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ORIGINAL ARTICLE

The efficacy of the prime-boost regimen for heterologous infectious bronchitis vaccines mandates the administration of homologous vaccines

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Abstract Infectious bronchitis virus (IBV) has been frequently reported in chickens worldwide, including in the Eastern Region of Saudi Arabia (ERS). Several IBV outbreaks were recently reported in chickens despite the massive use of various vaccines. Based on partial sequencing of the S1 gene, at least three genotypes were reported (CK/CH/LDL/97I, IS/720/99, and IS/Variant2/98) in the ERS with no available homologous vaccines. Herein, we tried to evaluate the protection provided by some selected commercial-available vaccines against these three genotypes. We divided the experimental chickens into eight groups. Representative isolates from these genotypes were inoculated into three groups of broiler chickens vaccinated with the H-120 vaccine at the age of 1 day and boosted with the 4/91 vaccine at the age of 14 days (challenged groups). One group of chickens had received the same protocol of IBV vaccines but was kept without infection to serve as a vaccine control group. The three isolates were inoculated into three other similar but unvaccinated groups of broiler chickens (infected groups). Group eight chickens were neither vaccinated nor infected and used as a negative control group. Evaluation of the protection induced by the tested vaccination schedule was assessed by several criteria, including the ability to reduce the severe clinical signs caused by IBV infection, changes in the body temperature of various groups of chickens, the reduction in the magnitude of IBV-induced lesions, and the reduction in the viral loads in tracheas of a different group of chicken. Monitoring the immune status of chickens was also recorded based on the hemagglutination inhibition antibodies in sera of various groups of chickens. Our results show clinical and tracheal protection against IBV/IS/Variant2/98-like and IBV/IS/720/99-like strains. Moderate protection was observed in the IBV/CK/CH/LDL/97I-like pressure. The kidneys of the challenged groups of chickens showed minimal or no gross lesions compared with the infected groups, even in those chickens challenged with the IBV/CK/CH/LDL/97I-like strain. In conclusion, this is the first study to perform the protectotyping of some IBV strains from Saudi Arabia. It demonstrated the proficiency of the investigated vaccination schedule in control of infection of broiler chickens with IBV/IS/Variant2/98 and IBV/IS/720/99 strains. It is highly recommended to introduce the homologous IBV/CK/CH/LDL/97I-based vaccine to the vaccination protocols of chickens in the ERS to match the circulating strains and ensure better protection.

Keywords IBV · Vaccine · Prime-boost · Antibody · Challenge

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Introduction

Infectious bronchitis (IB) is an acute, highly contagious respiratory disease of chickens (Gallus gallus) that is present virtually in all regions with the intensive poultry industry. In addition to respiratory involvement, IBV may also affect

the urogenitalandr alimentary tracts of chickens. It poses significant economic losses to the chicken industry by adversely affecting the body weight gain, the quality and quantity of the produced e and as acts as a predisposing factor for secondary infections [13]. IBV belongs to group III Coronaviruses of the family Coronaviridae and other viral diseases affecting several poultry spec,ies particularly turkey coronavirus (TCoV) [19–21]. It is an enveloped virus with a single-stranded positive-sense RNA genome 27.6 Kb in length. Four major structural proteins, including the Spike (S), the Envelope (E), the Membrane (M), and the Nucleocapsid (N) proteins, were encoded by the 3' end of the viral genome [12]. Numerous IBV types were reported worldwide, and several methods were used for IBV typing. Serotyping assembles IBV strains according to their relatedness in the virus neutralization test. Genotyping groups IBV strains according to the similarity in their genome sequence. Protectotyping clusters of IBV strains according to their liableness against complete immune components induced in chickens by other IBV strains. Though not the general role, it has been reported that strains of a single genotype/serotype may gather in different protectotypes [6].

Vaccination is the most efficient approach to control IBV infection. The main factors that hampered the success of IBV vaccines and protection are many IBV types, the continuous emergence of new viral strains/variants, and the poor cross-protection among various strains of IBV [9, 10]. Theoretically, complete protection against IBV infection is usually dependent on the administration of the homologous vaccine; the protection conferred by heterologous vaccines ranges from very poor to moderate protection, depending on the assigned criteria of the protectotyping. Therefore, to avoid/minimize the IBV vaccination failure, a homologous vaccine to the circulating field strains in chickenparticularcertain region should be carefully selected [11]. Another apapplying the prime-boost vaccination regimen using two different types of IBV strains. For example, priming of chicken with Mass type vaccine and boosting with 4/91 vaccine was reported to broaden the acquired protection against heterologous IBV strains [15, 37].

IBV was repeatedly reported in poultry of Saudi Arabia and, in particular, poultry of the ERS where the circulation of several genotypes including CK/CH/LDL/97I, 4/91, Mass, IS/720/99, IS/Variant2/9,8 and QX were reported [1, 2, 4, 6, 17, 24, 25]. Our previous work showed that IBV was detected at flock level seenin 42 out of 115 (36.52%) broiler flocks with respiratory involvement [35].the At bird level, seroprevalence against IBV in unvaccinated chicken flocks ranged between 14 and 54% [30]. The IBV/Mass and 4/91 vaccine strains have been used for many years to control the IBV infections among the chicken population in the ERS. The present study was designated to evaluate the level of chicken protection granted by the consecutive administration of some commercial IBV vaccines (Mass-H120 and 4/91) in a prime-boost regimen against some heterologous strains that are currently circulating in the ERS (CK/CH/LDL/97I, IS/720/99 and IS/Variant2/98).

Materials and methods

IBV isoaltes

We used three field IBV isolates (SA/IH16/13, SA/IC8/13, and SA/IH1/12). Thesebelonged belonging to lineages/genotypes GI-16/CK/CH/LDL/97I, GI-23/IS/720/99, and GI-23/IS/Var,iant2/98 respectively. The GenBank accession numbers of these isolates are (MH648701, MH648724, and MH449644) respectively. These isolates were isolated from some broiler poultry farmsand showed respiratory and renal involvement in the ERS. Isolation and identification of these isolates wdescribed out as previously described [5].

Quantitative estimation of IBV inoculum

The IBV titration and quantification were carried out in 10-days old embryonated specific-pathogen-free (SPF) eggs (Nile SPF, Egypt). The virus titers were calculated according to the Reed and Muench (1938) formula (35). The virus titer was expressed as an embryo infectious dose of 50% (EID50) /100 µl. The purified titrated allantoic fluids per each strain were used as inoculum for the challenge stuand as to prepare the antigens for the haemagglutination test.

The commercial IBV vaccines

Two live attenuated IBV vaccines, including the Izovac IB H120 vaccine (IZO, Spa Italy) and the NOBILIS®IB 4/91 (Intervet International B.V. Boxmeer, Holland) were used in this study. Vaccines were administered according to manufacturer instructions.

Embryonated chicken eggs and chickens

Fertile eggs from a commercial hatchery of a local chicken breed were obtained and incubated in the Virology Laboratory, College of Veterinary Medicine, King Faisal University. The hatched chicks were transferred to the isolation units in the Agriculture and Veterinary Training and Research Station (AVTRS), King Faisal University. Those chickens received the water and food ad libitum. Sex was not considered in the present study.

Experimental design

A total of 48 one-day-old chicks were used in this study. Chicks were divided into two equal divisions, vaccinated and

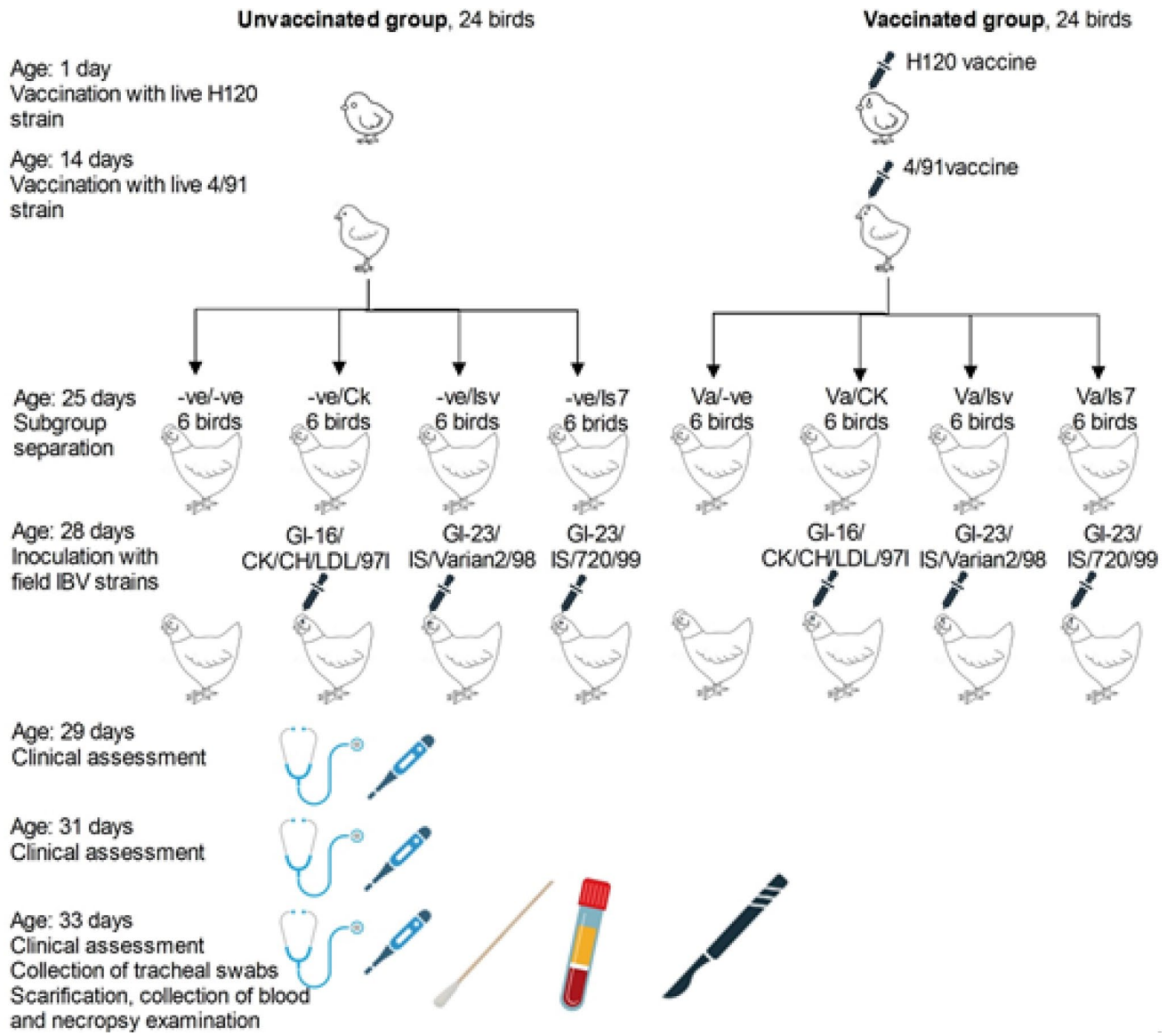


Fig. 1 Experimental design, 48 one-day-old chicks were divided into two divisions (vaccinated and unvaccinated,) each containing 24 chicks. Vaccination was performed intraocularly with live attenuated H120 at the age of 1 day and lived attenuated 4/91 periodat the age of 14 periodthe age ofage of 25 days, each division was further divided into four groups (each containing six chickenperiodt the age ofe of 28 days, three vaccinated groups were inoculated with field strains while the fourth was kept as a vaccine control group. Similarly, three unvaccinated groups were infected with the same field st,rains while the fourth group was left as negative control (unvaccinated/uninfected) group. Clinical assessment was performed at 1, 3, and 5 days post-inoculation (DPI). At the 5 DPI, a tracheal swab was collected from each chicken, chickens were sacrificed, blood was collected from each chicken, and necropsy findings were assessed

unvaccinated, and further divided intoeight8 experimen,tal groups as shown in Fig. 1 and Table 1.

The vaccinated 24 chicks receivedthe IBV/H120 vaccine at 1 day of age and then boosted with the 4/91 vaccine at 14 daysAccording to the producer's recommended dilution, both vaccines were administered via the intraocular route ministered via the intraocular route according to the producer's recommended dilution (103 and 103.6 EID50/dose for H120 and 4/91 vaccines, respectively). At 25 days of age, vaccinated chickens were randomly divided into four groups (6 chickens each). Chickens were housed in four isolated, separate units at the AVTRS. At 28 days of age, three groups of the vaccinated chickens were challenged with the field isolated IBV strains described above (herein referred to as challenged groups). The challenge dose was 103.0 EID50 per 100 µl per bird, as shown in Table 1. The inoculum was dispensed in both conjunctival sacs per chicken, approximately 50 µl per sac. The fourth group was not challenged and kept as a vaccine control (Va/−ve) group of chickens. Clinical observation and measuringof chicken's

Table 1 Experimental design and inoculated virusesare givenn ages of 1-day, 14-days, and 28-days

Group name	*Group abbreviationOne	Vaccine strain		Inoculated field strain (Lineage/Genotype)
		1 day	14 day	28 day
Vaccinated groups				
Challenged groups	Va/Ck	H120	4/91	SA/IH16/13 (GI-16/CK/CH/LDL/97I)
	Va/Isv	H120	4/91	SA/IH1/12 (GI-23/IS/Variant2/98)
	Va/Is7	H120	4/91	SA/IC8/13 (GI-23/IS/720/99)
Vaccine control	Va/−ve	H120	4/91	None
Unvaccinated groups				
Infected groups	−ve/Ck	None	None	SA/IH16/13 (GI-16/CK/CH/LDL/97I)
	−ve/Isv	None	None	SA/IH1/12 (GI-23/IS/Variant2/98)
	−ve/Is7	None	None	SA/IC8/13 (GI-23/IS/720/99)
Negative control	−ve/−ve	None	None	None

*Each groupcosixtainsn 6 birds

body temperaturen per group was performed on the 1st, then 3rd, and the 5th Day Post-Inoculation (DPI). Chickens were examined individually for the appearance of clinical signs and scored a on scale of 5 degrees (normal, mild, moderate, severe, death) according to [8, 29, 32] with modifications. Scoring was performed as followsy healthy birds with no respiratory sound for one minute after provoked movement (average, score 0), birds with any respiratory sound during 1 min immediately after provoked activity (mild, score 1), and birds with any rales audible from about 50 cm apart during one minute without provoked movement (moderate, score 2), birds with signs of gasping, coughing or rales audible from about 1.5 m without provoked movement (severe, score 3), birds found dead (score four until the end of the experiment). The chicken's body temperature was measured by inserting the thermometer probe (Hanna instrument, HI98511) about 2 cm in the cloaca. The remaining 24 one-day-old chicks in the non-vaccinated division of chickens were treated similarly, except for vaccination, as shown in Fig. 1 and includes 1. This division include three unvaccinated/infected groups which were used as positive control groups and one unvaccinated/uninfected group that served as a negative control group.

Collection and processing of samples

At the end of the 5th DPI, tracheal swabs were collected from each chicken and placed in 1 ml sterile phosphate-buffered saline (PBS) containingan antibiotic and antifungal cocktail. Chickens were then euthanized by cutting the jugular veins, and blood samples were collected for sera separation. All chickens were then examined individually for the presence of any gross tracheal, renal, splenic, or enteric lesions. Collected swabs and sera were stored at -80 °C until tested. Tracheal swabs in PBS were vigorously vortexed for one minute. The obtained fluid was used as a starting material for nucleic acid extraction. Collected sera were heat-inactivated at 56 °C for 30 min and tested for antibodies using the HI test.

Extraction of the total viral RNA and the Real-Time-RT-qPAccording to the manufacturer instructions, the total viral RNAs were extracted from the tracheal swab washes by IQeasy™ Plus Viral RNA Extraction Kit (Cat # 17153, iNtRON Biotmanufacturer'south Korea)onsThe Thermo Scientific Nanodrop 2000 Spectrophotometer measured the concentration of the extracted RNAsAccording to manufacturer instructions, the Reverse Transcription (RT) was performed using Reverse Transcription System (Cat # A3500, Promega, USA) using Reverse Transcription System (Cat # A3500, Promega, USA) according to manufacturer instructions. Quantitative PCR (qPCR) was performed using the primers and probe previously described by [21response2. Briefly, the reaction was performed using Quanti-Tect Virus Kit (Cat # 211011, Qiagen, Hilden, Germany) in a final volume of 20 µ. Each reaction includes 4 µl of QuantiTect Virus Master Mix, 1 µl of each primer (final concentration of 0.4 µM of each), 1 µl of the TaqMan probe (final concentration of 0.15 µM), 2 µl of the RT product, and 11 µl of RNase-free water. These reactions were peusing Agilent qPCR tubes using Agilent Stratagene Mx3000P instrumentCR tubes. The reaction parameters were as follows (95 °C/5 min, 40x (95 °C/15 s; 60 °C/45 s)). Data were analyzed using Agilent Mx3000P software. All samples were tested in two replicates to ensure the consistency and validation of the obtained results.

Preparation of the standard curve and quantification of the IBV viral load in tracheal swabs

To quantify the IBV viral load in tracheal swabs, a quantification curve was generated using a DNA amplicenclosed

Table 2 PCR primers used for quantification of viral load in tracheas

Name	Sequence 5′-3′	Product	References
Primers used for PCR to generate DNA amplicon			
N784	AATTTTGGTGATGACAAGATGA	403 bp (26,626–27,028)*	[16]
N1145	CATTGTTCCTCTCCTCATCTG		
PB			
AIBV-fr	ATGCTCAACCTTGTCCCTAGCA	130 pb (26,683–26,813)*	[30]
AIBV-as	TCAA-ACTGCGGATCA-TCACGT		
AIBV-TM	FAM-TTGGAAGTAGAGTGACGCCCA AACTTCA-BHQ1		

The qPCR targeted region is part of the DNA amplicon region used to generate DNAn

*Nucleotide position based on H120 genome, GB# FJ888351

amplicon was a 403 base pair (bp) of the N gene of IBV that enclose the sequence targeted by, the qPCR as shown in Table 2.

The H120 vaccine was grown in embryonated SPF eggs (Nile SPF, Egypt), and allantoic fluid was used as starting material for RNA extraction. Extraction of viral nucleic acid, RT-PCR reaction, and purification of PCR product was performed as previously described [6]. The Thermo Scientific Nanodrop 2000 Spectrophotometer measured the concentration of the purified DNA. The online Sequence Manipulation Suite (SMS) was used to calculate the molecular weight of the amplicon double-stranded DNA [8]. The online DNA Copy Number and Dilution Calculator calculated copy number/ng and dilution factor to prepare a solution containing 108 copies/µl (www.thermofisher.com). Tenfold-serial dilutiondraftedn prepared in Quanti-Tect Nucleic Acid Dilution Buffer and used to establish a standard curve. Amplification efficiency was calculated using the formula: Efficiency = −1 + 10(− 1/SThe generated standard curve converted sample Ct valuesnverted to logarithmic values (Log10) of copy number using GraphPad Prism version 9 (GraphPad Software San Diego, CA, USA). Any Cts lower than the quantification limit were arbitrary set at 101 copies.

The hemagglutination inhibition (HI) test

Preparation and titration of the HA antigen from the allawereic fluids of the IBV field isolate infectarried out as previously described [29]. The HI test was performed as per the OIE guidelines [32]. Each sample was tested in duplicate, including the negative and positive controls. The test was repeated for samples that generatedinconsistente results. The HI titers were expressed as Log2 geometric mean of reciprocal of the highest serum dicausesn that cause inhibition of the hemagglutination. The HI titer of the vaccine control (Va/−ve) and negative control (−ve/−ve) groups was measured three times using each IBV field isolate.

Statistical analysis

Prism was used for statistical analysis (GraphPad software version 9, San Diego, CA, USA). The median, 25% percentile, and 75% prepresentto express the results. Kruskal–Wallis was used to compare groups over three with Duncan Test variance analysis for quantitative results with non-parametric distribution of Levene's test. Significant was defined as a p-value of less than 0.05.

Results

Clinical observations on the IBV experimental chickens

The clinical signs were observed for 5 DPI and scored at 1st DPI, 3rd DPI, and 5th DPI. In challenged groups, symptoms were mild, observed after provoked movemmainlynd mostly commence at the 3rd DPI. In contrast, in the infected groups, signs erupted at the 1st DPI and were more severe as shown in Fig. 2A, with a noticeable decrease in feed consumption during the next couple of days. There was one death at the 5 DPI in CK/CH/LDL/97I-infected group. Neither clinical signs nor hostilere observed in the vaccine control and negative control groupssymptoms. Scores of clinical signs in infected groups were significantly higher than scores of the negative control and the vaccine control groups (Table 3).

Vsucceededion succeed in minimizing the score of clinical signs in challenged groups to the level that showed no significabetweenifference from the negative control and the vaccine control groups.

Monitoring of the body temperature of the IBV experimental chickens

IBV infection-induced increase in body temperature (Fig. 2B). Body temperatures of CK/CH/LDL/97I-infected and IS/720/99-infected groups were significantly higher than that of either the negative control or the vaccine control

Table 3 Statistical significance of differences, expressed as P-values, between scores of clinical signs, rectal temperatures, hemagglutination inhibition titers, and viral loads in the trachea of the experimental groups

Compared experimental groups	Group abbr	A score of clinical signs			Rectal temperature			HI titers			Viral load
		1st DPI*	3rd DPI	5th DPI	1st DPI	3rd DPI	5th DPI	Ck-Antigen[a]	Isv-Antigen	Is7-Antigen	
Negative control group vs. vaccine control group											
Unvaccinated/uninfected vs. vaccinated/unchallenged	−ve/−ve vs. Va/−ve	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	0.4157	> 0.9999
Negative control gr.oup vs infected groups											
Unvaccinated/Uninfected vs. Unvaccinated/Ck-Infected	−ve/−ve vs. −ve/Ck	> 0.9999	0.0013	0.0024	0.0011	0.21	0.2538	> 0.9999			< 0.0001
Unvaccinated/Uninfected vs. Unvaccinated/Is7-Infected	−ve/−ve vs. −ve/Is7	0.9757	0.0336	0.0034	0.0952	0.0066	0.0214			> 0.9999	< 0.0001
Unvaccinated/Uninfected vs. Unvaccinated/Isv-Infected	−ve/−ve vs. −ve/Isv	> 0.9999	0.1009	0.0104	0.2961	0.1687	0.0568		> 0.9999		< 0.0001
Negative control group vs. challenged groups											
Unvaccinated/Uninfected vs. Vaccinated/CK-Challenged	−ve/−ve vs. Va/Ck	> 0.9999	0.266	0.1808	0.1552	0.0212	0.1118	0.0599			< 0.0001
Unvaccinated/Uninfected vs. Vaccinated/Is7-Challenged	−ve/−ve vs. Va/Is7	> 0.9999	> 0.9999	0.3975	0.8113	0.0768	0.2113			0.0032	0.3586
Unvaccinated/Uninfected vs. Vaccinated/Isv-Challenged	−ve/−ve vs. Va/Isv	> 0.9999	> 0.9999	> 0.9999	0.1503	> 0.9999	> 0.9999		0.1313		> 0.9999
Infected vs. challenged groups											
Unvaccinated/Ck-Infected vs. Vaccinated/CK-Challenged	−ve/Ck vs. Va/Ck	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	0.0026			> 0.9999
Unvaccinated/Is7-Infected vs. Vaccinated/Is7-Challenged	−ve/Is7 vs. Va/Is7	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999			0.0028	0.037
Unvaccinated/Isv-Infected vs. Vaccinated/Isv-Challenged	−ve/Isv vs. Va/Isv	> 0.9999	> 0.9999	0.113	> 0.9999	> 0.9999	> 0.9999		0.0441		0.0045
Vaccine control group vs. challenged groups											
Vaccinated/Unchallenged vs. Vaccinated/CK-Challenged	Va/−ve vs. Va/Ck	> 0.9999	0.266	0.1808	0.141	0.0169	0.0699	> 0.9999			0.001
Vaccinated/Unchallenged vs. Vaccinated/Is7-Challenged	Va/−ve vs. Va/Is7	> 0.9999	> 0.9999	0.3975	0.7496	0.0625	0.1359			> 0.9999	> 0.9999
Vaccinated/Unchallenged vs. Vaccinated/Isv-Challenged	Va/−ve vs. Va/Isv	> 0.9999	> 0.9999	> 0.9999	0.1365	0.8711	> 0.9999		> 0.9999		> 0.9999
Vaccine control group vs. infected groups											
Vaccinated/Unchallenged vs. Unvaccinated/Ck-Infected	Va/−ve vs. −ve/Ck	> 0.9999	0.0013	0.0024	0.001	0.1741	0.1646	> 0.9999			< 0.0001
Vaccinated/Unchallenged vs. Unvaccinated/Is7-Infected	Va/−ve vs. −ve/Is7	0.9757	0.0336	0.0034	0.0861	0.0051	0.0125			0.3814	< 0.0001
Vaccinated/Unchallenged vs. Unvaccinated/Isv-Infected	Va/−ve vs. −ve/Isv	> 0.9999	0.1009	0.0104	0.2707	0.1393	0.0346		> 0.9999		0.0006

*Time of measuring the parameter

[a]The antigen used in the haemagglutination inhibition test

groups (Table 3). The body temperature of IS/Variant2/98-infected group was significantly higher than the body temperature of the vaccine control group. Vaccisucceedsucceededecreasing body temperature of IS/720/99-challenged and IS/Variant2/98-challenged groups to the level that showed no significant difference from hat of the negative control and the vaccine control groups. On the other hand, the body temperature of the CK/CH/LDL/97I-challenged group remained high and, like the CK/CH/LDL/97I-infected group, showed a significant difference from that of the negative control and the vaccine control groups.

Necropsy findings of the IBV experimental chickens

Necropsy examination of the infected groups of chickens revealed congestion and the presence of catarrhal exudates in the trachea. Congestion of the intestines and enlargement of the spleen were also reported. However, kidney lesions were the most prominent findings, especially in the case of the infected chickens with the CK/CH/LDL/97I-like strain. Kidneys were enlarged in size than usual, and pale-mottled color (Fig. 3). Necropsy examination of the challenged groups of chickens showed mild tracheal congestion and infiltration of exudates. Edema and congestion of kidneys in the challenged groups were also seen, especially in CK/CH/LDL/97I-challenged group. No apparent lesions were observed in the case of the vaccine control and the hostile control groups of chickens.

Monitoring the hemagglutination inhibition antibody titers

There was a significant increase in the titers of HI antibodies in all challenged grohen compared to the corresponding infected groups (Fig. 2C and Table 3). HI, titer in the IS/720/99-challenged group was also significantly higher than the HI titer of the negative control group. Minimal and insignificant differences in HI titers of the vaccine control group due to using hemagglutinin from the three field strains were also seen.

Quantification of the IBV load in tracheal swabs collected from the experimental chickens

Amplification of the ten-fold dilution series of the DNA amplicon presents linearity over the dilutions containing 102 to 107 copies/reaction (Supplementary Figures A and B). Higher dilutions of the DNA amplicon were detectable but out of linearity. Accordingly, viral load was quantified in the tracheas from chickens.

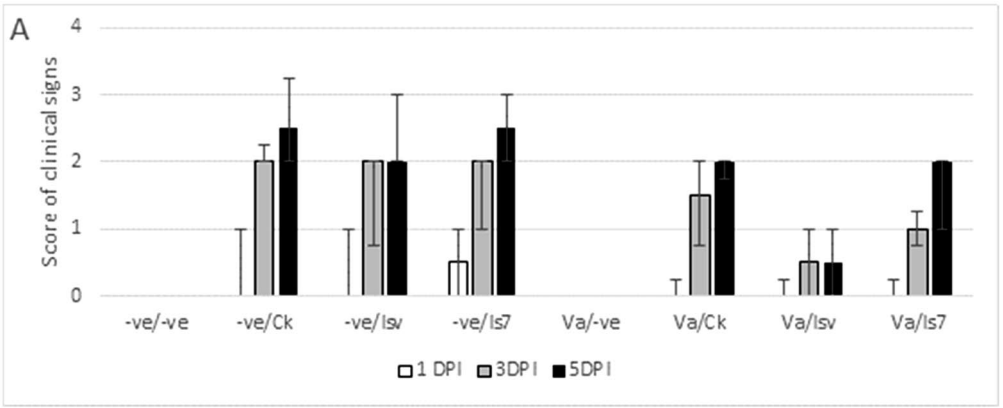
The IBV was detected within the quantification limit in samples from all experimental groups (Fig. 2D) except the IS/Variant2/98-challenged, the vaccine control, and the hostile control groups. The IBV was detected within the quantification limit in a sample from only one chicken and out of the quantification limit in samples from two- other chickens from the IS/Variant2/98-challenged group. Additionally, the IBV was detected but out of the quantification limit in samples from two chickens of the vaccine control group. The IBV was not seen in the negative control group of chickens.

Tracheal viral load in the infected groups was significantly higher that in the negative control and vaccine control groups (Table 3). Vaccination succeeds in minimizing tracheal viral load in IS/720/99-challenged and IS/Variant2/98-challenged groups to the levels that differ insignificantly from either the negative control or the vaccine control groups but are significantly different from that of the corresponding infected groups. On the other hand, tracheal viral load in CK/CH/LDL/97I-challenged group remained high and, like the CK/CH/LDL/97I-infected group, showed a significant difference from that of the negative control and the vaccine control groups.

Discussion

Active surveillance and protectotyping are essentialcontrollingcontrol of the continuously evolving IBV strains. In Saudi Arabia, several IBV genotypes were detected even in the presence of active vaccination programs. This highlights the importance of selecting the homologous IBV vaccines that match the field circulating strains [5, 6, 25]. Several IBV genotypes were recently detected and identified in t,his region including, but not limited to, CK/CH/LDL/97I, 4/91, Mass, IS/729/99, and IS/Variant2/98 [5, 6, 25]. The most commonly available commercial IBV vaccines belong to the Mass serotype (H120, Ma5) and 4/91 serotype. Unfortunately, there are no homologous commercial vaccines available in this region for the other three IBV genotypes. The main goal of this study was to evaluate the efficacy of some of the currently available commercial IBV vaccines in the protection against the three heterologous IBV types circulating in the ERS.

In the current study, we examined the efficacy of some commercial live attenuated IBV vaccines in a prime-post regimen (Mass and 4/91) in commercial broiler chickens. It was previously reported that the combination of some IBV antigenic-variable vaccines improved the protection rates provided against infection with IBV heterologous strains [37]. A previous study on the effects of variable sequential application of Mass (Ma5 or H120) and 4/91 vaccine,sands combining Ma5 and 4/91 vaccines was conducted [15]. This study showed that priming chickens with Mass type of vaccines followed by a booster with 4/91 vaccine provide superior chicken protection against IBV infection. This was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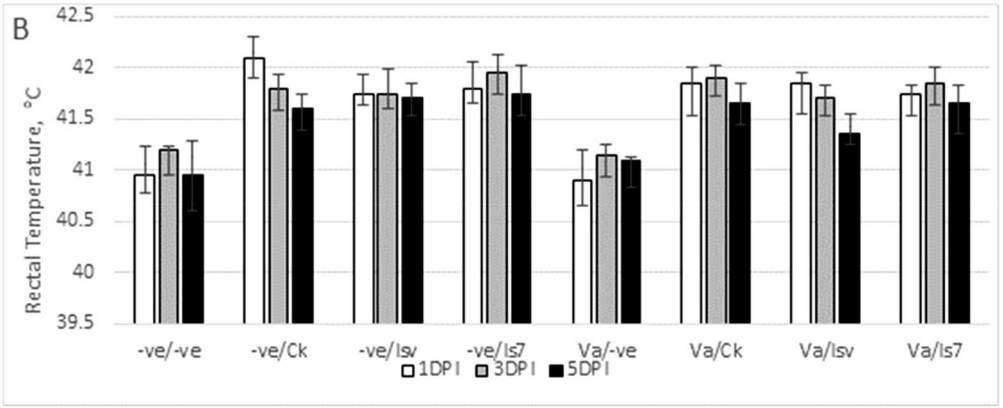
B
Rectal Temperature, °C
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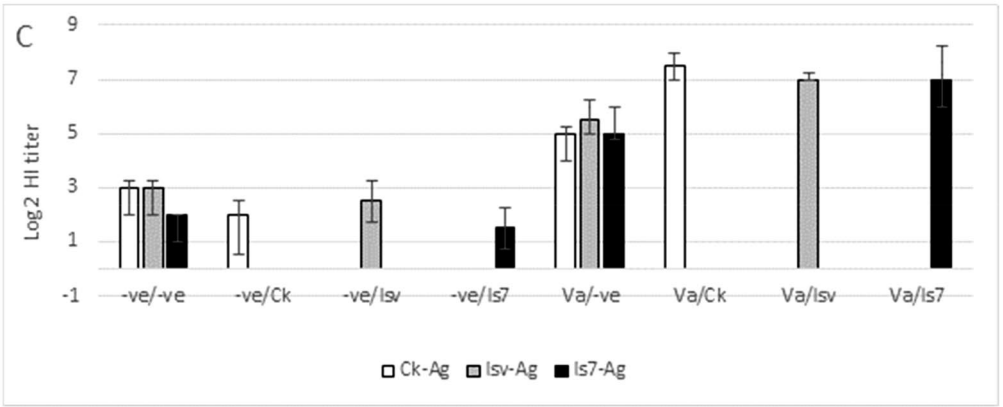
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-ve/Is7
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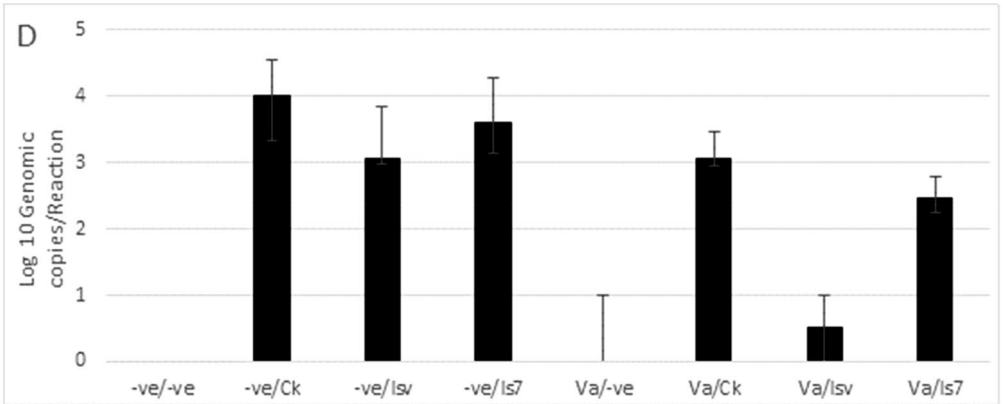
Va/Isv
Va/Is7
Ck-Ag
Isv-Ag
Is7-Ag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◂**Fig. 2** Evaluation of the protection conferred by the investigated vaccination schedule **A** median, 25% percentile, and 75% percentithe observed clinical signs scores signs at 1st, 3rd, and 5th Days Post Inoculation (DPI). **B** Median, 25% percentile, and 75% percentile of the body temperature (°C) of chickens at 1st, 3rd, and 5th DPI. **C** Median, 25% percentile, and 75% percentile of Log 2 Haemagglutination Inhibition (HI) titers of sera collected from experimental chickens at thhree 5th DPI. HI, titers were measured using 3 hemagglutinins derived from the 3 field isolates. Negative control and vaccine control groups werethreetested 3 times uthreeing the 3 hemagglutinins. **D** Median, 25% ercentile, and 75% percentile of Log 10 viral load (genome copy number per reaction) measured using qPCR in tracheal swabs from experimental chickens at the 5th DPI. Related data are presented in supplementary Table 1.

compared to priming chickens with 4/91, followed by boosting with the Ma5 vaccine. It was also much more efficient in protecting chickens than combining both vaccinessimultaneouslye or booster with the same initial vaccine [15]. Similarly, administration of Mass vaccine as eonerly as 1 day of age and revaccination with 4/91 vaccine at 14 daysan excellentd a good rate of protection against nephrogenic Brazilian IBV strains [38]. Similarly, the IBV vaccine combination provided high protection rates against the heterologous QX strains [7, 34, 37], though better protection was achieved with homologous QX vaccine [23]. In the current study, we decided to use the H120 rather than the Ma5 because the immunity driven by its administration is well characterized, and it was also shown to be safe at vaccination of day-old chickens [4]. We used the commercial broiler chickens in this study, rather than SPF chickens, to mimic the field conditions. Logically, the immune response against the used IBV vaccines in the SPF chickens was much more superior to the commercial chickens. This difference may be attributed to the maternally derived immunity that may interfere with the replication of the vaccine in the case of commercial chickens [37].

Our proposed vaccination schedule appears to provide high protection against the IS/Variant2/98-like strains. This was evident by the reduction in the severity of the developed clinical signs and necropsy lesions as well as a decrease in both the body temperature and the IBV-viral load in the trachea of the challenged group of chickens (Fig. 2D). This is in addition to the induction of production of circulating anti-IS/Variant 2/98 antibodies. These findings are in agreement with those reported previously [23]. The latter study vaccinated SPF chickens with the H120 and 1/96 strains at 1 and 14 days of age, respectively, then challenged them with the IS/1494/06 strain at age of 35 days. This vaccination/challenge protocol resulted in good protection manifested clinically with mild clinical and necropsy lesions, as well as resulted in a marked reduction in the IBV viral load in the tracheas of the infected chickens. This approach also resulted in a 69.2% protection score based on ciliostasis scoring [23]. Administration of the 4/91 vaccine to chickens at the 1 and 14 days old was reported to provide relatively lower protection compared to that provided by the administration of the Mass and 4/91 combination. This combination was not the only factor but also the sequential application of the vaccines, as priming chickens with the 4/91 type and boosting with the Mass type provide lower protection than that induced by either Mass prime and 4/91 boost or prime and boost with 4/91 vaccine [6]. In contrast, single or twice use of the homologous vaccine at the 1st or 1st and 14th day of age followed by the challenge at 2–3 weeks later was reported to provide complete protection from clinical signs, 73–85% protection scores based on ciliostasis and reduction in the virus shedding [4, 6].

Our vaccination protocol offered relatively good protection against the IS/720/99-like strain with regards to viral load, body temperature, induction of immune response, and protection from the severe clinical signs and lesions. This is in fine agreement with some previous studies [7] where both the H120 and CR88 vaccines were used to vaccinate some commercial broilers at 1 and 14 days of age respectively. Results of this experiment revealed a 60% ciliary protection rate, complete clinical protection, and variable protection from gross lesions [7]. On the other hand, it has been reported that similar vaccination/challenge programs of the SPF chickens provide complete clinical protection, 40% ciliary protection rate, and decrease in the rate of virus recovery from kidney up to 20%. This suggests the unsatisfactory protection against the challenged strain, EG/1212B, a IS/720/99-like strain [41]. Some non-consistent findings were reported after vaccination of SPF chicks with the H120 vaccine at 14 days old and then challenged with the IS/720/99 type. Based on virus re-isolation in embryonated SPF eggs, the tracheal protection rate of 36% was reported after the challenge with IS/720/99 strain [18] and 91%, together with 25% renal protection rate, was reported after challenge with IS/885 strain [31]. On the other hand, administration of the formalin-inactivated homologous vaccine in 14 days-old SPF chicks provided about 69% ciliary protection against challenge with EG/1212B, an IS/885 like strain at age of 42 days. The virus was also detected in tracheal and/or kidney tissues in 60% of the involved chickens [3, 31].

CK/CH/LDL/97I is a dominant IBV genotype in the ERS that was detected at a frequency of 28.6% of the total IBV detections. A mortalities of approximately 30% of the affected flocks as well as losses related to treatment, control programs and reduction of body weight was induced by this genotype [17, 36]. In the present work, based on the clinical signs protection scoring, the vaccinated-CK/CH/LDL/97I-challenged group remained at a good level of protection when compared with the corresponding infected group. Additionally, the vaccination scheme induces a significant rise in HI antibody titer. This contributed to the reduction in

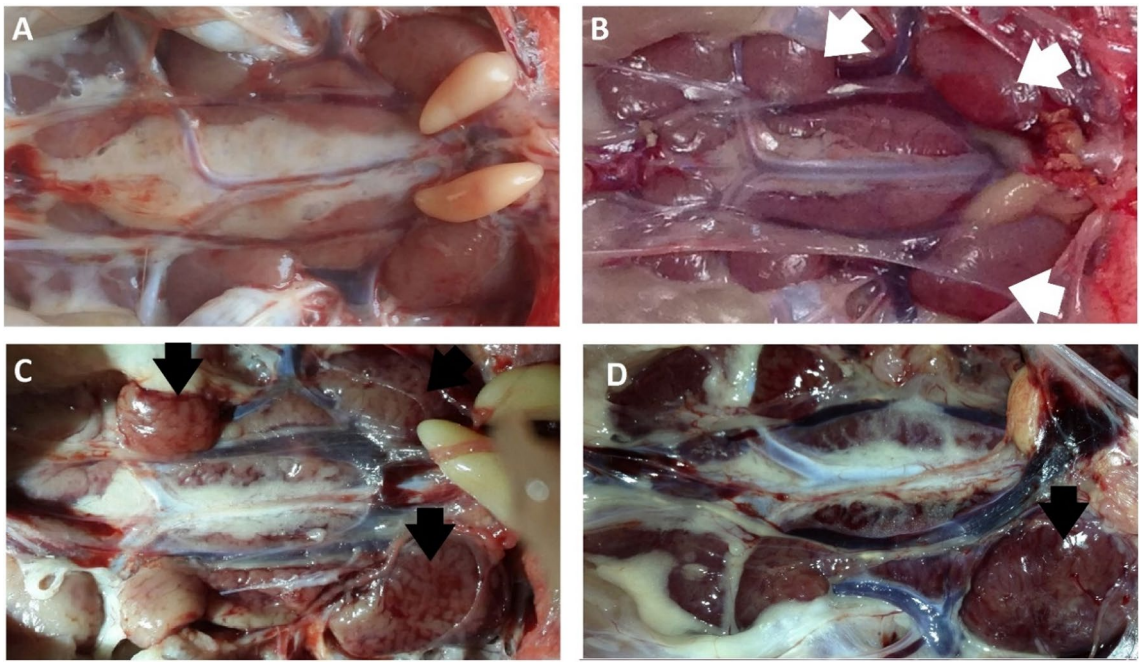

Fig. 3 Kidney lesions revealed by necropsy of **A** negative control (unvaccinated/ uninfected (−ve/−ve)) chicken, **B** vaccinated-CK/CH/LDL/97I-challenged (Va/Ck) chicken, **C** unvaccinated-CK/CH/LDL/97I-infected (−ve/Ck) chicken and unvaccinated-IS/720/99-infected (−ve/Is7). The whiten. White arrow in **B** shows the swollen lobules of tand he kidney, Black arrows in **C** an show hows swollen mottled kidney

the severity of renal lesions as well as resulted in a marked reduction in the mortality rates. On the other hand, vaccination was not able to provide a significant reduction in tracheal viral load or body temperature of the challenged group of chickens. Based on these findings, it is reasonable to state that the used vaccination program provides some protection against CK/CH/LDL/97-like strain, however, this protection was incomplete and relatively lower than that achieved with other investigated genotypes. This is compatible with our observation about the prevalence of CK/CH/LDL/97I- genotype in chickens in this region despite the use of Mass and 4/91 vaccines [5].

Ideally, complete protection against IBV infection usually requires the use of the homologous vaccine, while vaccination with a heterologous vaccine provides questionable protection [26, 27, 33]. Our protection study on the IBV/CK/CH/LDL/97I-like strain support this hypothesis. Previous studies showed that variable protection against IBV infection was provided by the administration of several heterologous vaccines and one candidate-homologous vaccine against CK/CH/LDL/97I strain [27]. This study showed that administration of a single homologous IBV vaccine dose to the SPF chickens resulted in the complete protection against CK/CH/LDL/97I strain [27]. However, the IBV was recovered from the tracheas of 50–100% of the chickens vaccinated with heterologous CK/CH/LDL/97I strains and from tracheas of 70% of the chicks vaccinated with H120 strain [27]. On the other hand, high protection rate against CK/CH/LDL/97I-like strain was also reported to be induced by heterologous vaccine strains. Both H120 and CR88 live attenuated vaccines were used to vaccinate commercial broiler chicks in a prime-boost regimen at (0 and 14) days of age, respectively. Chickens were then challenged with the IBV-Q1 strain, a CK/CH/LDL/97I-like strain, at 28 days of age. This was based on several criteria such as protection from the severe clinical signs, high ciliary protection (90%), and reduction in the viral loads from about 104.4 to around 101 equivalent units [14]. However, this partial disagreement may result from the use of different strains of vaccine, though of the same type (CR88 of the 4/91 type). In this context, it has been reported that strains of a single genotype/serotype may behave differently in protectotyping [39]. Vaccination of the SPF chicken with 4/91 vaccine at day 1 of age provided variably low protection against challenges with the IBV/GI-16 strain, CK/CH/LDL/97I type, at 21 days of age [22]. Similarly, both 4/91 and CR88 vaccine strains were reported to provide variable degrees of protection against the QX strain [8].

The systemic humoral immune response was observed in all challenged groups in the present study. The circulating antibody plays important role in kidneys protection as reported earlier [28]. In that study, kidney protection may be directly related to the effect of the circulating specific IBV antibodies. This was in the light of earlier study demonstrated that the high level of the HI antibody titers induced

by the heterologous IBV vaccine correlated well with the low recovery rate of IBV from the kidney or the genital tract. This was despite the lack of correlation with ciliary movement, reduction in the severity of the induced clinical signs, and virus recovery from the trachea [40]. Similar results were observed with nephropathy induced by CK/CH/LDL/97I-like strains [27].

Conclusions

In conclusion, our results demonstrate that vaccination of commercial broiler chickens in a prime-boost regimen using the Mass live attenuated vaccine at age of 1 day, and boosting with 4/91 live attenuated vaccine at 14 days of age provided a variable degree of protection against heterologous IBV strains. The protection rates were high against the IS/Variant2/98-like strain, good against IS/720/99-like strain, and moderate against CK/CH/LDL/97I-like strain. These data should help in the control of IBV in broiler chickens in a targeted area. Continuous monitoring of the circulating IBV strains in chickens in certain regions and preparation of some homologous vaccines to them is the main key role in the protection cycle against IBV natural infections.

Institutional review board statement

All handling and chicken experiments carried out in this study were conducted as per the instructions of the Animal Ethics protocols and the National Committee of Bio-Ethics, King Abdul-Aziz City of Science and Technology, Royal Decree No. M/59. The chicken experiments and protocols were reviewed and approved by the animal ethics committee of the deanship of scientific research, King Faisal University, Saudi Arabia (Approval No: KFU-REC/2020-12-35). All the necessary paperwork for sample collections was obtained.

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Author contributions "Conceptualization, MGH and AM; methodology, MGH, AK, AM; software, MGH, AK, AM; validation, MGH, AK, AM; formal analysis, MGH, AK, AM; investigation, MGH, AM; resources, MGH, AM; data curation, MGH, AK, AM; writing—original draft preparation, MGH, AK, AM; writing—review and editing, MGH, AK, AM; visualization, MGH, AK, AM; supervision, MGH, AM; Project administration, AM; funding acquisition, AM. All authors have read and agreed to the published version of the manuscript.

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Data availability All the data will be available upon request from the corresponding authors.

Declarations

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

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