



Letter

An Effective Mannosylated Chitosan Nanoparticle DNA Vaccine for FMD Virus

VIROLOGICA SINICA(2012)27: 372-375 Received: 2012-07-05, Accepted: 2012-11-07

Dear Editor,

We are reporting you an effective DNA vaccine for FMD virus, and it was prepared using mannosylated chitosan (MC) nanoparticles to form the FMDV-pVAC-VP1-OmpA complex particles. These particles were characterized for their physical properties like morphology, size and charge prior evaluating the actual vaccine delivery effect of MC-nanoparticles. The immunological evaluation indicated that the 20 µg of DNA vaccine complexed with MC nano- particles was found optimum in inducing the immune response in G pigs as measured by FMDV specific neutralising antibodies and Th1/Th2 responses using micro SNT and ELISA, respectively.

Foot and mouth disease (FMD) is a highly contagious, economically important, office international des epizooties notifiable transboundary viral disease of cloven-hoofed animals caused by FMD virus, an *Aphthovirus*. The disease is associated with high morbidity and less mortality in endemic countries with international trade restrictions. In a country where test and slaughter policy is difficult to follow, vaccination is a viable strategy for controlling the disease^[5]. The drawbacks of current inactivated virus vaccines are live virus handling, short duration of immunity, improper inactivation of virus with possible vaccine related outbreaks and poor elicitation of cell mediated immune response (CMI)^[12]. Among the available alternative strategies, the DNA vaccine is gaining importance^[1]. DNA immunization elicits CMI, humoral immune responses (HIR), antigen-specific cytotoxic T lymphocytes (CTL) and neutralizing antibodies^[5]. However in the case of FMD, DNA vaccines have been reported to provide partial protection, warranting improvement in the current DNA vaccine strategies^[4]. In particular, the immune response induced by DNA

vaccines can be improved using suitable delivery systems^[8]. Recently, the nanoparticle based techniques have emerged as potential DNA delivery systems as they stabilize DNA and also provide adjuvant effect^[1,3]. In this regard, chitosan (C) nanoparticles are beneficial compared to non viral vectors as they are inert, less toxic, rapidly biodegradable, and biocompatible^[2,3,6]. In view of these, the present study was conceptualized to prepare and physically characterize the mannosylated chitosan (MC)^[7,10] nanoparticles complexed with an FMDV DNA vaccine construct, FMDV-pVAC-VP1-OmpA and optimise dose of FMDV construct for immunization in G pigs.

Initially, the water soluble chitosan was prepared by the *N*-acetylation method^[7], dried at 50°C and stored at room temperature. Chitosan was mannosylated and the MC-DNA vaccine complex was prepared^[10]. FMD seronegative G pigs of either sex, each weighing 400–500 g were used. Recombinant DNA construct pVAC-VP1-*OmpA*, carrying the VP1 gene of FMDV type ‘O’ upstream of the *Omp A* gene of *S. typhimurium* in pVacl vector, was harvested using *E. coli* DH5a bacterial cultures. The plasmid DNA was confirmed by digesting with *EcoR* V and *Bgl* II and agarose gel electrophoresis for the presence of VP1-*OmpA* ~1.7 kb gene in the DNA vaccine construct. The endotoxin free vaccine construct was bulk prepared for *in vivo* immunization using a PerfectPrep™ Endofree Maxi kit (5PRIME, Germany). The concentration and purity of the plasmid DNA was determined by agarose gel electrophoresis and spectrophotometry (nanodrop). Later, the particle size and zeta potential of the MC nanoparticles and DNA loaded MC nanoparticles was measured in a Malvern Master sizer 2000 (Malvern Instruments, UK), and the mannosylation of chitosan was analysed by high resolution ¹H NMR spectra in an

NMR spectroscope (400 MHz Bruker, Germany, Ultrashield TM plus). Likewise, the DNA binding efficiency of MC was calculated as the ratio between the bound DNA and the total DNA used in the preparation of nanoparticles. The MC-DNA complex formation was confirmed by 1.0% agarose gel electrophoresis.

FMDV serotype 'O' at passage 5 in BHK₂₁ cells was titrated^[21] and whole virus (146S) inactivated antigen was prepared^[14]. Further, the optimal dose of MC-DNA vaccine was assessed in five groups (Group I-V) of G pigs with a group size of 8 animals. These groups were respectively immunized intramuscularly with 2, 5, 10, 20 and 50 µg of DNA vaccine complexed with MC nano-particles and observed over a period of 30 days. The animals were bled on 0, 4, 10, 16, 22 and 30 day post immunization (dpi) and serum was collected and heat inactivated. These sera were monitored for the presence of FMDV O serotype specific virus neutralizing antibody by SNT^[14] and whole IgG, IgG1 and IgG2 response by respective indirect ELISAs^[4]. The working dilutions of antigen, positive serum, negative serum and conjugate were determined by chequerboard titration. The peroxidase conjugated goat anti-guinea pig IgG1 and IgG2 were used at 1:5000, respectively.

The ~5.4 kb plasmid DNA (pVac-VP1- OmpA) extracted from positive *E.coli* and the recombinant plasmid DNA were intact upon electrophoresis. Further, the digestion of pVac-VP1-OmpA with *EcoR* V and *Bgl* II released a 1.7 kb VP1-OmpA fragment and a 3.7 kb vector fragment. However, pVac-VP1- OmpA linearized with *EcoR* V showed a single band of 5.4 kb. The chitosan used was soluble in water. The morphology of MC nanoparticles and MC DNA under scanning electron microscope appeared spherical with some irregularities. The NMR spectroscopy also revealed distinctness in the MC as compared to the chitosan nanoparticles. The particle size of the MC and MCDNA nanoparticles were 60-300 and 80-500 nm, respectively. The zeta potential of MC and MC-DNA nanoparticles were +32 to +60 mV, and +22 and +50 mV, respectively. Further agarose gel electrophoresis was carried out to determine the DNA loading efficiency and formation MC-DNA complex. Accordingly, MC-DNA complex remained inside the gel, whereas, un-bound plasmid DNA migrated down the gel lane indicating that the DNA bound to MC-DNA complex. In case of pVac-VP1-OmpA complexed with un-mannosylated chitosan, an amount

of DNA was migrating into the gel probably due to separation of weakly bound DNA. The results demonstrated that MC complexed better with DNA than unmannosylated chitosan. The DNA loading efficiency (expressed as the ratio between the bound DNA and the total DNA used to prepare nanoparticles) was 0.81 (81%).

The dose optimization studies in G pigs indicated that the virus neutralizing antibodies were induced in all immunized groups. But the group immunized with the MC-DNA complex containing 20 µg of DNA, showed a consistent rise in SN titres ranging from 0.6 to 1.8, respectively from pre-vaccination to 30 day post immunization (dpi) and sustained throughout the study period. Although the concentration greater than 20 µg (i.e. 50 µg) also induced SN antibody, the titres were not sustained beyond 16 dpi and it receded to 1.5 on 16 dpi onwards (Table 1). In order to monitor the Th2/Th1 responses elicited by different doses of MC-DNA vaccine complex, IgG1 and IgG2 responses were monitored in all the sera collected from G pigs by respective indirect ELISAs. The immune responses were represented in terms of OD_{492nm}. There was a graded increase in total IgG1 response from 0 to 30 dpi. The IgG1 response increased with an increase in dose of MC-DNA up to 20 µg, attaining a peak OD of 0.46±0.006 as observed and this was maintained up to 30dpi; whereas, G pigs immunized with a 50 µg dose showed an average OD of 0.38±0.006 on 30 dpi. Similarly, total IgG2 response showed an increasing trend from day 0 to 22 dpi with gradual decline thereafter. However, the peak titre was observed in the group immunized with 20µg dose both on 22 and 30 dpi (Table 2).

In the present study, chitosan and mannosylated chitosan were tested for effective delivery of a DNA vaccine cassette containing VP1 (immunodominant) region of FMDV along with full length outer membrane protein A (OmpA) gene of *S. typhimurium* cloned into pVAC plasmid DNA vector. Initially the morphology, size and charge of the MC nanoparticles prepared were conformed to the observations of other researchers^[8,13]. Incorporation of the FMDV-DNA construct into the nanoparticles reduced their positive charge, which is in accordance with *Bordetella bronchiseptica* dermonecrototoxin^[8] and *Treponema pallidum* Tp92 DNA vaccine vectored by MC/chitosan nanoparticles, respectively^[17]. Also, mannose receptor mediated uptake enhances the HLA class II restricted

Table 1. Serum neutralizing antibody titre against FMDV serotype 'O' in different immunized groups of Guinea pigs at different time intervals (values indicate the mean \pm SD)

Days post immunization	Immunization Groups (quantum of DNA in μ g)				
	2	5	10	20	50
0	0.6 \pm 0.08	0.6 \pm 0.08	0.6 \pm 0.08	0.6 \pm 0.08	0.6 \pm 0.08
4	0.6 \pm 0.08	0.6 \pm 0.08	0.9 \pm 0.09	0.9 \pm 0.09	0.6 \pm 0.09
10	0.9 \pm 0.09	0.9 \pm 0.09	0.9 \pm 0.09	0.9 \pm 0.09	0.9 \pm 0.09
16	0.9 \pm 0.09	0.9 \pm 0.09	0.9 \pm 0.09	1.2 \pm 0.09	1.2 \pm 0.09
22	1.2 \pm 0.09	1.2 \pm 0.09	1.2 \pm 0.12	1.5 \pm 0.09	1.4 \pm 0.08
30	1.2 \pm 0.09	1.2 \pm 0.09	1.2 \pm 0.09	1.8 \pm 0.09	1.5 \pm 0.09

Table 2. FMDV Type O specific IgG1 and IgG2 antibody response of various immunized groups of guinea pigs at different time intervals in the respective indirect ELISAs. The values indicate the mean \pm SD at OD_{492nm}.

Days post immunization	ELISA (IgG1/IgG2)	Immunization Groups for IgG1 and IgG2 (quantum of DNA in μ g)				
		2	5	10	20	50
0	IgG1	0.10 \pm 0.00	0.11 \pm 0.00	0.11 \pm 0.00	0.11 \pm 0.00	0.11 \pm 0.00
	IgG2	0.12 \pm 0.01	0.12 \pm 0.01	0.12 \pm 0.01	0.12 \pm 0.01	0.12 \pm 0.01
4	IgG1	0.12 \pm 0.01	0.14 \pm 0.01	0.15 \pm 0.01	0.15 \pm 0.01	0.15 \pm 0.01
	IgG2	0.24 \pm 0.01	0.29 \pm 0.00	0.29 \pm 0.01	0.30 \pm 0.01	0.30 \pm 0.01
10	IgG1	0.18 \pm 0.01	0.20 \pm 0.00	0.23 \pm 0.01	0.28 \pm 0.00	0.23 \pm 0.01
	IgG2	0.33 \pm 0.01	0.36 \pm 0.01	0.39 \pm 0.01	0.46 \pm 0.01	0.36 \pm 0.01
16	IgG1	0.21 \pm 0.00	0.22 \pm 0.00	0.26 \pm 0.01	0.37 \pm 0.01	0.24 \pm 0.01
	IgG2	0.36 \pm 0.01	0.37 \pm 0.01	0.40 \pm 0.01	0.54 \pm 0.00	0.39 \pm 0.02
22	IgG1	0.25 \pm 0.01	0.30 \pm 0.00	0.31 \pm 0.01	0.42 \pm 0.01	0.35 \pm 0.01
	IgG2	0.38 \pm 0.01	0.39 \pm 0.00	0.44 \pm 0.00	0.61 \pm 0.01	0.43 \pm 0.01
30	IgG1	0.28 \pm 0.01	0.35 \pm 0.01	0.36 \pm 0.01	0.46 \pm 0.01	0.38 \pm 0.01
	IgG2	0.28 \pm 0.01	0.33 \pm 0.01	0.38 \pm 0.01	0.57 \pm 0.01	0.36 \pm 0.01

antigen presentation by cultured DCs^[16]. In general, a higher dose of DNA vaccine is required to induce a good immune response, but in the present experiment 20 μ g was found optimum. This is true to the statement that the delivery with cationic polymers such as MC nanoparticles reduces the quantity of DNA antigen due to the effective targeting and presentation through mannose receptors on the APCs for better immunity. The presence of positive charge on these particles facilitates the formation of polyelectrolyte complexes with negatively charged nucleic acids^[6,11]. Similarly after complexing chitosan/MC with DNA construct, the zeta potential of these complexes remained positive that might have provided a more stable colloidal dispersion^[13] as chitosan derivatives have already been proved to enhance the immunity of DNA vaccine encoding hepatitis B virus core^[9]. Furthermore, it is also possible to select the type of immune response essential to help the host to engender the type of

protective immune response with the minimum possible antigen dose. It is warranted that the results in G pigs (an FMD animal model) needs to be evaluated in the natural host, bovine for its ultimate use. MC as a potent adjuvant can be assessed with other DNA vaccines and bacterial/viral antigens.

Acknowledgments

The authors thank the Director, IVRI, Izatnagar and Joint Director, IVRI, Bangalore for providing the necessary facility to carry out the work. The first author thanks the Department of Animal Husbandry, Government of Orissa, India, for sponsoring to complete master degree in Veterinary Immunology of the Institute. Also, the help extended by Dr Govindaraju, Assistant Professor in the physical characterization of nanoparticles at Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore, Karnataka, India is duly acknowledged.

Raj Kishore Nanda, Bedaso Mammo Edao, Irshad Ahmad Hajam, Shanmugam Chandra Sekar, Kondabattula Ganesh, Veerakyathappa Bhanuprakash and Subodh Kishore ✉

FMD Quality Control and Quality Assurance Laboratory, Indian Veterinary Research Institute, H A Farm, Hebbal, Bangalore 560 024, Karnataka, India

✉ Corresponding author.

Phone: +91-80-23410908, Fax: +91-80-23412509,

Email: subodhkishore@gmail.com

References

1. Apostolopoulos V, Xiang S D, Selomulya C, *et al.* 2010. Delivery of DNA vaccines: an overview on the use of biodegradable polymeric and magnetic nanoparticles. *Wiley Interdiscip Rev Nanomed Nanobiotechnol*, 2(3): 205–218.
2. Bozkir A, Saka O M. 2004. Chitosan nanoparticles for plasmid DNA delivery: effect of chitosan molecular structure on formulation and release characteristics. *Drug Deliv*, 11: 107–112.
3. Centelles M N, Qian C, Campanero M A, *et al.* 2008. New methodologies to characterize the effectiveness of the gene transfer mediated by DNA-chitosan nanoparticles. *Int J Nanomed*, 3(4): 451–460.
4. Chinsangaram J, Beard C, Mason P W, *et al.* 1998. Antibody response in mice inoculated with DNA expressing foot-and-mouth disease virus capsid proteins. *J Virol*, 72: 4454–4457.
5. Doel T R. 2003. FMD Vaccines. *Virus Res*, 91: 81–89.
6. Hashimoto M, Morimoto M, Saimoto H, *et al.* 2006. Gene transfer by DNA/mannosylated chitosan complexes into mouse peritoneal macrophages. *Biotechnol Lett*, 28: 815–821.
7. Jang M K, Jeong Y I, Cho C S, *et al.* 2002. The preparation and characterization of low molecular and water soluble free-amine chitosan. *Bull Korean Chem Soc*, 23: 914–916.
8. Jiang H L, Kang M L, Quan J S, *et al.* 2008. The potential of mannosylated chitosan microspheres to target macrophage mannose receptors in an adjuvant-delivery system for intranasal immunization. *Biomaterials*, 29: 1931–1939.
9. Jiang L, Qian F, He X, *et al.* 2007. Novel chitosan derivative nanoparticles enhance the immunogenicity of a DNA vaccine encoding hepatitis B virus core antigen in mice. *J Gene Med*, 9: 253–264.
10. Kim T H, Jin H, Kim H W, *et al.* 2006. Mannosylated chitosan nanoparticle-based cytokine gene therapy suppressed cancer growth in BALB/c mice bearing CT-26 carcinoma cells. *Mol Cancer Ther*, 5: 1723–1732.
11. Lu S, Song X, Cao D, *et al.* 2004. Preparation of Water- Soluble Chitosan. *J Appl Polymer Sci*, 91: 3497–3503.
12. McCullough K C, Sobrino F. 2004. Immunology of Foot and Mouth Disease. In: *Foot and Mouth Disease current perspectives*. Sobrino F, Domingo E, Ed. England: Horizon Bioscience, pp173–222.
13. Mady M M, Darwish M M, Khalil S, *et al.* 2009. Biophysical studies on chitosan coated liposomes. *Eur Biophys J*, 38: 1127–1133.
14. OIE Manual. 2008. *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*, Chapter No.2.1.5. (Revised version May 2012), 6th Edition, Volume 1 and 2.
15. Reed L J, Muench H A. 1938. A simple method of estimating 50% end points. *Am J Hyg*, 27: 493–497.
16. Tan M C, Mommaas A M, Drijfhout J W, *et al.* 1997. Mannose receptor-mediated uptake of antigens strongly enhances HLA class II-restricted antigen presentation by cultured dendritic cells. *Eur J Immunol*, 27: 2426–2435.
17. Zhao F, Wu Y, Zhang X, *et al.* 2011. Enhanced immune response and protective efficacy of a *Treponema pallidum* Tp92 DNA vaccine vectored by chitosan nanoparticles and adjuvanted with IL-2. *Hum Vaccine*. 1: 7(10).