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Inactivated infectious bronchitis virus vaccine encapsulated in chitosan nanoparticles induces mucosal immune responses and effective protection against challenge

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ABSTRACT

Avian infectious bronchitis virus (IBV) is one of the most important viral diseases of poultry. The mucosa of upper respiratory tract, specially the trachea, is the primary replication site for this virus. However, conventional inactivate IBV vaccines usually elicit reduced mucosal immune responses and local protection. Thus, an inactivated IBV vaccine containing BR-I genotype strain encapsulated in chitosan nanoparticles (IBV-CS) was produced by ionic gelation method to be administered by oculo-nasal route to chickens. IBV-CS vaccine administered alone resulted in markedly mucosal immune responses, characterized by high levels of anti-IBV IgA isotype antibodies and IFNγ gene expression at 1dpi. The association of live attenuated Massachusetts IBV and IBV-CS vaccine also induced strong mucosal immune responses, though a switch from IgA isotype to IgG was observed, and IFNγ gene expression peak was late (at 5 dpi). Efficacy of IBV-CS was evaluated by tracheal ciliostasis analysis, histopathology examination, and viral load determination in the trachea and kidney. The results indicated that IBV-CS vaccine administered alone or associated with a live attenuated heterologous vaccine induced both humoral and cell-mediated immune responses at the primary site of viral replication, and provided an effective protection against IBV infection at local (trachea) and systemic (kidney) sites.

1. Introduction

The avian infectious bronchitis virus (IBV) variants are continuously emerging in different regions of the world and can break the immunity elicited by commercial anti-IBV vaccines formulated with classical viral strains [1–3]. Thus, live attenuated vaccines formulated with regionally important variants have been produced in several countries to control the infection caused by new IBV variants [1,3]. Live attenuated vaccines of the Massachusetts (Mass) serotype are commonly used in Brazil, but outbreaks by indigenous strains from BR-I genotype are continuously occurring and affecting respiratory and uro-genital tracts of poultry [4]. Additionally, previous studies demonstrated that only a partial cross-protection was provided by live attenuated Massachusetts vaccines against experimental and field infections with IBV BR-I variant strains [5–7].

The use of live attenuated vaccines presents several risks, such as virulence reversion, recombination with virulent field strains, slight tissue injuries that sometimes facilitate the development of more severe secondary infections [8].

Abbreviations: AF, allantoic fluid; BPL, beta-propiolactone; CEF, Chicken Embryo Fibroblast; CMI, cell-mediated immune; IB, infectious bronchitis; IBV, infectious bronchitis virus; IBV-CS, inactivated IBV vaccine from BR-I genotype encapsulated in chitosan nanoparticles; L+Nano, chickens vaccinated with a live attenuated IBV vaccine strain H120 followed by vaccination with IBV-CS; Mass, Massachusetts; Nano, chickens vaccinated only with IBV-CS; NC, non-vaccinated and non-challenged chickens; nm, nanometers; NV, non-vaccinated and challenged chickens; PDI, polydispersity index; SEM, scanning electron microscope; SPF, specific pathogen-free; TEM, transmission electron microscope; TPP, sodium tripolyphosphate.

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Inactivated IBV vaccines have been also routinely used in layer/breeder type chickens; however, poor mucosal immune responses have been induced, especially those mediated by IgA antibodies and cytotoxic T-lymphocytes (CD8+) [3,9,10]. High antigen levels and multiple immunizations are usually required for inactivated IBV vaccines to elicit strong immune responses. Moreover, local injuries are frequently found when it is used by the recommended administration routes (intra-muscular or subcutaneous) [8].

Currently, despite these disadvantages, live attenuated and inactivated IBV vaccines comprise the majority of commercial-available approaches for Infectious Bronchitis (IB) prevention [1,3,8]. Thus, new formulations of IB vaccines are widely sought [8]. In this context, chitosan nanoparticles have been used as both vaccine delivery systems and mucosal adjuvants [11,12].

In addition, few studies have demonstrated so far that chitosan particles carrying virus particles or viral antigens are efficient for inducing mucosal immune protection against avian respiratory pathogens [13–15]. As IBV initially invades and replicates in the epithelia of the respiratory mucosa [3], the use of a vaccine that is able to induce IBV-specific antibodies and cell-mediated immune (CMI) responses at the primary site of viral replication, can allow for a more effective protection against IBV infection and can also prevent the systemic dissemination of this infection and the development of more severe lesions [10,16,17].

The aim of this study was to develop and evaluate the efficacy of an inactivated vaccine formulated with a BR-I genotype strain of IBV encapsulated in chitosan nanoparticles (IBV-CS) administered by mucosal route in chickens. The antibody and CMI responses elicited by this vaccine and the protective immunity were determined in vaccinated chickens.

2. Materials and methods

2.1. Ethics statement

All procedures with experimental chickens were approved by the Animal Ethics Committee of Universidade Estadual Paulista (Protocol Number: 010140/14) in accordance with ethical principles and guidelines of animal experimentation adopted by Brazilian College of Experimentation.

2.2. Virus

A Brazilian variant strain of IBV (IBV/Brazil/PR05; NCBI accession n° GQ169242) was used in this study. The strain was propagated and titrated in 10-day-old specific pathogen-free (SPF) embryonated chicken eggs [18] and an infective titer of $10^{8.285}$ Embryo Infectious Dose (EID_{50})/ml of the virus was obtained in allantoic fluid (AF).

The infected AF was treated with beta-propiolactone (BPL) [19] to inactivate the virus (Supplementary data – 1). It was stored at −70 °C until processing.

2.3. Optimization of method to produce IBV-CS nanoparticles

Chitosan (Medium weight molecular; 75–85% deacetylation – Sigma-Aldrich, St. Louis, MO, USA) was dissolved in a 3% acetic acid solution (60.05 M – Sigma-Aldrich, St. Louis, MO, USA). The chitosan solution and sodium tripolyphosphate (TPP – Sigma-Aldrich St. Louis, MO, USA) were dissolved in ultrapure water for a final stock solution of 0.2% (w/v, 2 mg/ml). Different chitosan concentrations and TPP were tested. Fixed volumes were used for chitosan (5 ml) and AF (600 μL), while variable amounts of TPP were used (Table 1).

Nanoparticle size and polydispersity index (PDI) were measured on the ZetaSizer Nano ZS90 particle analyzer (Nano Series, Malvern Instruments Ltd, Worcestershire, UK). The encapsulation efficiency was assessed by protein quantification using Bradford technique.

2.4. Production of IBV-CS vaccine

The IBV-CS vaccine given to chickens was produced by ionic gelation method [14]. Briefly, 600 μL of the infected AF was added drop wise in 5 ml of 0.05% chitosan pH 4.5 at maximum stirring. Then, 1 ml of 0.1% TPP was added drop wise in the solution under magnetic stirring and incubated for 10 minutes (min) at room temperature.

IBV-CS was precipitated by centrifugation at 10,000*g* at 4 °C for 30 min and the supernatant was tested for non-encapsulated virus quantification. IBV-CS was re-suspended in 1 ml of ultrapure water. It was lyophilized and stored at 4 °C until use.

2.4.1. In vitro characteristics of IBV-CS

Morphological characteristics of IBV-CS were examined by transmission electron microscopy (TEM) and scanning electron microscope (SEM) (Supplementary data – 2) in the Multi-User Laboratory of Electron Microscopy (LMMC) at the University of São Paulo (USP – Ribeirão Preto).

The encapsulation efficiency was assessed by protein quantification using the Bradford technique. Size, PDI, and Zeta potential were measured on the ZetaSizer Nano ZS90 particle analyzer.

2.4.2. Citotoxicity of IBV-CS

The *in vitro* cytotoxicity of IBV-CS was evaluated in Chicken Embryo Fibroblast (CEF) culture [20] (Supplementary data – 3).

The viability of non-treated cells (control) was set at 100%, and the relative cell viability treated with IBV-CS was calculated using the following equation: [A]test/[A]control × 100.

2.5. Efficacy of IBV-CS vaccine

102 1-day-old SPF chickens were randomly divided into four groups. These groups were housed in separated positive pressure isolators. On the first day of age, the chicks of L + Nano group (n =

Table 1
Different conditions of preparation for optimization of nanoparticles.

Formulation	Chitosan concentration – 5 ml (%)	TPP concentration (%)	TPP volume (ml)	Viral encapsulation (%)	RT-PCR	Nanoparticles size (nanometers)	PDI
1	0.2	0.2	1	80	✔	399	0.437
2	0.2	0.1	1	84	✔	402	0.481
3	0.1	0.1	1	80	✔	388	0.444
4	0.1	0.1	2	82	✔	339	0.398
5	0.05	0.05	2.5	75	✔	228	0.432
6	0.05	0.1	1	85	✔	256	0.307
7	0.05	0.1	2.5	75	✔	241	0.383
8	0.05	0.1	1.5	82	✔	293	0.342

PDI: Polydispersity index; ✔: Confirmation of viral encapsulation by the detection of the viral genomic RNA by RT-PCR.

30) were vaccinated with H120 commercial attenuated strain of IBV by oculo-nasal route (according to the manufacturer's recommendations). At 14 days of age, chickens from the L + Nano and Nano groups (n = 30 for each group) received 100 μL of IBV-CS (containing $10^{8.285}$ EID_{50} of the virus) by oculo-nasal route. At 31 days of age, the vaccinated groups (L + Nano and Nano groups), along with a third non-vaccinated group (NV group, n = 21) were challenged with $10^{4.5}$ EID_{50}/bird of IBVPR-05 strain by oculo-nasal route. A fourth group (NC group, n = 21) was mock immunized with 100 μL of chitosan-free nanoparticles with no virus at 14 days and 100 μL of DMEM culture medium at 31 days of age, both by oculo-nasal route. During the experiment, the chickens were monitored daily in regard to the presence of clinical changes and/or macroscopic lesions. Chickens from all experimental groups were euthanized and necropsied at 1, 5 and 11 days post-infection (dpi).

At 1, 5 and 11 dpi, tracheal and renal samples were immediately frozen in liquid nitrogen and kept at −70 °C until processing by RNA extraction. Lachrymal samples were also collected and kept at −20 °C until processing for quantification of anti-IBV IgA and IgG isotypes. Ciliostasis analysis, microscopic lesions and quantification of IBV viral load were performed using tracheas collected at 5 dpi. Kidneys at 5 dpi were also processed for quantification of IB viral loads and examination of microscopic lesions.

2.5.1. Evaluation of tracheal ciliostasis

Samples from the proximal, medial and distal regions of the trachea were evaluated for ciliostasis (nine tracheal rings per bird) at 5 dpi and ciliary activity was scored as recommended by Andrade et al. [21] and Darbyshire and Peters [22] (Supplementary data – 4). The protection score for each group was calculated according to the formula used by Jackwood et al. [23], and a score ≥50% was considered protected.

2.5.2. Microscopic pathological alterations

Tracheas and kidneys were routinely processed at 5 dpi and stained with hematoxylin and eosin (H&E) to histopathology examination. Absence of tissue damage was classified as 0, while mild, moderate and severe were classified as 1, 2, and 3 respectively [24].

2.5.3. RNA extraction

Total RNA extractions from the tracheas and kidneys were performed immediately using the QIAzol Lysis Reagent (Qiagen, Crawley, West Sussex, UK) followed by RNA purification using RNeasy mini kit (Qiagen, Crawley, West Sussex, UK) according to the manufacturer's instructions. Extracted RNA was quantified at 260 nm using a spectrophotometer (Nanodrop® ND 1000 – Thermo Fisher Scientific, Waltham, Massachusetts, USA). The RNA quality was analyzed on a 1% agarose gel or was checked with Agilent RNA 6000 Nano Kit (Agilent Technologies, South Queensferry, UK) in an Agilent 2100 Bioanalyzer instrument (Agilent Technologies, South Queensferry, UK) for determination of RIN (RNA Integrity Number). The RNA was stored at −70 °C until use for RT-qPCR.

2.5.4. Quantification of IBV replication

Absolute quantification of the viral RNA in trachea and kidney (5 dpi) was performed by quantitative real-time reverse transcription-PCR (RT-qPCR) using AgPath-IDTM One-Step RT-PCR kit (Ambion, Applied Biosystems, Foster City, California, US), primers and probes for amplification of 3′UTR region of IBV, as previously described by Chousalkar et al. [25]. The RT-qPCR reactions were carried on the Applied Biosystems 7500 Realtime PCR system (Applied Biosystems, Foster City, Califórnia, US). The reaction mixture and cycling are described in the Supplementary data (number 5). Cq (Cycle quantification) results were used to calculate the Log of RNA copies (Log10) using the linear equation from a standard curve.

2.5.5. Quantification of IFNγ gene expression

The relative quantification of IFNγ gene expression in trachea (1, 5 and 11 dpi) and kidney (5 and 11 dpi) were determined by RT-qPCR, as previously described by Okino et al. [26]. After extraction from trachea and kidney samples, the RNA was submitted to cDNA synthesis with the reverse transcription (RT) technique using Oligo DT (Invitrogen, Carlsbad, California, USA) and MMLV kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

RT-qPCR was performed using QuantiFast SYBR Green PCR kit (Qiagen, Crawley, West Sussex, UK). The oligonucleotides TOP2B and IFNγ were previously described by Okino et al. [26]. The oligonucleotide GAPDH (F: AGCTGAATGGGAAGCTTACTGG; R: GCAGGTCAGGTCAACAACAGAG) was designed using Primer3 [http://frodo.wi.mit.edu] software, spanning exons according to gene sequences from Ensembl [http://ensembl.org] (Supplementary tables – Table 1). All reactions were set up on the 7500 Real-Time PCR System (Applied Biosystems, Foster City, California, USA). The reaction mixture and cycling are described in the Supplementary data (number 6). The Cq values obtained for IFNγ gene were normalized to GAPDH and TOP2B reference genes. The relative expression of IFNγ gene was measured as fold change relative to mock infected birds (negative control group) [27].

2.5.6. Evaluation of mucosal anti-IBV antibody responses

The IgA and IgG anti-IBV antibodies levels were measured from lachrymal secretion samples by the sandwich-ELISA-concanavalin A (S-ELISA-ConA) technique following all procedures as reported previously by Bronzoni et al. [28]. For this, anti-chicken IgA and anti-chicken IgG peroxidase conjugates (Bethyl Laboratories, Montgomery, Texas, US) were used. The lachrymal samples were diluted in PBS (1:25) and the reagents used were 1.5 mg/mL of Concanavalin A, viral antigen (IBV/Brazil/PR05 strain) diluted at 1:2 and anti-chicken IgA (1:2000) or anti-chicken IgG (1:1000) peroxidase conjugates.

2.6. Statistical analysis

The comparisons of viral load and IgA and IgG antibodies levels between the experimental groups were performed using Kruskal-Wallis test followed by Dunn's test. Tukey's Multiple Comparison Test was used for the analysis of the levels of IFNγ gene expression and ciliostasis scores. Correlations between the immune response parameters and the tracheal pathology parameters were estimated using the Spearman method. In all tests the 95% confidence interval (CI) was adopted, and those values with descriptive levels below or equal to 0.05 were considered statistically significant. The data was analyzed using Prism v.6.0 software (GraphPad Software, Inc., La Jolla, CA).

3. Results

3.1. Optimization of the preparation of IBV-CS nanoparticles

The protocols used to optimize the preparation of chitosan nanoparticles are described in Table 1. One of the eight formulations tested was chosen to be the IBV-CS vaccine administered to chickens. According to the results, the formulation 6 generated a better encapsulation efficiency, nanoparticle size, and PDI and it was selected for the formulation of IBV-CS vaccine.

3.2. IBV-CS vaccine in vitro characteristics

The average encapsulation efficiency of nanoparticles given to chickens was 85%, the size of 286 nanometers (nm), the PDI of 0.311 and the Zeta potential of +19.9 mV. The nanoparticles were spherical in shape as indicated by the electron micrographs (Supplementary Fig. 1).

3.3. In vitro cytotoxicity and in vivo toxicity of IBV-CS vaccine

The cytotoxicity of the IBV-CS vaccine was tested on CEF. The CEF survival rate inoculated with IBV-CS vaccine was 88.1% ± 3.2 3, and no significant changes in cell morphology were observed in comparison to the control cells, demonstrating low cytotoxicity of this vaccine formulation.

In addition, no local or systemically clinical manifestation or macroscopic lesions were observed after administration of IBV-CS vaccine by oculo-nasal route.

3.4. Efficacy of IBV-CS vaccine

Two groups of chickens received different vaccine protocols with IBV-CS. One group was vaccinated with only one dose of IBV-CS vaccine (Nano group), and the other group was vaccinated with a priming dose with live attenuated Massachusetts vaccine followed by a secondary dose with an IBV-CS (L + Nano group). The pathological alterations in trachea and kidney, as well as the local immune responses were evaluated after challenge with a virulent IBV/PR05 strain, as described below.

3.4.1. Tracheal ciliostasis

Inhibition of the tracheal ciliary activity was measured at 5 dpi. Chickens from Nano and L + Nano groups were protected against challenge in terms of sustainment of tracheal ciliary activity, showing protection tracheal ciliary scores of 65% and 66.7%, respectively. On the contrary, the non-vaccinated and challenged chickens (NV group) showed a protection tracheal ciliary score of 25% and were not considered protected against the challenge. The non-vaccinated and non-challenged chickens (NC group) showed full integrity of ciliary activity.

In addition, the Nano and L + Nano groups demonstrated lower tracheal ciliostasis scores than the NV group ($P \leq 0.05$ – Fig. 1A); however, all the three challenged groups had higher tracheal ciliostasis scores when compared to NC group ($P \leq 0.05$).

3.4.2. Viral loads

IBV-specific RT-qPCR was used for monitoring the presence of IBV genomes in trachea and kidney samples at 5 dpi (Fig. 1B). Vaccinated chickens from the Nano and L + Nano groups showed significantly lower viral loads ($P \leq 0.05$) in the trachea and kidney compared to NV group. In addition, no IBV RNA copies were detected in kidney samples of chickens from the Nano group, while IBV RNA was detected in lower number of copies from 2 out of 10 kidney samples collected from the L + Nano group. No IBV RNA copy was detected in the kidney samples from the NC group.

3.4.3. Microscopic pathological alterations

The microscopic alterations in trachea and kidney were evaluated at 5 dpi (Table 2). Approximately 70% of chickens from the

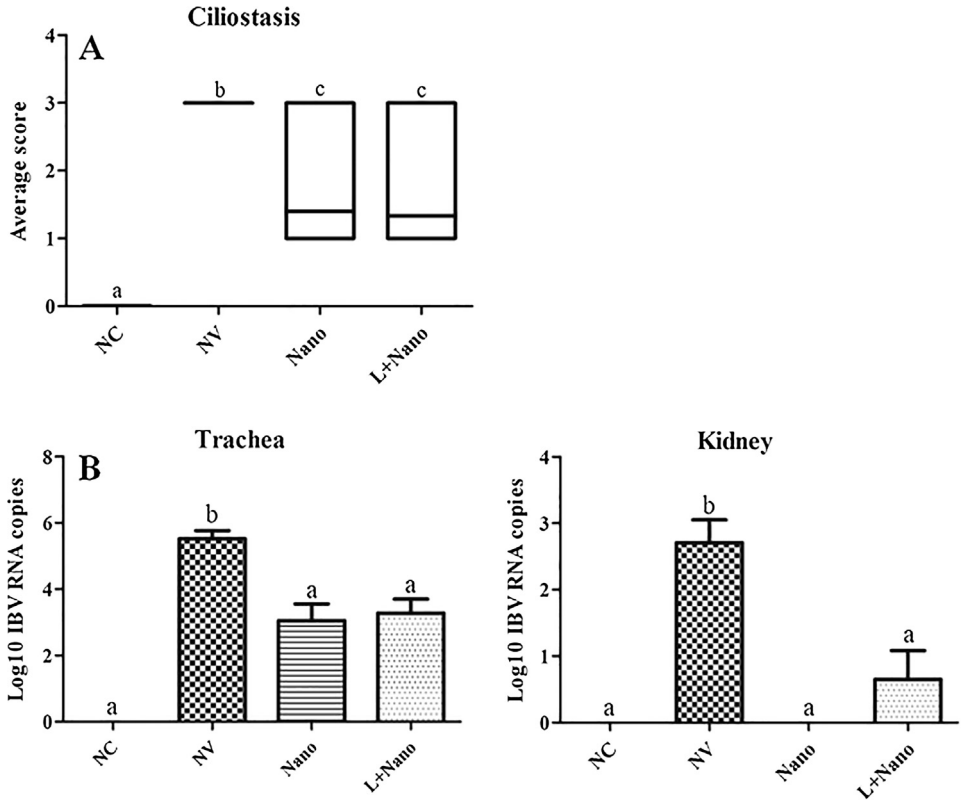

Fig. 1. Pathological alterations in chickens from the NC, NV, Nano and L + Nano groups at 5 dpi. (A) Mean ciliostasis score in the trachea and (B) viral loads measured as Log10 of IBV RNA copy numbers by real time RT-qPCR in trachea and kidney from 7 to 10 chickens of each group. Chickens from Nano and L + Nano groups were vaccinated with IBV-CS (oculo-nasal route) associated or not with a live attenuated Massachusetts IBV vaccine, respectively, and challenged with $10^{4.5}$ EID_{50}/bird of IBVPR-05 strain. Comparison between groups was performed and significant differences are represented by different letters for each group ($P \leq 0.05$). NC: non-vaccinated and non-challenged chickens; NV: non-vaccinated and challenge chickens; Nano: chickens vaccinated with IBV-CS (at 14 days of age); L + Nano: chickens vaccinated with live attenuated vaccine (at 1 day of age) followed by vaccination with IBV-CS (at 14 days of age); dpi: days post-infection.

Table 2
Histopathology developed after challenge with IBVPR05 strain in trachea and kidney of chickens from the NC, NV, Nano and L + Nano groups.

Groups	Histopathology scores							
	Trachea				Kidney			
	0	1	2	3	0	1	2	3
NC	7/7*	–	–	–	7/7	–	–	–
NV	–	–	1/7	6/7	–	3/7	3/7	1/7
Nano	2/10	4/10	4/10	–	4/10	3/10	3/10	–
L + Nano	5/10	2/10	3/10	–	2/10	4/10	4/10	–

The mean score per chicken was evaluated; *N° of Chickens presenting lesion score/Total N° tested; NC: negative control group; NV: non-vaccinated and challenged group; Nano: vaccinated with IBV-CS group; L + Nano: vaccinated with a live attenuated vaccine followed by vaccination with IBV-CS group.

Nano and L + Nano groups showed no relevant microscopic lesions or had mild lesions in the trachea and kidney, which ranged from histopathology score of 0–1. In contrast, all chickens from the NV group showed more severe microscopic lesions ranging from moderate to severe scores (2–3) in trachea, and from mild to severe scores in kidney (1–3). The major histological lesions in the trachea were epithelial cell losses, ciliary loss, lymphoid infiltration, congestion, and reduction of mucous glands. In the kidney the more characteristic lesions were degeneration of tubular cubic cells, tubular necrosis, perivascular inflammation with predominance of mononuclear cells, mononuclear inflammation, and vascular congestion (Supplementary Fig. 2). No microscopic alterations in tracheal and kidney samples were observed in chickens from the NC group.

3.4.4. Cell-mediated immune (CMI) responses

The CMI responses were evaluated by measuring the expression of INFγ gene in trachea (1, 5 and 11 dpi) and kidney (5 and 11 dpi) samples and the results are presented in the Fig. 2A. Chickens from the Nano group demonstrated an early and up-regulated expression of INFγ gene in the trachea at 1 dpi, differing significantly ($P \leq 0.05$) from the other experimental groups (NC, NV and L + Nano groups). At 5 dpi, birds from the L + Nano group showed a greater increase in the expression of IFNγ gene in the trachea, differing statistically from the NC, NV and Nano groups ($P \leq 0.05$). The expression of IFNγ gene dropped markedly to basal levels in the trachea samples from all challenged groups at 11 dpi ($P \geq 0.05$). No relevant changes were observed in the expression of IFNγ gene in kidney samples, either at 5 or 11 dpi ($P \geq 0.05$).

In addition, the levels of IFNγ gene expression in the trachea did not showed significant correlation with pathological alterations in trachea at 5 dpi (ciliostasis and viral load) ($P \geq 0.05$; Supplementary tables – Table 2).

3.4.5. Mucosal antibody immune-responses

The IBV-specific IgA and IgG antibody titers were measured as S/P levels in lachrymal secretion samples using a S-ELISA-ConA technique (Fig. 2B). Chickens from the Nano group developed higher levels of anti-IBV IgA antibodies in the mucosa at 1 dpi that were statistically different from the NV group ($P \leq 0.05$). At 5 dpi, chickens from the Nano and L + Nano groups increased their levels of IgA anti-IBV antibodies that were totally or partially different from those exhibited by NV and NC groups ($P \leq 0.05$). At 11 dpi, IgA anti-IBV levels increased markedly in chickens from the NV group and declined slightly in the Nano and L + Nano groups, differing significantly from the NC group ($P \leq 0.05$).

Chickens from the L + Nano group produced higher anti-IBV IgG levels than chickens from the NC and NV groups ($P \leq 0.05$) at 1 dpi. A further increase in the levels of IgG anti-IBV antibodies was detected at 5 dpi in chickens from Nano and L + Nano groups, that differed partially or totally from the NV and NC groups ($P \leq 0.05$). All challenged groups vaccinated or not against IBV showed high levels of IgG anti-IBV antibodies at 11 dpi, which were partially or totally different from the NC group ($P \leq 0.05$).

Additionally, significant negative correlations were observed between the IgA and IgG anti-IBV antibody levels detected in the lachrymal secretion and the pathological alterations in trachea at 5 dpi ($P \leq 0.05$; Supplementary tables – Table 2). Thus, correlation coefficients of $r = -0.4809$ for ciliostasis and $r = -0.4994$ for viral load, or $r = -0.4052$ for ciliostasis and $r = -0.4421$ for viral load were found for IgA and IgG antibodies, respectively.

4. Discussion

Vaccination is one of the most effective approaches to afford effective protection against IBV infection [3,8]. The World Organization for Animal Health (OIE – 2013) recommends that the epidemiology of distribution of IBV serotypes should influence the choice of a vaccine strain-specific for use in each country or geographic region [8,35]. However, the use of an inactivated vaccine for each region would be safer, since it contains inactivated virus and does not offer risks of genetic recombination or mutation that causes the emergence of new IBV genotypes [8,29]. The IBV-CS formulated in this study is an interesting model for inactivated vaccine that could be used for other IBV variants, since it was formulated with an inactivated Brazilian IBV genotype strain which provided an effective protection against challenge and presented a set of advantages, such as easy formulation and administration (mucosal route), low-cost, and non-toxicity.

The ionic gelation method used to formulate IBV-CS did not use harmful organic solvents, heat, or vigorous agitation that usually damage protein antigens present in the virus suspension [15]. The shape, surface charge, and size of the nanoparticles are important parameters during the interaction between antigens and the antigen presenting cells (APCs) [12]. They can be optimized by using different concentrations of chitosan and TPP, pH, and chitosan molecular weight [30,31]. IBV-CS comprised particles <286 nm with zeta potential of +19.9 mV and spherical shape. It is well known that cationic particles <500 nm help to increase antigen contact area with the mucous membranes and are more efficient to induce antigen uptake by mucosal APCs, mainly the dendritic cells, while spherical particles are more effective in inducing antibody responses [12,32].

The two vaccination regimes (Nano and L + Nano groups) were investigated in this study and resulted in an effective protection against challenge with homologous and virulent IBV strain from BR-I genotype. These findings were based on the significant reduction in tracheal ciliostasis, low viral loads, and histological lesions in the trachea and kidney of IBV-CS vaccinated chickens when compared to the NV group.

Interestingly, the chickens that received only the IBV-CS vaccine (Nano group) reached a similar protection status compared to those birds that received two anti-IBV vaccines (L + Nano). However, the kinetic of antibody and CMI responses were different

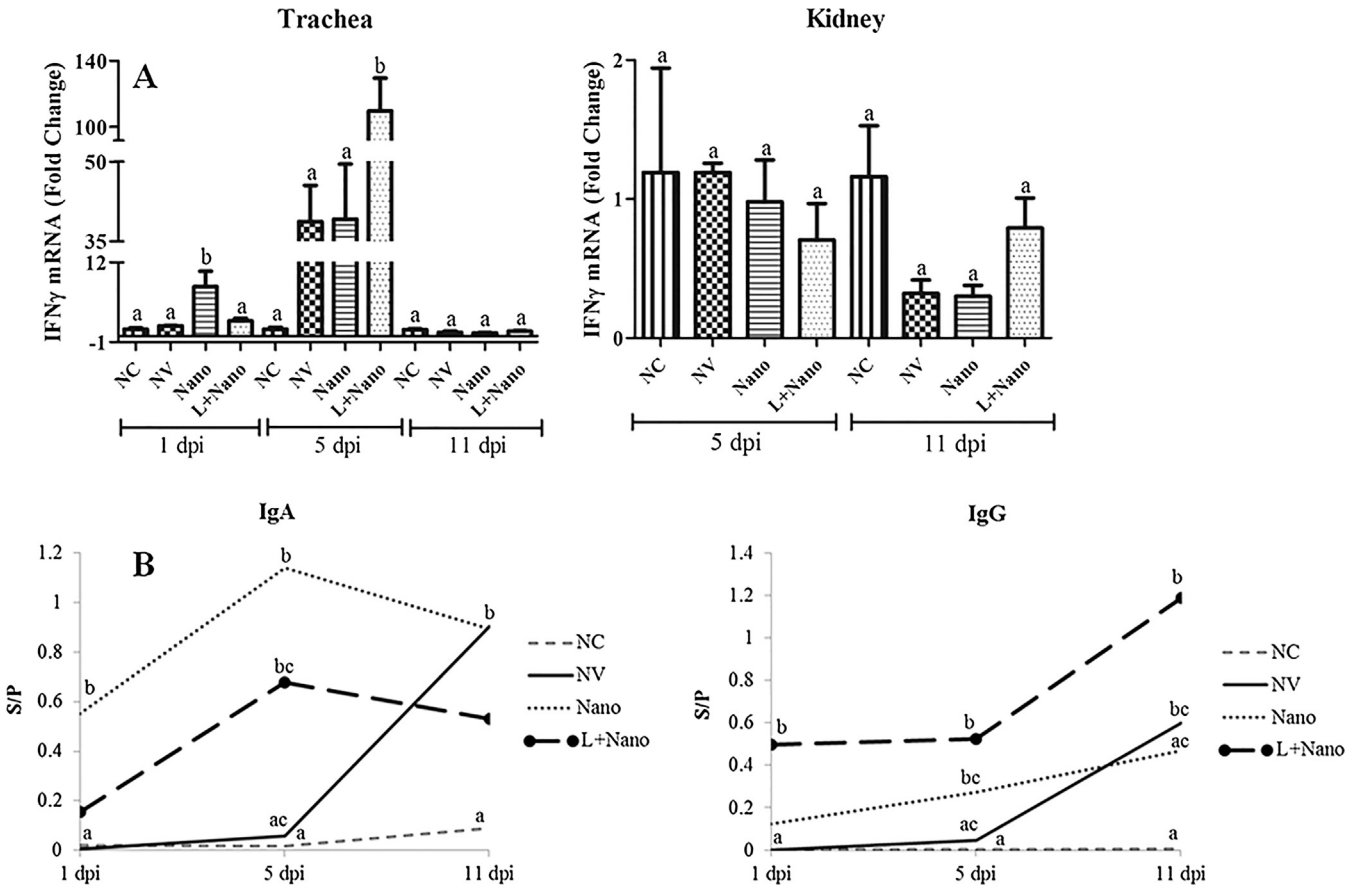

Fig. 2. Evaluation of the cell-mediated (CMI) and humoral (RIH) immune responses in chickens from the NC, NV, Nano and L + Nano groups. (A) CMI responses measured as mean fold changes in the mRNA expression of IFNγ in trachea (1, 5 and 11 dpi) and kidney (5 and 11 dpi). (B) RIH measured as mean Sample/Positive (S/P) values for the levels of anti-IBV IgA and IgG antibodies in lachrymal secretion at 1, 5 and 11 dpi. Chickens from Nano group were vaccinated only with IBV-CS (by oculo-nasal route; at 14 days of age); while chickens from L + Nano were vaccinated with live attenuated Massachusetts IBV (by oculo-nasal route at 1 day of age) and IBV-CS (by oculo-nasal route; at 14 days of age) vaccines. Chickens from NV, Nano and L + Nano groups were challenged with $10^{4.5}$ EID_{50}/bird of IBVPR-05 strain at 31 days of age. Comparison between groups was performed and significant differences are represented by different letters for each group ($P \leq 0.05$). NC: non-vaccinated and non-challenged chickens; NV: non-vaccinated and challenge chickens; Nano: chickens vaccinated with IBV-CS; L + Nano: chickens vaccinated with live attenuated vaccine followed by vaccination with IBV-CS; dpi: days post-infection.

between these two groups of immunized chickens. We assumed that these differences may have been influenced by factors such as the vaccination regime and the type of vaccine used (live attenuated and/or inactivated), age of vaccination, and the major cell type involved in antigen recognition [16,32].

Chitosan nanoparticles have been used as adjuvant for mucosal vaccines due to their ability to increase both cellular and humoral immune responses and to elicit a balanced Th1/Th2 response, specially at mucosal sites [14,15,33,34]. Indeed, our study demonstrated that both vaccinated groups with IBV-CS (Nano and L + Nano groups) developed at mucosal sites of upper respiratory tract a marked memory of anti-IBV IgA and IgG antibodies production, as well as a CMI memory responses characterized by an early increase in IFNγ gene expression in the trachea, when compared with chickens from the NV group. Thus, mucosal immune responses at respiratory tract mediated by IgA and IgG antibodies and CMI responses were effectively elicited by IBV-CS vaccine. These responses are crucial to restrict IBV replication and to protect the trachea against the development of ciliostasis, microscopic lesions, and viral replication, as reported previously [10,16,17]. In fact, in this study the mucosal IgA and IgG anti-IBV antibody responses demonstrated a significant negative correlation with virus clearance and reduction of pathological alterations in the trachea, and might act as an efficient protective mechanism against IBV infection at the mucosal site [17].

In conclusion, IBV-CS induced stronger mucosal immune responses with early and marked induction of IFNγ gene expression and production of IgA and IgG anti-IBV antibodies, when it was used alone or associated with a live attenuated vaccine. Additionally, the IBV-CS vaccine conferred effective protection against challenge with a virulent IBV strain from BR-I genotype. This study has also provided promising results for the further development of mucosal inactivated vaccines with other IBV strains, or even other avian viruses, encapsulated in chitosan nanoparticles.

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Conflict of interest

The authors declare that there is no conflict or financial interests.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2018.03.065.

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