), with a score of 0.8184. When the threshold was adjusted to high (0.89) no palmitoylation sites were predicted.

Sites of S1 subunit of Spike gene under Selective Pressure

The number of sites under positive and negative selection are summarized in Table 3. Positive selection was identified by at least two models in 10 positions, and they occurred mainly inside

the RBD and HVR-1 and HVR-2 (amino acids 24, 38, 55, 94, 119, 140 and 250) (Fig. 2). None of these are potential glycosylation, phosphorylation or palmitoylation sites. The dN/dS ratio of 4/91 strains were 0.6332, indicating that the S1 region of the IBV genome of this strain had evolved mainly under negative selection.

Epitopes prediction of S1 subunit

A total of 14 linear epitopes with a score above 0.5 were identified in the S1 subunit protein model. Seven of these epitopes corresponded with the HVR1, HVR2, and HVR3 regions (Table S6). Epitopes located between amino acid positions 55-73, 88-100 and 110-156 were visualized in Chimera, using the homology model obtained in Swiss-Model, as they contained codons predicted to be under positive selection (aa 55, 94, 119 and 140) and are located inside HVR regions (Fig. 3).

Discussion

This study molecularly characterizes circulating IBV variants in Costa Rica after the introduction of the 4/91 live-attenuated vaccine in 2017, which was implemented to control a GA13-like (GI-17) outbreak in 2016. We analyzed a total of 177 samples collected in 2023 from broilers, layers and breeding birds showing IBV symptoms. Seven complete S1 sequences were obtained and characterized.

The use of 4/91 live-attenuated vaccine has proven to be effective protecting against heterologous IBV variants and displacing wild variants (Fan et al., 2018), particularly when combined with Ma5, following the protectotype strategy (Terregino et al., 2008). The introduction of this vaccine may have successfully displaced the GA13-like variant, as no samples related to this variant were identified in our study. Nonetheless, it is important to note that processes unrelated to vaccination, such as genetic drift and natural selection can cause certain strains to become less common or even disappear over time (Jackwood et al., 2012). The high similarity observed between the 2023 isolates and the 4/91vaccine strain suggests that 2023 isolates might be cases of vaccine reisolation. However, the samples used in this study were obtained from birds that had not received the 4/91 vaccine, according to the reports provided (Table S4). Additionally, they were collected from outbreaks in commercial flocks exhibiting clinical signs of disease and that tested negative for other common diseases, such as Influenza and Newcastle Disease. A

similar case was reported in Chile by Guzmán et al., (2019), who investigated the impact of the introduction of the 4/91 vaccine on circulating IBV variants. Before the vaccine introduction, the 4/91 wild variant was not reported in Chile. Six years after the vaccine's introduction, they found that the vaccine had displaced GI-16 variants and replace them with 4/91 strains closely related to the vaccine. Some isolates showed only four amino acid changes at positions 55, 95, 181 and 303. These isolates were not considered cases of vaccine reisolation, as they originated from outbreaks with clinical signs and from unvaccinated birds.

Whether the amino acid changes observed in this study at positions 55, 95, 508, and 530 are linked to the clinical signs seen in birds requires further investigation. However, some studies have reported the same amino acid changes without evidence of their role in disease. Callison et al., (2001) noted that the amino acids at positions 95, 508 and 530 differed between the UK/4/91 isolate (referred to as "4/91 pathogenic") and its embryo-passaged attenuated derivative (referred to as "4/91 vaccine"). Later, Cavanagh et al. (2005) determined that the substitution at position 95 occurred as an adaptation to embryo passages. Specifically, embryo-adaptated strains exhibit an alanine (A) in this position, whereas chicken-adapted strains have a serine (S), however it was reported that this change does not alter pathogenicity in chickens. The presence of serine in our isolates suggests that field isolates have adapted to propagate in chickens and are not vaccine reisolates, despite their high homology. In Japan, Shimazaki et al. (2009) isolated the 4/91 strain JP/Wakayama-2/2004, which shared 99.4% amino acid identity with the vaccine strain and exhibited the same amino acid changes at positions 55, 508, and 530 as the 2023 Costa Rican sequences. This strain was found to have low pathogenicity, similar to the vaccine strain. However, neither study provided details about the age or breed of the birds, nor the pathogenicity experimental conditions, limiting the ability to draw definitive conclusions.

The presence of the 4/91 vaccine in unvaccinated flocks observed in this study has been reported previously (Guzmán et al., 2019). Legnardi et al. (2019) conducted a large-scale diagnostic survey in Poland from 2017 to 2019 and found the presence of 4/91 vaccine in unvaccinated farms. Out of 589 samples analyzed it their study, 224 sequences were identified as 4/91 vaccine or vaccine-derived, of which 73% were isolated from flocks where 4/91 vaccine was not applied. 793B vaccine strains has shown to have the capacity to persists longer and to reach higher titers compared to other live-attenuated vaccines such as Mass-like vaccines in field conditions (Tucciarone et al., 2018). This requires increased biosecurity measures, as unintentional

exposure of susceptible flocks to vaccine could lead to development of disease and result in "rolling reactions"(Bhuiyan et al., 2021).

With the exception of two predicted phosphorylation changes, the mutations found in our sequences have no apparent impact on post-translational modifications. Protein phosphorylation plays a vital role in modulating protein activity, signal transduction and cellular behavior (Bhuiyan et al., 2023). In coronaviruses, phosphorylation has been primarly associated with the nucleocapsid (Bhuiyan et al., 2023). Wilbur et al. (1986) experimentally detected phosphorylation sites in the nucleocapsid of a coronavirus that causes mouse hepatitis, but their study did not examine the spike gene region. More recent studies in SARS-CoV-2 have experimentally identified phosphorylation sites in the spike protein (Bouhaddou et al., 2020; Örd et al., 2020; Yin et al., 2024). To our knowledge, phosphorylation in the IBV spike protein has not yet been experimentally identified, but other *in silico* studies have also predicted its presence (Ali et al., 2022; Hussain et al., 2012; Marandi & Davachi, 2018). The two phosphorylation changes predicted in this study are inside the RBD and interestingly, the p.Ala95Ser substitution that has been related with the different adaptation between embryos and chickens, creates a phosphorylation site.

The mean dN/dS ratio obtained for 4/91-like variants (0.6332), suggests that the S1 subunit is primarily evolving under negative selection. This value is similar to the dN/dS ratio obtained by Jackwood & Lee, (2017) for Mass-like variants (0.681). In contrast, the value obtained for Ark-like variants (1.230) indicates evolution under positive selection. The authors attributed this result to the prolonged circulation of the Ark-type virus in the field and the high presence of subpopulations in the vaccine, with polymorphisms in the S1 spike protein that can sometimes lead to adaptation and generation of infection in birds. Although the presence of subpopulations with similar behavior has not been described in the 4/91 vaccine, the mean dN/dS ratio calculated in this study indicates low adaptation of the 4/91-like virus, and thus amino acid changes are not being conserved frequently. Analysis with further 4/91-like sequences is required to corroborate these results.

Positive selection was identified in 10 codons, six of which are located in the S1 N-terminal domain (19-237 aa), encompassing most of the receptor-binding domain, HVR-1 and HVR-2. HVR regions are known to be associated with epitopes for neutralization (Moore et al., 1997). Four codons under positive selection (55, 94, 119 and 140) are located within three predicted epitope regions located in HVRs (55-73, 88-100, 110-156), which highlights the importance of

mutations on these positions as they may be involved in immune evasion. Sives et al. (2023) experimentally identified 4/91 vaccine epitopes using CLIPS arrays as a tool for mapping antigenic regions, from which 7 out of 11 match or overlap our predicted epitopes, including modelled region 110-156. Epitope regions 55-73 and 110-156 have been also detected *in silico* in QX-like variants (Parvin et al., 2021). Monitoring changes within these regions in further studies is important, as they could potentially affect the replication and pathogenicity of this variant.

Overall, our study recovered 4/91-like sequences, showing a high percentage of nucleotide and amino acid identity with the 4/91 live-attenuated vaccine. The absence of GA-13-like variants may be attributed to the vaccine's introduction; however, natural processes such as genetic drift and natural selection could also have played a role. Further research is needed to assess the impact of the observed amino acid changes on pathogenicity, and a complete genome analysis is required to determine whether changes outside the S1 region are contributing to disease development. Alternatively, the symptoms observed during this study could be caused by another unidentified pathogen. As isolations were obtained from unvaccinated flocks, biosecurity measures are needed to prevent outbreaks in the future. An adequate vaccination program is important not only for its immediate clinical benefits, but also to hinder viral evolution. Ineffective vaccination is a major long-term threat, as it might take to an increase in viral circulation and the possible emergence of new variants.

Tables and Figures

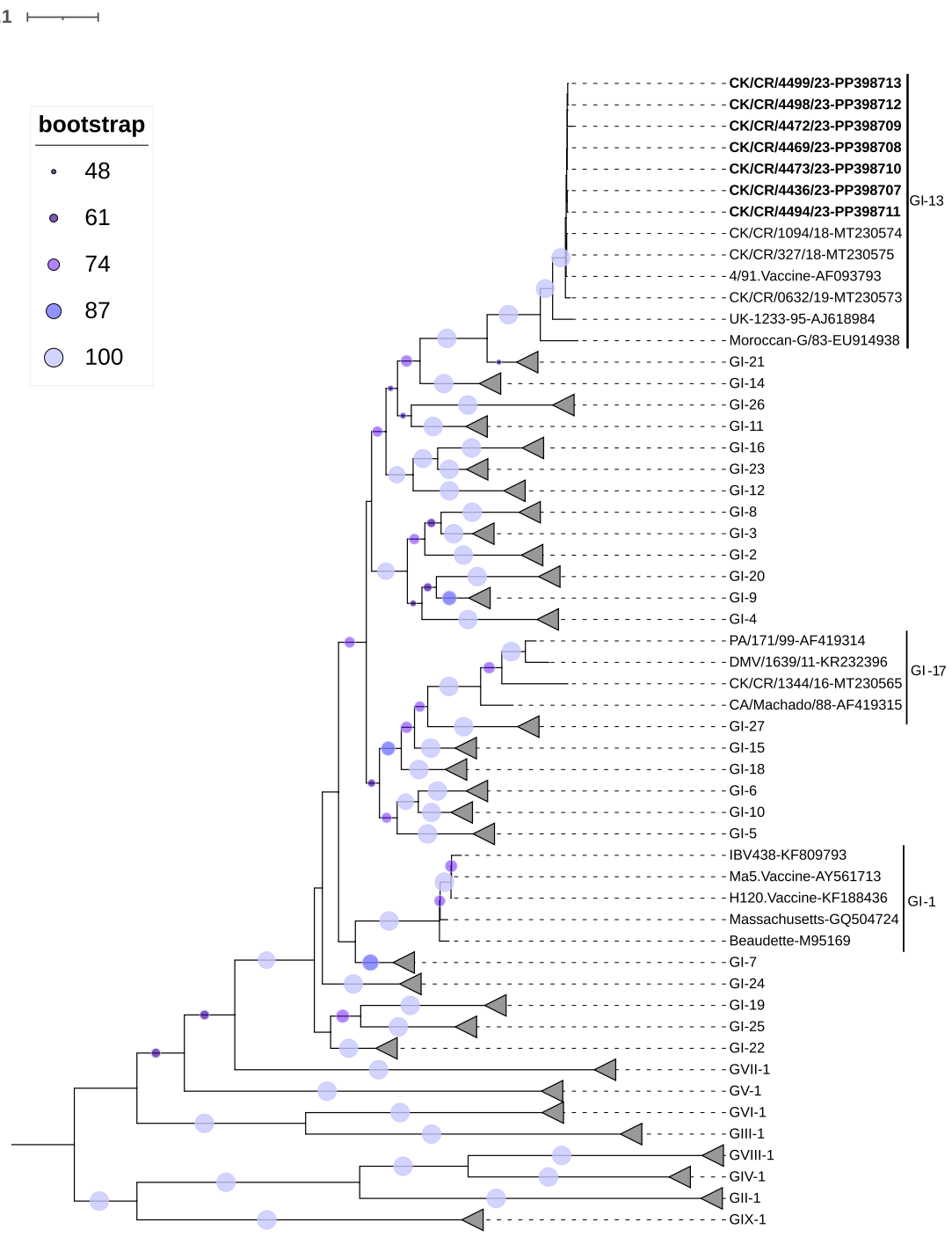
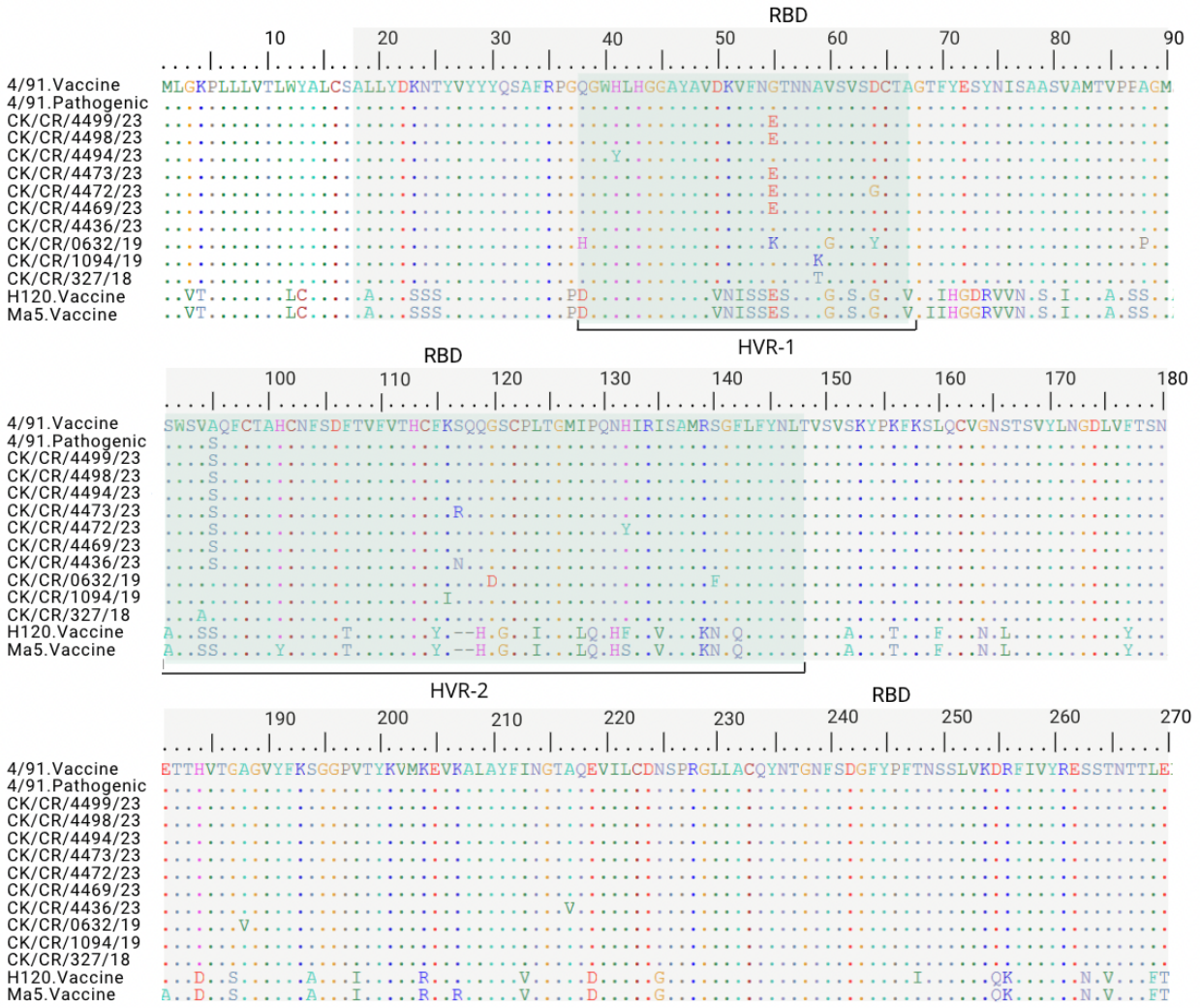


Figure 1. Phylogenetic tree based on the nucleotide sequence of the S1 region of the IBV spike protein gene. The tree was constructed using the Maximum Likelihood with 1,000 non-parametric bootstrap iterations. IBV isolates used in this study are shown in bold.


RBD
10
20
30
40
50
60
70
80
90
4/91.Vaccine
4/91.Pathogenic
CK/CR/4499/23
CK/CR/4498/23
CK/CR/4494/23
CK/CR/4473/23
CK/CR/4472/23
CK/CR/4469/23
CK/CR/4436/23
CK/CR/0632/19
CK/CR/1094/19
CK/CR/327/18
H120.Vaccine
Ma5.Vaccine
HVR-1
RBD
100
110
120
130
140
150
160
170
180
HVR-2
RBD
190
200
210
220
230
240
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260
270

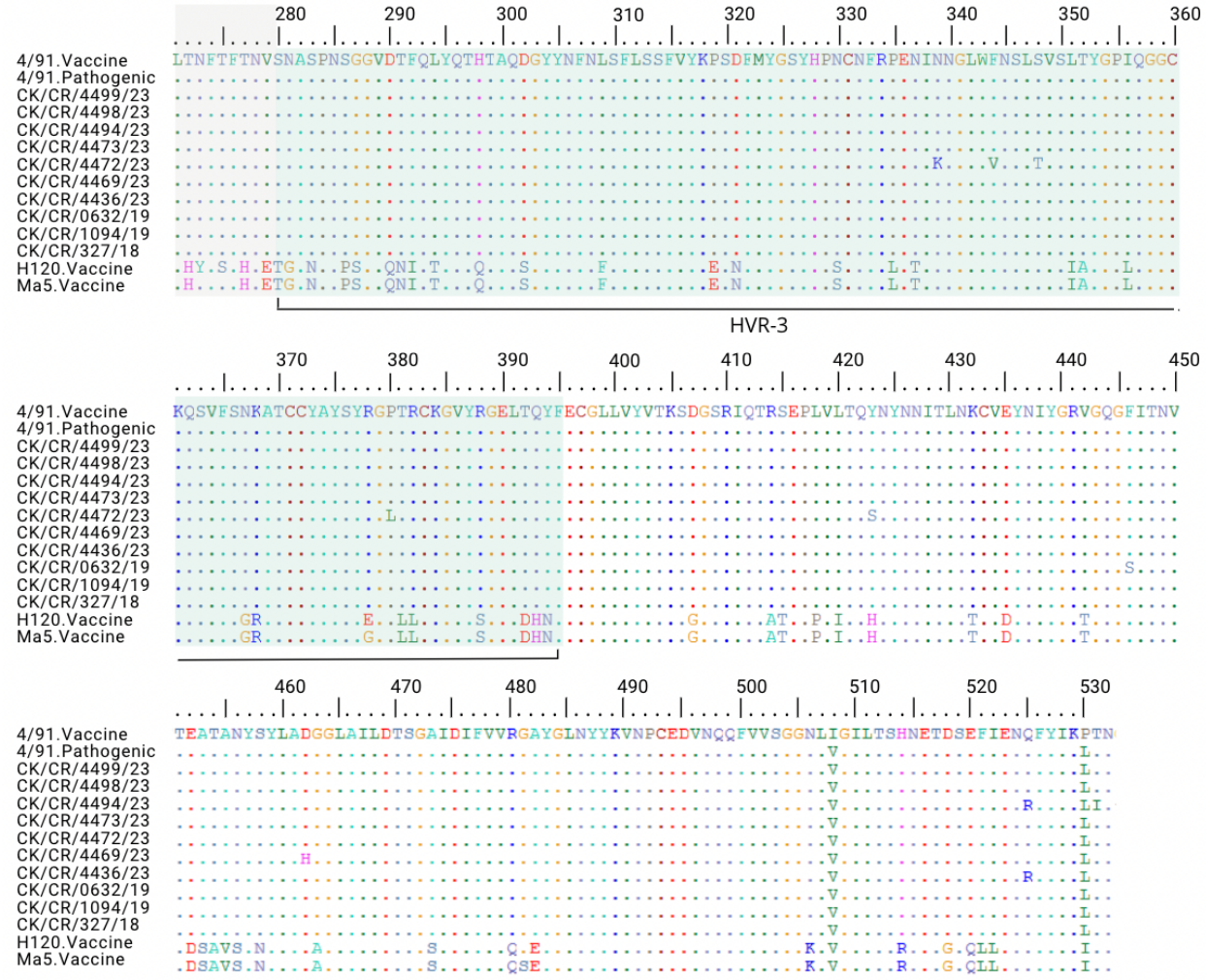

Figure 2. Sequence alignment of amino acids in the S1 subunit of the IBV Spike protein from 2023 isolates, 4/91 sequences and 2018-2019 isolates. Sequences were aligned with IBV 4/91 vaccine as reference. The dots indicate identical residues with 4/91 vaccine variant at that position. Section highlighted in gray indicates RBD region.

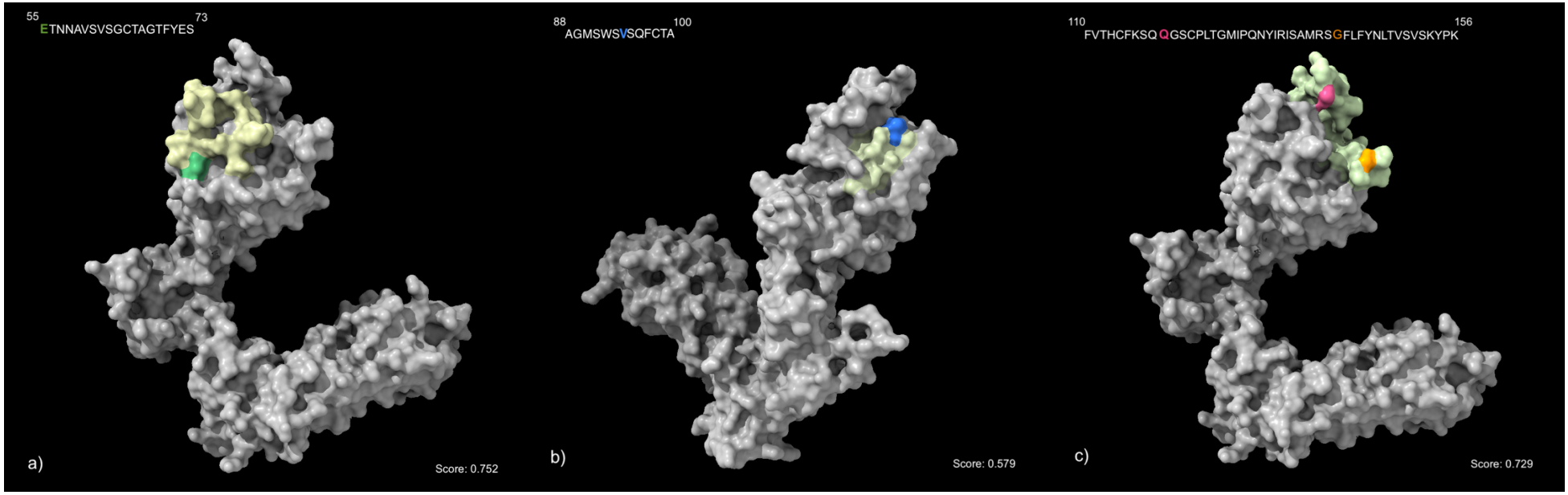

Figure 3. Structural view of three predicted linear epitopes of the S1 subunit of IBV, containing codons under positive selection. Epitopes are shown in yellow a) codon 55 is shown in green, b) codon 94 is shown in blue, c) codon 119 is shown in red and aa 140 is shown in orange.

Table 1. Nucleotide and amino acid sequence identities in the S1 region of the spike protein gene of IBV 2023 isolates compared to 4/91 sequences and 2018-2019 isolates.

Amino acid similarity % / Nucleotide similarity %		1	2	3	4	5	6	7	8	9	10	11	12	13	14
4/91.Vaccine	1		99.44	99.25	99.26	98.87	99.07	97.96	99.07	98.89	97.96	99.26	99.26	74.91	74.54
4/91.Pathogenic	2	99.81		99.81	99.81	99.44	99.63	98.51	99.63	99.44	98.14	99.44	99.44	75.28	74.91
CK/CR/4499/23	3	99.69	99.88		100	99.25	99.81	98.69	99.81	99.25	98.13	99.25	99.25	75.56	75.19
CK/CR/4498/23	4	99.63	99.81	100		99.25	99.81	98.70	99.81	99.26	98.14	99.26	99.26	75.46	75.09
CK/CR/4494/23	5	99.62	99.81	99.69	99.69		99.06	97.93	99.06	99.25	97.56	98.87	98.87	74.62	74.25
CK/CR/4473/23	6	99.69	99.88	99.88	99.88	99.69		98.51	99.63	99.25	97.95	99.07	99.07	75.56	75.19
CK/CR/4472/23	7	99.13	99.32	99.32	99.32	99.12	99.32		98.51	97.96	97.03	97.96	97.96	74.91	74.54
CK/CR/4469/23	8	99.69	99.88	99.88	99.88	99.69	99.88	99.32		99.07	97.96	99.07	99.07	75.28	74.91
CK/CR/4436/23	9	99.63	99.81	99.69	99.63	99.75	99.69	99.13	99.69		97.59	98.89	98.89	74.91	74.54
CK/CR/0632/19	10	99.19	99.25	99.25	99.25	99.06	99.25	99.69	99.25	99.06		97.96	97.96	74.72	74.35
CK/CR/327/19	11	99.75	99.81	99.69	99.69	99.62	99.69	99.12	99.69	99.62	99.19		99.44	74.72	74.72
CK/CR/1094/18	12	99.69	99.75	99.62	99.62	99.56	99.62	99.06	99.62	99.56	99.12	99.81		74.91	74.54
H120	13	78.71	78.84	78.81	78.77	78.54	78.87	78.58	78.82	78.71	78.46	78.65	78.58		97.95
Ma5	14	78.22	78.34	77.31	78.28	78.04	78.37	78.08	78.33	78.22	77.96	78.15	78.08	98.26	

Table 2. Prediction of conserved serine, threonine and tyrosine phosphorylation sites in S1 glycoprotein of 2023 isolates, 4/91sequences (vaccine and pathogenic) and 2018-2019 isolates

Serine phosphorylation sites	Threonine phosphorylation sites	Tyrosine phosphorylation sites
YYYQS[32]AFRP	YDKNT[25]YVYY	SALLY[21]DKNT
NNAVS[61]VSDC	VFNGT[56]NNAV	DKNTY[26]VYYY
TFYES[73]YNIS	CTAGT[69]FYES	AGTFY[71]ESYN
ISAAS[80]VAMT	SVAMT[84]VPPA	FYESY[74]NISA
GMSWS[93]VAQF	FSDFT[108]VFVT	STSVY[170]LNGD
HCNFS[105]DFTV	FYNLT[148]VSVS	KALAY[211]FING
HCFKS[117]QQGS	DLVFT[178]SNET	SDGFY[245]PFTN
HIRIS[136]AMRS	TSNET[182]THVT	AQDGY[304]YNFN
NLTVS[150]VSKY	GGPVT[199]YKVM	SSFVY[317]KPSD
PKFKS[159]LQCV	SSTNT[267]TLEL	SDFMY[324]GSYH
CVGNS[166]TSVY	LTNFT[275]FTNV	VSLTY[353]GPIQ
LCDNS[226]PRGL	SVSLT[352]YGPI	KCVEY[436]NIYG
VYRES[263]STNT	YRGPT[381]RCKG	EYNIY[439]GRVG
YRESS[264]TNTT	RGELT[392]QYFE	YGLNY[487]YKVN
VSNAS[283]PNSG	SRIQT[413]RSEP	ENQFY[527]IKPT
LSFLS[313]SFVY	PLVLT[421]QYNY	
SFLSS[314]FVYK	QGFIT[448]NVTE	
VYKPS[320]DFMY	IGILT[512]SHNE	
FMYGS[326]YHPN	SHNET[517]DSEF	
LWFNS[346]LSVS	PTNGT[534]RRSR	
FNSLS[348]VSLT		
GCKQS[366]VFSN		
CYAYS[376]YRGP		
KSDGS[409]RIQT		
QFVVS[503]GGNL		
GILTS[513]HNET		
NETDS[519]EFIE		
GTRRS[537]RR--		

Table 3. Selective pressure analysis on 4/91 S1 glycoprotein

Mean dN/dS	0.6657			
Model	FEL	FUBAR	SLAC	MEME
Number of positve selection sites	6	19	3	15
Number of negative selection sites	53	22	11	-
Positively selected sites (by at least two models)	3(G/V/D) – 24(N/D/G) – 38(Q/H/R/P/L/S) – 55(G/E/R/K/V/A) – 94(V/A) – 119(Q/L/P/H/A) – 140(S/F/D/G/N) – 250(S/A/H) – 521(F/P/L/Q/S) – 530(L/P/T)			

Table S1. List of primers used in this study, binding site for each primer and size of PCR amplified band.

Primers	Sequence direction 5' – 3'	Location[A]	Band size (bp)
N784	ATT TTG GTG ATG ACA AGA TGA	26681-26701	402
N1145	CAT TGT TCC TCT CCT CAT CTG	27062-27082	
NewS1Oligo5	TGA AAC TGAA CAA AAG AC	20281-20299	1700
S1Oligo3	CCA TAA GTA ACAT AAG GRC RA	22002-22020	

Table S2. IBV sequences obtained from GenBank for Phylogenetic Analysis.

Strain	**Accesion Number**	**Classification**
Beaudette	M95169	GI-1
Massachusetts	GQ504724	GI-1
H120.Vaccine	KF188436	GI-1
IBV438	KF809793	GI-1

Ma5.Vaccine	AY561713	GI-1
Holte	GU393336	GI-2
Iowa97	GU393337	GI-2
SDW	DQ070840	GI-2
Gray	L14069	GI-3
JMK	L14070	GI-3
PA/5344/98	AY789947	GI-3
56GX-98I	KC577394	GI-4
GX2-98	AY251816	GI-4
Holte	L18988	GI-4
N1/62	U29522	GI-5
Armidale	DQ490205	GI-5
Ck/CH/Shaanxi/2012/WN	KC478648	GI-5
VicS	U29519	GI-6
V5/90	U29520	GI-6
N2/75	U29523	GI-6
TP/64	AY606320	GI-7
SC1203	KC478591	GI-7
T07/02	AY606322	GI-7
L165	JQ964061	GI-8
L613	JQ964066	GI-8
L919	JQ964071	GI-8
ARK99	M99482	GI-9
ck/CH/LSD/110712	JQ739363	GI-9
IBV/CAL99	JF774063	GI-9
B	AF151954	GI-10
K43	AF151958	GI-10
T6	AF151960	GI-10
UFMG/G	JX182775	GI-11
AR/11/ER/33	KM658244	GI-11
IBV/Brazil/NUP/0616	KY465749	GI-11
D3896	X52084	GI-12
D207	M21969	GI-12
NGA/295/2006	FN182276	GI-12
Moroccan-G/83	EU914938	GI-13
4/91.Vaccine	AF093793	GI-13

UK-1233-95	AJ618984	GI-13
CK/CR/4436/23	PP398707	GI-13
CK/CR/4469/23	PP398708	GI-13
CK/CR/4472/23	PP398709	GI-13
CK/CR/4473/23	PP398710	GI-13
CK/CR/4494/23	PP398711	GI-13
CK/CR/4498/23	PP398712	GI-13
CK/CR/4499/23	PP398713	GI-13
CK/CR/0632/19	MT230573	GI-13
CK/CR/327/18	MT230575	GI-13
CK/CR/1094/18	MT230574	GI-13
B1648	X87238	GI-14
NGA/324/2006	FN182277	GI-14
B4	FJ807932	GI-15
EJ95	FJ807933	GI-15
K281-01	AY257062	GI-15
IZO 28/86	KJ941019	GI-16
AR/09/BA/29	KM658246	GI-16
AR/11/BA/28	KM658255	GI-16
CA/Machado/88	AF419315	GI-17
DMV/1639/11	KR232396	GI-17
PA/171/99	AF419314	GI-17
CK/CR/1344/16	MT230565	GI-17
JP8127	AY296744	GI-18
48SD-96VI	KC577388	GI-18
JP8443	AY296745	GI-18
58HeN-93II	KC577395	GI-19
SAIBK2	KU317090	GI-19
gammaCoV/Ck/Italy/966/2013	KU934151	GI-19
Qu_mv	AF349621	GI-20
Qu16	AF349620	GI-20
Spain/97/314	DQ064806	GI-21
UK/L-633/04	DQ901376	GI-21
Spain/99/326	DQ064812	GI-21
40GDGZ-97I	KC577382	GI-22
66GD-98VI	KC577397	GI-22

HN08	GQ265940	GI-22
variant 2	AF093796	GI-23
Israel/720/99	AY091552	GI-23
Eg/1265B/2012	KC533682	GI-23
V13	KF757447	GI-24
V25	KF757451	GI-24
IBV422	KF809791	GI-24
DMV/5642/06	EU694402	GI-25
GA/60173/2007	JN160805	GI-25
GA/10216/2010	KM660636	GI-25
NGA/N544/2006	FN182269	GI-26
NGA/N545/2006	FN182270	GI-26
NGA/BB91/2007	FN182266	GI-26
Georgia 08	GU301925	GI-27
GPL8225	GU437858	GI-27
GPL8264	GU437864	GI-27
D1466	M21971	GII-1
V1397	M21968	GII-1
N1/88	U29450	GIII-1
V18/91	U29521	GIII-1
V6-92	DQ490219	GIII-1
AR/6386/97	AF274436	GIV-1
GA/5381/99	AF274439	GIV-1
CU82616	AF317212	GIV-1
N4/02	DQ059618	GV-1
N5/03	DQ059619	GV-1
N4/03	DQ059620	GV-1
TC07-2	GQ265948	GV1-1
K119/09	JF804680	GV1-1
K273/09	JF804687	GV1-1
gammaCoV/ck/China/I0636/16	MH924835	GVII-1
GX-NN130021	KM365468	GVII-1
Mex-12	ON470391	GVIII-1
Mex-3009	ON470392	GVIII-1
Mex-56-7	ON470394	GIX-1
Mex-14P	ON470393	GIX-1

Table S3. Sequences used for Selective Pressure Analysis of 4/91 like sequences

Reference Sequence	**Accesion Number**	**Country**
4/91	MG407590	Poland
IBV/ck/MEX/2592/21	OM912692	Mexico
IBV/ck/MEX/2826/21	OM912701	Mexico
IBV/ck/MEX/2354/20	OM912682	Mexico
IBV/ck/MEX/2753/21	OM912686	Mexico
IBV/ck/MEX/2523/21	OM912695	Mexico
IBV/ck/MEX/2721/21	OM912691	Mexico
IBV/ck/MEX/2819/21	OM912700	Mexico
IBV/ck/MEX/2725/21	OM912689	Mexico
IBV/ck/MEX/2833/21	OM912702	Mexico
IBV/ck/MEX/1619/19	OM912697	Mexico
4/91Pathogenic	AF093794	United Kingdom
4/91Attenuated	AF093793	United Kingdom
FR-CR88061-88	AJ618986	United Kingdom
FR-85131-85	AJ618985	United Kingdom
UK-1233-95	AJ618984	United Kingdom
CK/CH/GX/NN1306	KX107723	China
CK/CH/GD/LZ1311	KX107666	China
CK/CH/GX/GL1311-2	KX107701	China
4/91Vaccine	KF377577	China
CK/CH/GX/GL1301-1	KX107692	China
CK/CH/HuB/HC1408-2	KX107759	China
CK/CH/JS/ZJ1502	KX107826	China
CK/CH/FJ/PT1301	KX107649	China
CK/CH/HuB/HC1303-1	KX107740	China
CK/CH/HuB/HC1303-2	KX107741	China
CK/CH/HuB/HC1303-4	KX107743	China
CK/CH/HuB/HC1303-3	KX107742	China
CK/CH/HuB/WH1304-1	KX107779	China
CK/CH/HuB/HC1304-1	KX107744	China
CK/CH/HuB/HC1304-2	KX107745	China
CK/CH/HuB/HC1402-3	KX107755	China

IBV/India/ck/01/23	OR824985	India
CK/CR/4436/23	PP398707	Costa Rica
CK/CR/4473/23	PP398710	Costa Rica
CK/CR/4469/23	PP398708	Costa Rica
CK/CR/4498/23	PP398712	Costa Rica
CK/CR/4494/23	PP398711	Costa Rica
CK/CR/4472/23	PP398709	Costa Rica
CK/CR/327/18	MT230575	Costa Rica
CK/CR/1094/18	MT230574	Costa Rica
CK/CR/0632/19	MT230573	Costa Rica
ck/Malaysia/8530/2016	OR509347	Malaysia
ck/Malaysia/13161/2015	OR509319	Malaysia
ck/Malaysia/3912/2016	OR509320	Malaysia
ck/Malaysia/8529/2016	OR509346	Malaysia
ck/Malaysia/7626/2016	OR509339	Malaysia
ck/Malaysia/7972/2016	OR509340	Malaysia
ck/Malaysia/10839/2015	OR509316	Malaysia
ck/Malaysia/1881/2015	OR509312	Malaysia

Table S4. S1 sequences obtained in this study during 2023, length of the fragment obtained and information recopilated about birds and 4/91 vaccine application.

Isolate	Acc-Number	Sequence length (pb)	Chicken Type	Location	Clinical Sings	Age (days)	Vaccinated with 4/91
CK/CR/4436/23	PP398707	1617	Broiler	Alajuela	Respiratory	40	No
CK/CR/4469/23	PP398708	1615	Broiler	Alajuela	Respiratory	36	No
CK/CR/4472/23	PP398709	1615	Broiler	Alajuela	Respiratory	36	No
CK/CR/4473/23	PP398710	1609	Broiler	Alajuela	Respiratory	36	No
CK/CR/4494/23	PP398711	1598	Broiler	Alajuela	Respiratory	41	No
CK/CR/4498/23	PP398712	1616	Broiler	Alajuela	Respiratory	36	No
CK/CR/4499/23	PP398713	1609	Broiler	Alajuela	Respiratory	36	No

Table S5. Predicted N-glycosylation sites in S1 subunit of the spike protein gene of IBV 2023 isolates compared to 4/91sequences and 2018-2019 isolates.

Isolate	Position																
	54	75	103	146	165	180	214	239	249	266	273	278	308	427	449	515	532
	NGTN	NISA	NFSD	NLTV	NSTS	NETT	NGTA	NFSD	NSSL	NTTL	NFTF	NVSN	NLSF	NITL	NVTE	NETD	NGTR
4/91.Vaccine	0.725	0.701	0.695	0.811	0.617	0.582	0.659	0.585	0.635	0.629	0.611	0.540	0.549	0.608	0.623	0.563	0.605
4/91.Pathogenic	0.725	0.701	0.695	0.811	0.617	0.582	0.659	0.585	0.635	0.629	0.611	0.540	0.549	0.608	0.623	0.545	0.619
CK/CR/4499/23	0.663*	0.701	0.695	0.811	0.617	0.581	0.659	0.584	0.634	0.629	0.611	0.539	0.549	0.607	0.622	0.545	0.587
CK/CR/4498/23	0.663*	0.701	0.695	0.811	0.617	0.582	0.659	0.585	0.634	0.629	0.611	0.540	0.549	0.607	0.622	0.545	0.605
CK/CR/4494/23	0.725	0.701	0.695	0.811	0.617	0.581	0.658	0.584	0.634	0.628	0.611	0.538	0.548	0.606	0.621	0.528	---
CK/CR/4473/23	0.663*	0.701	0.695	0.811	0.617	0.582	0.659	0.585	0.634	0.629	0.611	0.539	0.549	0.607	0.623	0.545	0.587
CK/CR/4472/23	0.669*	0.701	0.695	0.811	0.617	0.582	0.659	0.585	0.634	0.629	0.611	0.540	0.549	---	0.622	0.546	0.605
CK/CR/4469/23	0.663*	0.701	0.695	0.811	0.617	0.582	0.659	0.585	0.634	0.629	0.611	0.540	0.549	0.608	0.622	0.546	0.605
CK/CR/4436/23	0.725	0.701	0.695	0.811	0.617	0.582	0.705	0.585	0.635	0.629	0.611	0.540	0.549	0.608	0.623	0.530	0.613
CK/CR/0632/19	0.682+	0.701	0.695	0.811	0.617	0.573	0.659	0.585	0.635	0.629	0.611	0.540	0.549	0.608	0.609	0.546	0.619
CK/CR/1094/18	0.703	0.701	0.695	0.811	0.617	0.582	0.659	0.585	0.635	0.629	0.611	0.540	0.549	0.608	0.623	0.546	0.619
CK/CR/327/18	0.715 0.601 57**	0.701	0.697	0.811	0.617	0.582	0.659	0.585	0.635	0.629	0.611	0.540	0.549	0.608	0.623	0.546	0.619

*NETN is in position 54.

+NKTN is in position 54.

** Only isolate CK/CR/327/18 has NNTV is in position 57.

Table S6. Predicted epitopes of S1 subunit.

Start	End	Peptide	Score	HVR
55	73	ETNNAVSVSGCTAGTFYES	0.752	1
88	100	AGMSWSVSQFCTA	0.579	2
110	156	FVTHCFKSQQGSCPLTGMIPQNYIRISAMRSG FLFYNLTVSVSKYPK	0.729	2
190	196	VYFKSGG	0.608	-
238	244	GNFSDGF	0.620	-
261	271	RESSTNTTLEL	0.585	-
275	297	TFTNVSNASPNSGGVDTFQLYQT	0.815	3
313	328	SSFVYKPSDFMYGSYH	0.549	3
351	370	LTYGPIQGGCKQSVFSNKAT	0.710	3
375	402	YSYRGLTRCKGVYRGELTQYFECGLLVY	0.844	3
420	432	LTQSNYNNITLNK	0.637	-
448	465	TNVTEATANYSYLADGGL	0.759	-
478	489	VVRGAYGLNYYK	0.728	-
499	538	QFVVSGGNLVGILTSHNETDSEFIENQFYIKLTNGTRRSR	0.730	-

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Data availability S1 sequences were uploaded to GenBank under accession numbers: PP398707, PP398708, PP398709, PP398710, PP398711, PP398712, PP398713.

Declarations

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

Ethics approval All procedures performed in animals were in accordance with the ethical standards of the ‘‘Comite´ Institucional de Cuido y Uso de Animales’’ (CICUA) of the University of Costa Rica.

CONCLUSIONES

1. Las secuencias obtenidas en 2023 muestran un alto porcentaje de identidad tanto a nivel de nucleótidos como de aminoácidos con la secuencia de la vacuna atenuada 4/91.
2. La relación entre los cambios de aminoácidos observados y la aparición de síntomas en las aves requiere mayor investigación. Es necesario un análisis del genoma completo para determinar si los cambios fuera de la región S1 contribuyen al desarrollo de la enfermedad.
3. Las predicciones de sitios de glicosilación y palmitoilación, se mantuvieron conservadas entre las variantes 4/91-CR 2023, 4/91-CR 2018-2019, y variante vacunal y patogénica. En cuanto a la fosforilación, se predijeron dos cambios en serinas.
4. Los análisis de selección indican que la variante está evolucionando bajo selección negativa.
5. Se predijeron diez sitios bajo selección positiva, principalmente localizados entre el RBD y las regiones HVRs.
6. La presencia de variantes altamente similares a la vacuna atenuada 4/91 en aves no vacunadas requiere de atención y subraya la necesidad de reforzar las medidas de bioseguridad en las granjas.

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ANEXO

Diseño de iniciadores para detección de variantes GA13-CR

Debido al alto costo y al tiempo que requiere el proceso de propagación viral en huevos embrionados SPF, se diseñaron iniciadores dirigidos a una región conservada de la secuencia que codifica la subunidad S1 de la proteína espicular de las variantes GA13-CR. El objetivo fue detectar si los diagnósticos positivos para IBV estaban relacionados con las variantes GA13-CR, causantes del último brote en el país, evitando la necesidad de realizar pasajes en embriones y secuenciación de la región S1.

1. Las secuencias S1 de variantes GA13-CR y GA-13/14255/14 (Georgia 13 USA) (Tabla 1) fueron alineadas en Geneious 11.0.20.1 software (Kearse et al., 2012) utilizando MAFTT. Se obtuvo la secuencia consenso.

2. El diseño de los iniciadores se realizó sobre la secuencia consenso en Primer3Plus https://www.primer3plus.com/index.html (Untergasser et al., 2013).

3. La selección de iniciadores se realizó con base a los valores de CG, tamaño y Tm (Tabla 2). El tamaño del producto fue 535 pb.

4. Para determinar su funcionamiento se realizaron pruebas *in silico* en Genome Browser https://genome.ucsc.edu/. Las amplificaciones fueron positivas para las secuencias Georgia 13 Costa Rica y USA, con el fragmento esperado y negativas para secuencias pertenecientes a otros linajes (4/91, Massachusetts, Beaudette, Spain97314, Variant2, ARK99 y V13).

5. Se realizaron extracciones de ARN a líquidos alantoideos almacenados a -80 °C obtenidos de huevos embrionados infectados con las variantes GA13-CR, provenientes del brote que

ocurrió en el país en 2016 (Villalobos-Agüero et al., 2021), y de los cuales previamente se había determinado la presencia de esta variante por secuenciación de la S1. Con estas extracciones se realizaron RT-PCR utilizando los iniciadores GA13-CR para determinar su funcionamiento experimentalmente (ver programa en Tabla 3).

6. Se realizaron pruebas de sensibilidad con diluciones seriadas de la extracción de ARN ($1:10^1 – 1:10^8$) (Fig. 1).

Tabla 1. Secuencias de la región S1 de variantes Georgia 13 utilizadas para el diseño de iniciadores.

Secuencia	Números de accesión
GA-13/14255/14	KM087780
CK/CR/491/17	MT230572
CK/CR/1160/16	MN757859
CK/CR/175/17	MT230563
CK/CR/186/17	MT230569
CK/CR/1298/16	MT230566
CK/CR/1167/16	MT230567
CK/CR/1329/16	MT230568
CK/CR/307/17	MT230571
CK/CR/1344/16	MT230565
CK/CR/185/17	MT230570
CK/CR/0068/17	MT230562
CK/CR/176/17	MT230564

Tabla 2. Características de iniciadores seleccionados.

	5’ – 3’	3’ – 5’
Secuencia	CGGAGTTGGCATGTGTCCTT	AGCACTAATATCACCGCCGG
Tm	60.6 °C	60.6 °C
GC	55%	55%
Longitud	20 pb	20 pb

Tabla 3. Programa de termociclador utilizado en RT-PCR para GA13-CR.

Etapa	Temperatura (°C)	Tiempo
Trascripción Reversa	52	30 min
Activación Inicial	95	15 min
Desnaturalización	95	30 seg.
Alineamiento	65.3	40 seg. x40
Extensión	72	20 seg
Extensión final	72	5 min

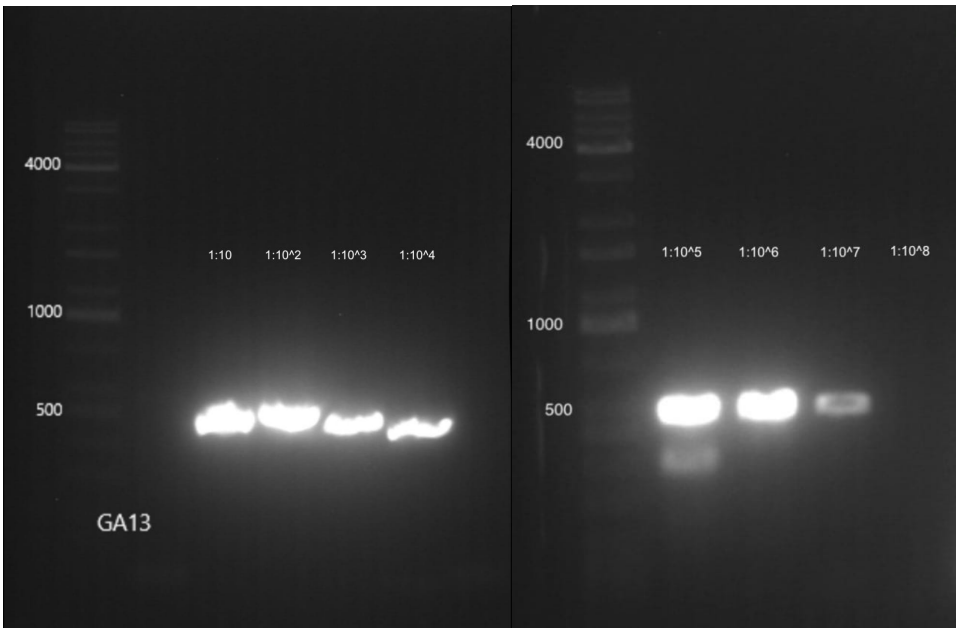

Figura 1. Controles de sensibilidad de los iniciadores diseñados para detectar por medio de RT-PCR a la variante GA13. A partir de la dilución seriada $1/10^8$ el resultado es negativo.