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

Priscila Diniz Lopes [a,*], Cintia Hiromi Okino [b], Filipe Santos Fernando [a], Caren Pavani [a], Viviane Mariguela Casagrande [a], Renata F.V. Lopez [c], Maria de Fátima Silva Montassier [a], Helio José Montassier [a]

[a] Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista "Júlio de Mesquita Filho" (UNESP), Campus de Jaboticabal, Via de Acesso Prof. Paulo Donato Castelane, S/N – Vila Industrial, Jaboticabal, São Paulo CEP: 14884-900, Brazil
[b] Embrapa Pecuária Sudeste, Empresa Brasileira de Pesquisa Agropecuária (Embrapa), Rodovia Washington Luiz, Km 234 s/n – Fazenda Canchim, São Carlos, São Paulo CEP: 13560-970, Brazil
[c] Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo (USP), Avenida Bandeirantes, 3.900 – Monte Alegre, Ribeirão Preto, São Paulo CEP: 14040-900, Brazil

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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.

* Corresponding author.
*E-mail address:* prisciladiniz_vet@yahoo.com.br (P.D. Lopes).

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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.

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