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Communication

[*Comunicação*]

Construction and characterization of a recombinant vaccine encoding the nucleocapsid protein gene of avian infectious bronchitis virus

[*Construção e caracterização de uma vacina recombinante codificando o gene da proteina do nucleocapsideo do virus da bronquite infecciosa das galinhas*]

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The infectious bronchitis (IB) is a highly contagious poultry disease caused by infectious bronchitis virus (IBV), a member of *Coronaviridae* family, which causes significant economic impact on the worldwide poultry industry. IBV is an enveloped virus which has a genome of single stranded RNA positive-sense of 27.6kb, encoding four major structural proteins, known as spike (S), nucleocapsid (N), membrane (M) and envelope (E) (Cavanagh, 2007). The IBV N, a major structural protein, is produced abundantly during infection. It is highly conserved, sharing 94–99% identity among various strains, readily inducing antibody, as well as immunity by cytotoxic T lymphocytes (Seo *et al.*, 1997). In this study, a replication-defective human adenovirus vector expressing N gene of IBV was constructed and evaluated in SPF chickens for the immune responses and protective efficiency against IBV challenge.

The study was conducted in accordance to Brazilian and International Standards on animal welfare being evaluated and approved by Embrapa's Swine and Poultry Ethics Committee on Animal Utilization (CEUA) under protocol number 010/2010. Initially the reference IBV strain Massachusetts/41 (M41) was propagated at 37ºC in the allantoic cavities of 9-day-old Specific Pathogen Free (SPF) embryonated chicken eggs (Vallo Biomedia). Total RNA was isolated from IBV infected allantoic fluid using Trizol reagent (Invitrogen®) according to the manufacturer's instructions. Complete N gene (1233 bp accession number GQ504724.1) was amplified by RT-PCR using the following primers: 5'–CCATCGATGTCATGGCAAGCGGTAAGGCA–3' (forward) and 5'–TCTAGATCAAAGTTCATTCTCTCCTAGGGCTG– 3' (reverse) (*Cla*I and *Xba*I restriction sites underlined). The PCR products were cloned into TOPO vector (Invitrogen®) and all plasmids were sequenced to confirm gene insertion. The N gene cloned was removed by digestion with *Cla*I and *Xba*I, purified gel and ligated into similarly digested pAd5-Blue adenovirus vector (provided by Dr. Marvin J. Grubman, Plum Island Animal Disease Center), under transcriptional control of the human cytomegalovirus (CMV) early promoter. The resulting adenoviral plasmid (pAd5_N) was linearized with *Pac*I, purified by ethanol precipitation, and used to transfect HEK-293A cells. The cells were transfected in 6-well plates with 2µg of linearized plasmid DNA using Polyfect Transfection Reagent (Qiagen®) following the manufacturer's instructions. Wells were examined daily until viral plaque formation. Plaques were individually picked and used to infect a 150mm flask of HEK-293A cells as described by Moraes *et al.* (2001) to generate recombinants adenovirus, designated rAd5_N. The expression of N protein was demonstrated in HEK-293A by immunoperoxidase monolayer

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assay. Briefly, 48 hours after rAd5_N infection cells were washed with PBS-T and fixed with 4% paraformaldehyde solution for 30min at room temperature, followed by incubation with mouse monoclonal antibody anti-N of IBV (Prionics®) for 1h at 37°C. The cells were rinsed and incubated with anti-mouse conjugated with peroxidase (Sigma®) at 37°C for 30 minutes. The cells were then washed with PBS-T, incubated with AEC (3-amino-9-ethyl-carbazole peroxide substrate) solution for 10min at room temperature, washed again and visualized in an inverted microscope. The results indicated that the cells infected by rAd5-N shown intense red staining, due to the presence of the expressed protein (Figure 1).

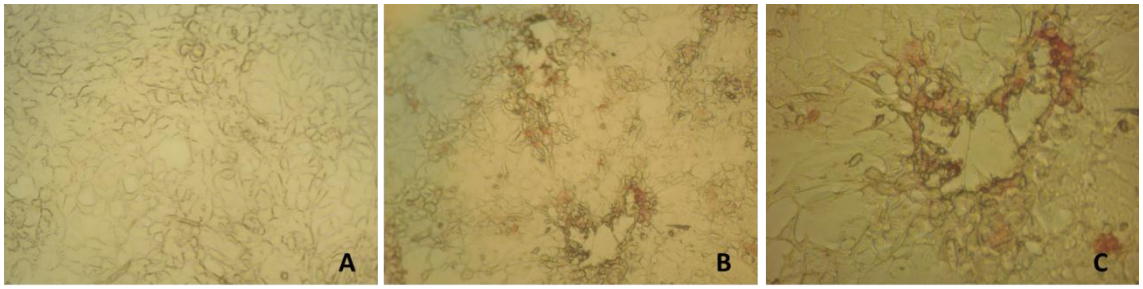

Figure 1. Immunoperoxidase monolayer assay of HEK293A cells infected with rAd5_N using mouse monoclonal antibody anti-N of IBV. A) Cell negative control, 20X magnification; B) rAd5_N inoculated cells, 10X magnification; C) rAd5_N inoculated cells, 40X magnification.

For large-scale preparation, the recombinant adenovirus was propagated in multiple 150mm flasks of HEK-293A cells and purified by sedimentation through a cesium chloride gradient (23000 x *g*, 15°C, 20h). Virus titers were determined in HEK-293A cells and expressed as $TCID_{50}$ (tissue culture infectious dose) and the final product was used in immunization strategies of SPF chickens at a dose of $10^7 TCID_{50}$/0.1mL. The chickens were housed in positive-pressure isolators and divided randomly into seven groups (*n*= 10), which were vaccinated with Ad5_N, Ad5 or H120 (live attenuated commercial vaccine, strain Massachusetts) and challenged through the nasal-ocular route according to the strategies summarized in Table 1.

Table 1. Immunization strategies and serum sample collection

Group	Vaccination		IBV Challenge		Serum samples collection
	1 day old	14 days	21 dpv*	35 dpv*	Age of birds (days)
G1 – negative control	-	-	-	-	1
G2 – positive control	-	-	M41	-	26
G3	Ad5_N	-	M41	-	21;26
G4	Ad5_N	Ad5_N	-	M41	7;14;21;40
G5	H120	-	M41	-	21;26
G6	H120	Ad5_N	-	M41	7;14;21;40
G7	Empty Ad5	-	-	-	26

*dpv: days post vaccination

Specific antibodies for IBV were measured by enzyme-linked immunoassay kit (IDEXX IBV antibody ELISA kit), according to the manufacturer's instructions. Pre-vaccination sera were collected for the control chickens. Blood was also collected before and after booster vaccination as well as after the challenge. Chickens were challenged at 21 (G2, G3, G5) or 35 (G4 and G6) days post vaccination (dpv), with 10^3 EID_{50} of IBV M41 strain, through a nasal-ocular route and examined daily for the clinical signs of BI. Five days after the challenge, birds were euthanized and immediately necropsied, followed by collection of trachea and

lung tissues for histopathological analysis. Trachea samples were carefully removed, and transverse sections were made resulting in 3 portions (proximal, medial and distal), making a total of 9 rings from each bird. Each trachea portion was individually examined by microscopy for evidence of ciliary activity and the scores were determined according to previously described recommendations with some modifications (Darbyshire, 1980): zero, all cilia beating; 1, 75% beating; 2, 50% beating; 3, 25% beating; and 4, none beating (100% ciliostasis). The final score zero, 1 or 2 was deemed to have been protected by the vaccine against the challenge strain. Total RNA extractions from the proximal third from tracheal samples of experimentally infected chickens were performed using Trizol Reagent (Invitrogen) followed by purification using RNeasy Mini Kit (Qiagen). The real-time PCR using the SYBR Green I marker was used for the relative quantification of mRNA to IFNγ as described by Okino *et al*. (2014). The relative expression of the IFNγ gene in the tracheal samples of infected chicks was quantified as the fold change relative to the mock infected group and the gene expression from each sample were standardized using the Cq value of the GAPDH mRNA for the same sample, according to the recommendations (Pfaffl, 2001). Absolute quantification of viral load of IBV from total extracted RNA, was performed using AgPath ID One-step RT-PCR kit (Ambion®) and primers and probe previously described (Chousalkar *et al*., 2009) for 3´UTR of IBV. The standard curve was constructed using RNA transcripted from plasmidial DNA containing 3´UTR fragment of IBV.

The recombinant vaccine Ad5_N do not induce detectable antibodies to IBV in chickens three weeks after inoculation and there was no specific antibody response in the group of chickens receiving two doses of recombinant Ad5_N. On the other hand, there was specific antibody response elicited by commercial vaccine since the 7th-day after first inoculation (in group 6). Tracheal fragments examined by optical microscopy showed ciliary loss, degeneration and necrosis of epithelial cells, glandular degeneration, inflammatory cell presence and epithelial hyperplasia. No pathologic alterations of trachea were observed in the uninfected group (G1) and in the empty vector (G7), without IBV gene. In addition, chickens vaccinated with recombinant vaccine showed similar score of lesions to the challenge control group (M41 only), indicating that the vaccine seemed to be ineffective. Meanwhile, in G5, where the birds were vaccinated with the commercial vaccine, the scores of lesions were similar to those observed in the negative control group, suggesting that in the experimental conditions used in this study, the commercial vaccine was able to induce protection against to homologous IBV challenge. In lung fragments analyzed, it was observed that few birds in groups 3 and 4 (5/20) had mild heterophils infiltration of the bronchial mucosa, stressing that the election of tissue for this analysis should be the trachea. In ciliostasis evaluation, all chickens that received one or two doses of Ad5_N vaccine shown ciliostasis score 3, indicating that those cilia were no longer functional and, as consequence, birds were not protected against the challenge.

The results of qPCR to evaluate the relative expression of IFNγ in tracheal samples at 5 days post IBV challenge showed that the mean level of IFNγ was not significantly higher in chickens inoculated with recombinant Ad5_N vaccine as compared to that of the control group (data not shown). Similarly, the quantification of viral load in tracheal samples at 5 days after the challenge showed that the vaccine rAd5_N did not provide protection against virulent challenge, as 100% of chickens in G3 and G4 had virus present in trachea (data not shown).

Studies performed in order to increase the efficiency of immunization procedures to control IBV infection had demonstrated that the utilization of such type of vaccines are able to protect birds from IBV field variants. Such studies employed the recombinant vaccine approach in order to develop a vaccine against IBV and the results have been variable, reporting protection induced by S1 gene, with partial or complete protection in vaccinated chickens (Wang *et al.*, 2009; Zhang *et al.*, 2012). In the present study, a replication defective human Ad5 vector expressing the complete N gene of IBV was constructed and the immune response was analyzed in SPF chickens. The recombinant vaccine provided no significant protection against the IBV challenge. Although the N protein is recognized as immunogenic, the recombinant vaccine Ad5-N does not cause

detectable positive serological responses by ELISA in vaccinated chickens. Our observations are in agreement with some previous studies that report the development of experimental DNA vaccines, where despite the recognized immunogenic, the N protein did not result in a detectable proliferative response to IBV in vaccinated chickens (Boots *et al*, 1992) and vaccine provided no significant protection against the challenge (Ignjatovic, *et al.*, 1994). However, the use of N of IBV has been demonstrated in other studies using DNA vaccines, with protection rates ranging between 50 and 86% (Tang *et al.*, 2008; Yan *et al.*, 2013).

The use of the N gene has been widely demonstrated in studies using DNA vaccines, with protection rates ranging between 50 and 86% (Tang *et al.*, 2008; Yan *et al.*, 2013). Our observations are in agreement with some previous studies that report the development of experimental DNA vaccines, where despite the recognized immunogenic, the N protein did not result in a detectable proliferative response to IBV in vaccinated chickens and vaccine provided no significant protection against the challenge (Ignjatovic, *et al.*, 1994).

In conclusion, our results indicate that construction and *in vivo* evaluation of a recombinant adenovirus expressing N gene of IBV was ineffective because it did not induce sufficient protection to vaccinated chickens challenged with IBV. Further studies should be conducted in order to construct and evaluate recombinant adenovirus expressing other important IBV genes, as S1 gene, with different immunization protocols and combinations, in order to develop a better alternative vaccine against the IB.

Keywords: infectious bronchitis virus, nucleocapsid protein, adenovirus vector, immune responses

RESUMO

A vacinação é a forma mais utilizada para prevenir a bronquite infecciosa causada pelo vírus da bronquite infecciosa das galinhas (IBV). Contudo, as vacinas convencionais são incapazes de diferenciar aves infectadas de vacinadas. No presente trabalho foi construído, caracterizado, e avaliado como candidato vacinal, um adenovírus recombinante expressando o gene N do IBV. O gene N foi clonado em um adenovírus humano tipo 5 defectivo e transfectado para as células HEK-293A para gerar rAd5_N. Após o vetor ser obtido como esperado e a confirmação da expressão da proteína N em HEK-293ª, foi realizada inoculação pela via oculo-nasal na dose de 10^7TCID_{50}/0,1mL para imunização de galinhas livres de patógenos específicos (SPF). A resposta imunológica do Ad5_N e a proteção contra o desafio ao IBV foram avaliadas e comparadas com uma vacina viva comercial. Não foram detectados anticorpos anti-IBV em aves vacinadas com o Ad5_N. A vacina comercial induziu anticorpos detectáveis a partir do 7º dia pós-vacinal. Em aves vacinadas com o Ad5_N não houve aumento na expressão de IFNγ. Neste estudo, o rAd5_N obtido não conferiu proteção contra desafio com IBV-M41. Os resultados indicam a necessidade de avaliar adenovírus recombinantes expressando outros genes do IBV.

Palavras-chave: vírus da bronquite infecciosa, nucleoproteína, adenovírus, resposta imune

REFERENCES

CAVANAGH, D. Coronavirus avian infectious bronchitis virus. *Vet. Res*., v.38, p.281-297, 2007.

CHOUSALKAR, K.K.; CHEETHAM, B.F.; ROBERTS, J.R. LNA probe-based real-time RT-PCR for the detection of infectious bronchitis virus from the oviduct of unvaccinated and vaccinated laying hens. *J. Virol. Methods*, v.155, p.67-71, 2009.

DARBYSHIRE, J. H. Assessment of cross-immunity in chickens to strains of avian infectious bronchitis virus using tracheal organ cultures. *Avian Pathology*, v.9, p.179-184, 1980.

IGNJATOVIC, J.; GALLI, L. The S1 glycoprotein but not the N or M proteins of avian infectious bronchitis virus induces protection in vaccinated chickens. *Arch. Virol.*, v.138, p.117-134, 1994.

MORAES, M.P.; MAYR, G.A.; GRUBMAN, M.J. pAd5-Blue: direct ligation system for engineering recombinant adenovirus constructs. *BioTechniques*, v.31, p.1050-1056, 2001.

OKINO, C.H.; SANTOS, I.L.; FERNANDO, F.S.; ALESSI, A.C. *et al*. Inflammatory and cell-mediated immune responses in the respiratory tract of chickens to infection with avian infectious bronchitis virus. *Viral Immunol.*, v.27, p.383-391, 2014.

PFAFFL, M.W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.*, v.29, p.2002-2007, 2001.

SEO, S.H.; WANG, L.; SMITH, R.; COLLISSON, E.W. The carboxyl-terminal120-residue polypeptide of infectious bronchitis virus nucleocapsid induces cytotoxic t lymphocytes and protects chickens from acute infection. *J. Virol.*, v.71, p.7889-7894, 1997.

TANG, M.; WANG, H.; ZHOU, S.; TIAN, G. Enhancement of the immunogenicity of an infectious bronchitis virus DNA vaccine by a bicistronic plasmid encoding nucleocapsid protein and interleukin-2. *J. Virol. Methods*, v.149, p.42-48, 2008.

WANG, Y.F.; SUN, Y.K.; TIAN, Z.C.; SHI, X.M. *et al*. Protection of chickens against infectious bronchitis by a recombinant fowlpox virus co-expressing IBV-S1 and chicken IFN gamma. *Vaccine*, v.27, p.7046-7052, 2009.

YAN, F.; ZHAO, Y.; HU, Y.; QIU, J. *et al*. Protection of chickens against infectious bronchitis virus with a multivalent DNA vaccine and boosting with an inactivated vaccine. *J. Vet. Sci.*, v.14, p.53-60, 2013.

ZHANG, X.; WU, Y.; HUANG, Y.; LIU, X. Protection conferred by a recombinant Marek's disease virus that expresses the spike protein from infectious bronchitis virus in specific pathogen-free chicken. *Virol. J.*, v.9, p.85, 2012.