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Humoral Immune Response Induced by PLGA Micro Particle Coupled Newcastle Disease Virus Vaccine in Chickens

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Abstract

This experiment was conducted for evaluating the humoral immune responses induced by Poly Lactide-co-Glycolide Acid (PLGA) microspheres coupled inactivated Newcastle Disease Virus (NDV) vaccine in comparison to an 'in-house' prepared inactivated and a live commercial vaccine. PLG microparticles containing inactivated NDV were prepared by a double emulsion technique based on solvent evaporation method. The size of the NDV coupled PLG microparticles was determined by Electron Microscopy. NDV coupled PLG microparticles were spherical having smooth surface, hollow core inside with no pores on the surface. The experiment was conducted in four groups of chickens (n=15). The encapsulation efficiency of NDV coupled PLG microparticles was determined by protein estimation and HA activity in elute. The mean (± SE) size of PLG microspheres was found to be 2.409 ± 0.65 μm. The mean percent of encapsulation efficiency of PLG microspheres coupled to NDV was assessed based on the total protein content and HA activity in elute was found to be 8.03 ± 0.50 and 12.5 ± 0.00, respectively. In conclusion, the results of the experiment showed that PLGA coupled NDV vaccine elicited stronger and prolonged humoral immune response in chickens, in comparison to the other tested vaccines, as assessed by haemagglutination inhibition and enzyme linked immuno sorbent asaay titers.

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Introduction

At micro or nano scales, the physical, chemical and biological properties of materials differ fundamentally from those of the corresponding bulk materials. Hence this enables unique interaction with biological systems at the molecular level. Nanoparticles have been prepared from a variety of compounds such as natural or synthetic polymers (Praveen *et al*., 2004; Coombes *et al*., 1999), chitosan (Kato *et al*., 2003), gelatin (Leong *et al*., 1998), calcium phosphate (He *et al*., 2000 and 2002) and so on. The nano or micro particle based vaccine delivery systems offer a number of distinct advantages over conventional vaccines, including prolonged responses, induction of cell mediated and mucosal immunity and a relatively higher intracellular antigen uptake (Nixon *et al*., 1996).

Polymer particles are prepared either from natural or synthetic polymers. Natural polymers (*i.e.* proteins or polysaccharides) have not been widely used for this purpose since they vary in purity and often require cross linking that could denature the embedded antigen. Consequently, synthetic polymers have received significantly more attention in this area. The most widely used polymers for particle-based delivery of vaccines have been Poly Lactic Acid (PLA), Poly Glycolic Acid (PGA), and their copolymer, Poly Lactide-co- Glycolide Acid (PLGA). These polymers are known for both their biocompatibility and resorbability through natural pathways (Hans and Lowman, 2002).

Newcastle Disease (ND) is an economically important avian disease, next only to avian influenza that has considerable impact on the poultry industry world wide. This is not only due to the devastation caused by ND virus but also the economic impact that may ensue due to trading restrictions and embargoes placed on countries where outbreaks have occurred. The disease has a wide variation of severity, ranging from asymptomatic infection to 100% mortality. Both live attenuated and inactivated vaccines are used routinely to protect chicken against ND during the entire life of bird. In this context, PLGA based delivery of Newcastle Disease Virus (NDV) was attempted and the humoral immune responses elicited by this vaccine was studied in comparison with a commercial live vaccine and an 'in house' prepared killed vaccine.

Materials and Methods

PLGA microspheres

PLGA copolymer 50,50, (Resomer RG 503, MW 34,000 Cat No. P2191), dichloromethane (DCM) (Cat. No. D7566) and poly vinyl alcohol (PVA) (87-89% hydrolyzed, MW 13,000 ± 23,000, Cat No. P8136) were all obtained from Sigma-Aldrich, USA (St.Louis,MO).

Newcastle disease virus (NDV)

The velogenic NDV isolate, originally obtained from Institute of Veterinary Preventive Medicine (Ranipet, India) and maintained in the Department of Animal

Biotechnology (Madras Veterinary College, Chennai, India) was used for preparation of PLGA coupled vaccine.

Chickens and embryonated chicken eggs

Apparently healthy one-day old commercial layer chickens were procured from Venkateshwara Hatcheries (Pune, India). Ten-day-old Embryonated Chickens Eggs (ECE) from unvaccinated flocks were procured from Poultry Research Station Nandanam (Chennai, India) and used for NDV propagation. They were randomly checked for the presence of Haemagglutination Inhibition (HI) antibodies against NDV in their yolk samples and were found to be negative.

Titration and inactivation of NDV

The infectivity titre was determined by inoculating serial 10-fold dilutions (10^{-1} to 10^{-13}) of virus in the form of allantoic fluids, in to ECE. The end point titre was expressed as 50 percent Embryo Infective Dose (EID_{50}) per mL as calculated by the method of Reed and Muench (1938). Haemagglutination (HA) test was performed following the procedure specified in the Terrestrial Manual (OIE, 2004).

Infective allantoic fluid containing NDV having HA titre 2^{11} and EID_{50} $10^{10.45}$ was treated with formalin at the final concentration of 1:1000 and complete inactivation was achieved after 16 hours at 37°C. Inactivation of NDV was checked by two serial passages in ECE, resulting in live embryos with no HA activity in their allantoic fluids. This inactivated allantoic fluid containing NDV was used for coupling with PLGA particles.

Preparation of PLG particles coupled with NDV

PLG microparticles containing inactivated NDV were prepared by a double emulsion technique based on the solvent evaporation method as described by Greenway *et al*. (1998) with some modifications. Briefly, an aqueous solution of infective allantoic fluid containing NDV (25 mL) was emulsified with 10 mL of a 6% solution of PLG 50:50 in dichloromethane, using a homogenizer (Silent Crusher M, Heidolph, Germany) for 5 mins at 10,000 RPM to produce the primary Water-in-Oil-in-Water (W/O/W) emulsion. The resulting water-in-Oil (W/O) emulsion was then emulsified for 10 mins at high speed with 80 mL of 2% Polyvinyl Alcohol (PVA) solution to produce a primary W/O/W emulsion. The primary W/O/W emulsion was then stirred magnetically for approximately 18 hrs at room temperature to allow solvent evaporation. The microspheres were isolated by centrifugation at 10,000 RPM for 10 mins, washed three times in distilled water and freeze-dried. The microspheres were stored in a desiccator at a temperature below 5ºC.

Electron microscopy

PLGA microparticles were dispersed in distilled water, coated over carbon conducting tape and air dried under ambient conditions. Over that gold was coated using ion sputter coating instrument (HITACHI, Tokyo, Japan) under a high vacuum, 0.1 Torr, high voltage, 15 KV and 50 mA for 60 seconds. The coated samples were examined using scanning electron microscope (HITACHI, S-3400 N Model, Tokyo, Japan).

Ten μL of the pre-lyophilized aqueous dispersion of PLGA particles followed by one drop of 1% phosphotungstic acid were put on a formvar coated copper grid (1% solution of formvar was prepared in spectroscopic grade chloroform) and air dried in a vacuum desiccator. The dried grid was then examined under transmission electron microscope (PHILIPS, TECNAI 10, Netherlands).

Assessment of encapsulation efficiency of PLGA microspheres coupled to NDV

The amount of protein coupled to the PLGA microspheres was determined by dissolving 20 mg of the microspheres in 0.5 mL of 0.1M NaOH followed by incubation at 37ºC for 30 min. After elution, the supernatant was collected and analyzed for protein content using Bicinchonic Acid (BCA) protein estimation kit (Bangalore Genei, India) and HA activity. Encapsulation Efficiency (EE %) was calculated based on protein content and HA activity separately, using the formula:

$$EE\ \% = \frac{\text{protein content in eluate}}{\text{protein content in sample added}} \times 100$$

Preparation of 'in house' NDV inactivated vaccine

Oil-based NDV inactivated vaccine was prepared as an emulsion of water-in-oil at 1:3 ratio (OIE, 2004). Briefly, nine volumes (27 mL) of highly refined paraffin oil and one volume (3 mL) emulsifying agent such as sorbitane monoleate (Span 80, Cat No, SL6S56169, SD Fine chemicals) was mixed with aqueous phase containing 10 ml of inactivated allantoic fluid containing NDV and 400 μL polyoxyetylene sorbitane monoleate (Tween 80, Cat No, 39436I05, SD Fine chemicals, Mumbai, India) and homogenized.

Experimental design

Groups of 15, 4-days old chickens each were used with the following treatments: (1) PLGA micro particles coupled with NDV vaccine inoculated intranasally and intraocularly (100 μL), (2) 'In-house' prepared oil adjuvant inactivated NDV vaccine injected intramuscularly (1 mL), (3) Commercial live vaccine (RDV 'F') inoculated intranasally and intraocularly at manufacturer's recommended dose (100 μL) [Venkateshwara Hatcheries, Pune, India.], and (4) Unvaccinated control.

These chickens were boosted with the same vaccine at three weeks post-initial vaccination. Serum was collected from all the chicks every week up to six weeks

and used in HI and Enzyme Linked Immunosorbent Assay (ELISA) for assessing the humoral antibody induced by these vaccines.

Assessment of humoral immunity

The HI test was performed on individual sera taken every week after immunization following the procedure specified in OIE (2004). Indirect ELISA (single serum dilution) was performed as per the method described by Kirubaharan *et al.* (2008). The log ELISA titers were arrived by applying the following formula, where corrected absorbance was obtained by taking the mean value of two replicates of a sample and subtracting it with conjugate control.

Log_{10} titer = (Corrected absorbance + 0.4208) / 0.1838

Titer = antilog (log_{10} titer)

Statistical analysis

Data were analyzed with one-way ANOVA and student 't' test using SPSS (1996) software.

Results

Surface morphology and size of NDV coupled PLGA microspheres

The surface morphology of PLGA micro spheres were spherical having smooth surface, hollow core in side with no pores on the surface (Figure 1). They were present in clusters adhering to the allantoic fluid proteins. Transmission electron micrographs also revealed individual smooth particles with hollow core (Figure 2). The mean size of PLGA micro spheres was determined using in-built software of SEM. The mean (± SE) size of PLGA micro spheres was found 2.409 ± 0.65 μm (n = 16).

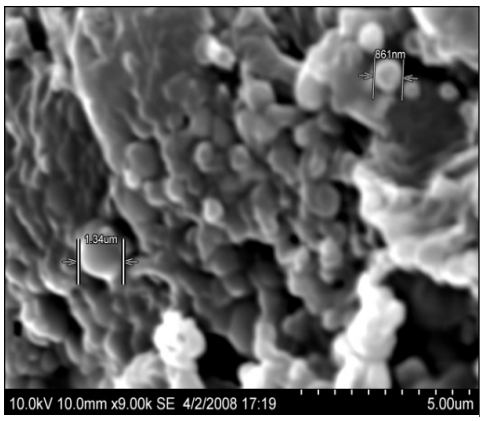

Figure 1. Scanning electron micrograph of lyophilized PLGA microparticles coupled to NDV vaccine (× 9.0 k).

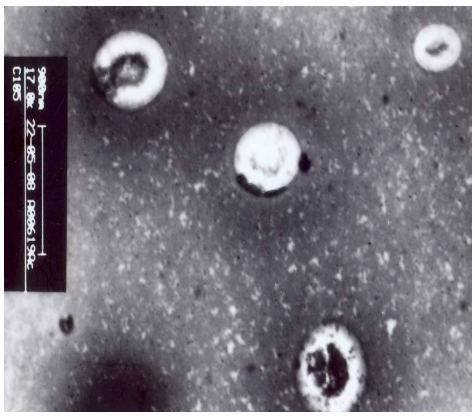

Figure 2. Transmission electron micrograph of PLGA microparticles coupled to NDV vaccine (× 15.0 k).

Encapsulation efficiency of PLGA microspheres coupled to NDV

The mean (± SE) percentage of Encapsulation Efficiency (EE %) of PLGA micro spheres obtained from three different trials are given in Table 1. Humoral immune responses of PLGA micro spheres coupled NDV vaccine. The mean (± SE) HI log_2 titers and mean (± SE) ELISA log_{10} titers induced by PLGA micro spheres coupled NDV vaccine in comparison to a commercial (RDV 'F') and 'in house' prepared NDV inactivated vaccines are shown in Table 2 and Table 3, respectively. At the 2nd week PV, PLGA coupled NDV vaccines had significantly higher HI titers than RDV 'F' vaccinated chicken. Maximum HI titre was seen in PLGA coupled NDV vaccinated group at 3 weeks PV ($log_2$8.0). Similarly, after booster vaccination also, PLGA coupled NDV vaccine induced maximum HI titers.

Chicks vaccinated with the 'in house' prepared inactivated vaccine showed a booster effect with higher HI and ELISA titers post secondary vaccination at the 3rd week PV, PLGA coupled NDV vaccines had significantly higher ELISA titers than RDV 'F' or the inactivated vaccinated chickens. Maximum ELISA titers were seen in PLGA coupled NDV vaccinated group at 3 weeks PV (log_{10}8.05). PLGA vaccine group had higher ELISA titers compared to RDV 'F' group at 2 weeks post secondary vaccination as seen in 3 weeks post primary vaccination.

Table 1. Encapsulation efficiency of NDV[1] coupled PLGA[2] micro spheres based on protein content and HI[3] activity of the eluate

Parameter	Mean ± SE (n=3)
Infective allantoic fluid added	10408.10 ± 380.01
Eluate from coupled PLGA micro spheres	986.02 ± 80.01
Encapsulation efficiency based on protein content in eluate (%)	8.03 ± 0.50
HA titer in	
Infective allantoic fluid added	1024.00 ± 0.00
Eluate from coupled PLGA micro spheres	128.00 ± 0.00
Encapsulation efficiency based on HA activity in eluate (%)	12.50 ± 0.00

[1]Newcastle Disease Virus; [2]Poly Lactide-co-Glycolide Acid;
[3]Haemagglutination Inhibition

Table 2. Mean (± SE) HI[1] log_2 titers in the serum of chickens vaccinated with PLGA[2] micro spheres coupled NDV[3] vaccine in comparison with a commercial and 'in house' prepared NDV inactivated vaccine

Weeks Post Vaccination	Chickens Vaccinated with			
	PLGA Coupled NDV	In House Prepared NDV Inactivated Vaccine	RDV 'F'	Uninfected Allantoic Fluids (Control)
1*	2.85 ± 0.26^{b}	3.83 ± 0.17^{a}	2.83 ± 0.31^{b}	3.16 ± 0.31^{ab}
2	5.14 ± 0.34^{a}	2.67 ± 0.33^{c}	4.00 ± 0.26^{b}	2.00 ± 0.26^{c}
3	8.00 ± 0.82^{a}	3.50 ± 0.50^{b}	3.83 ± 048^{b}	1.83 ± 0.17^{c}
4#	6.71 ± 0.75^{a}	6.33 ± 0.88^{a}	4.66 ± 0.42^{b}	0.50 ± 0.50^{c}
5	7.42 ± 0.48^{a}	8.17 ± 0.95^{a}	4.16 ± 0.40^{b}	1.16 ± 0.17^{c}
6	8.71 ± 0.52^{a}	7.17 ± 0.60^{b}	4.16 ± 0.40^{c}	1.50 ± 0.22^{d}

[1]Haemagglutination Inhibition; [2]Poly Lactide-co-Glycolide Acid; [3]Newcastle Disease Virus.
*Age at vaccination-4 day old. #Booster dose given after three weeks.
Means bearing different superscript differ significantly along row at *P<0.05*, one way ANOVA.

Table 3. Mean (± SE) ELISA log_{10} titers in the serum of chickens vaccinated with PLGA[1] micro spheres coupled NDV[2] vaccine in comparison with a commercial and 'in house' prepared NDV[2] inactivated vaccine

Weeks Post Vaccination	Chickens Vaccinated with			
	PLGA Coupled NDV	In House Prepared NDV Inactivated Vaccine	RDV 'F'	Uninfected Allantoic Fluids (Control)
1*	1.47 ± 0.02^{b}	1.65 ± 0.07^{ab}	1.68 ± 0.10^{ab}	1.72 ± 0.05^{a}
2	4.02 ± 0.72^{a}	2.03 ± 0.03^{b}	3.40 ± 0.45^{a}	1.98 ± 0.02^{b}
3	8.05 ± 0.93^{a}	2.88 ± 0.16^{bc}	3.70 ± 0.25^{b}	1.64 ± 0.17^{c}
4#	5.61 ± 0.62^{a}	4.45 ± 0.73^{ab}	3.33 ± 0.30^{b}	1.87 ± 0.05^{c}
5	4.50 ± 0.35^{a}	4.46 ± 0.54^{a}	3.11 ± 0.26^{b}	1.78 ± 0.05^{c}
6	4.38 ± 0.20^{a}	4.39 ± 0.55^{a}	3.80 ± 0.17^{a}	1.90 ± 0.02^{b}

[1]Poly Lactide-co-Glycolide Acid; [2]Newcastle Disease Virus; *Age at vaccination-4 day old; #Booster dose given after three weeks.

Means bearing different superscript differ significantly along row at *P<0.05*, one way ANOVA.

Discussion

Newcastle disease is one of the major constraints of poultry production in India. The disease is being controlled by extensive use of live vaccines during the growing period followed by inactivated vaccines before the point of laying. Any technology that could increase the magnitude of immune response induced by the NDV vaccines or prolong its duration is needed to reduce the number of vaccinations given to the birds. In turn this would reduce the cost of vaccination and stress to the bird.

One such advancement in antigen delivery is the use of nano and micro particles as adjuvants. Hence, in the present study, this technology was explored by using PLGA micro spheres. These were coupled to NDV vaccine and the micro particle adjuvanted vaccine was assessed for its potential in eliciting humoral immune responses in vaccinated chickens, in comparison to an 'in house' prepared inactivated oil-adjuvant vaccine and a commercial live vaccine.

PLGA is biodegradable and is widely used for vaccine and drug delivery (Hilbert *et al.*, 1999). Several reports have demonstrated the utility of PLGA micro spheres in coupling with peptide, protein, virus and other macromolecules (Feng *et al.*, 2006; Delgado *et al.*, 1999; Eldridge *et al.*, 1991). The size of the PLGA micro particles also varies with the method of preparation and sizes in the range of 1 – 3 μm have been reported earlier (Evans *et al.*, 2004; Dandapat *et al.*, 2003a; Eldridge *et al.*, 1991). PLGA micro particles have been used to couple egg drop syndrome-76 and Infectious Bursal Disease (IBD) viruses (Dandapat *et al.*, 2003a and 2003b) and the coupled vaccines were shown to be more potent than the conventional vaccines.

For coupling of viruses to nano particles, several workers have used only partially purified pelleted viruses (Dandapat *et al.*, 2003a; Greenway *et al.*, 1998) or plasmid DNA expressing the immunological gene (Santander *et al.*, 2007).

However, in this study the inactivated allantoic fluid containing NDV was used directly for coupling with PLGA particles. This was done primarily with the view of commercial applicability of the prepared vaccine under the field conditions. However, this resulted in lowered encapsulation efficiency, all hovering around 10% level. Dandapat *et al.* (2003a) used 4.5 mg of IBD pelleted virus of which only 420 – 510 µg/mL of protein was present in the hydrolysate of different micro sphere preparations.

As expected, the in-house prepared inactivated oil adjuvant vaccine stimulated higher HI and ELISA titers following the booster vaccination only since inactivated vaccines require prior priming to stimulate adequate immune response. PLGA coupled to NDV vaccine produced very high HI titers of 28.0 and 28.71 at 3 weeks post vaccination and post booster vaccination, respectively. Such titers are never induced by RDV 'F' live vaccination during their primary response. Only HI titers of 25.0 to 26.0 are normally seen at 3-4 weeks post vaccination (Jayawardane and spradbrow, 1995). Protection against NDV is highly correlated with the humoral antibody responses commonly estimated by the HI test. The reciprocal HI titers required to protect against mortality induced by NDV is around 25 to 26, while the titers required to protect against egg drop is 29 (Allan *et al.*, 1978). Routinely, under the field conditions, a second dose of NDV live vaccine is given to the chickens at about 3 – 4 weeks of age. Judging by the high HI titers induced by the PLGA coupled NDV vaccine following primary vaccination, this second dose may not be required and could be deferred by another 3 weeks. Thus, the PLGA micro particle coupled NDV could off set the increased cost of the vaccine by increasing the magnitude of the humoral immune responses it induces.

However, more work needs to be done on increasing the poor encapsulation efficiency of the PLGA coupled NDV vaccine still retaining the use of infected allantoic fluids as a source of virus. This could facilitate the further large-scale usage of such technologies under the field conditions.

References

Allan WH, Lancaster JE, & Toth B. 1978. Newcastle disease vaccine – Food and Agricultural Organization of the United Nations, Rome.

Coombes AGA, Lavellea EC & Davis SS. 1999. Biodegradable lamellar particles of poly (lactide) induce sustained immune responses to a single dose of adsorbed protein. Vaccine, 17: 2410-2422.

Dandapat S, Banerjee DR, Dash BB, Kataria JM & Yadav MP. 2003a. Immunogenicity of single intranasal dose of IBD vaccine based on poly-lactide-co-glycolide (PLG) microparticles in broiler chickens. Indian Journal of Comparative Microbiology Immunology and Infectious Diseases, 24: 173-179.

Dandapat S, Banerjee DR, Kataria JM, Ram GC & Yadav MP. 2003b. Induction of mucosal immunity in poultry after oral immunization with single dose of

microencapsulated EDS-76 Virus. Indian Journal of Comparative Microbiology Immunology and Infectious Diseases, 24: 181-185.

Delgado A, Lavelle EC, Hartshorne M & Davis SS. 1999. PLG microparticles stabilized using enteric coating polymers as oral vaccine delivery systems. Vaccine, 17: 2927-2938.

Eldridge JH, Staas JK, Meulbroek JA, Tice TR & Gilly RM. 1991. Biodegradable and biocompatible poly (D,L-lactic-co-glycolic acid) micro spheres ad an adjuvant for Staphylococcal Enterotoxin B toxoid which enhances the level of toxin neutralizing antibodies. Infection and Immunity, 59: 2978-2986.

Evans JT. Ward JR. Kern J & Johnson ME. 2004. A single vaccination with protein-microspheres elicits a strong CD8 T-cell-mediated immune response against Mycobacterium tuberculosis antigen Mtb8.4. Vaccine, 22: 1964–1972.

Feng L, Rong X, Zhou XJ, Maitani Y, Wang SC, Jiang Y & Nagai T. 2006. Pharmaceutical and immunological evaluation of a single-dose hepatitis B vaccine using PLGA micro spheres. Journal of Controlled Release, 112: 35–42.

Greenway TE, Eldridge JH, Ludwig G, Stass JE, Smith JF, Gilly RM & Michalek SM. 1998. Induction of protective immune responses against Venezuelan equine encephalitis (VEE) virus aerosol challenge with microencapsulated VEE virus vaccine. Vaccine, 15: 1314-1323.

Hans ML & Lowman AM. 2002. Biodegradable nanoparticles for drug delivery and targeting. Current Opinion in Solid State and Materials Science, 6: 319–327.

He Q, Mitchell AR, Johnson SL, Wagner BC, Morcol T & Bell SJD. 2000. Calcium phosphate nano particle adjuvant. Clinical Diagnostic Laboratory Immunology, 7: 899–903.

He Q, Mitchell AR, Johnson SL, Wagner BC, Morcol T & Bell SJD. 2002. Calcium phosphate nano particles induce mucosal immunity and protection against Herpes Simplex Virus type 2. Clinical Diagnostic Laboratory Immunology, 5: 1021-1024.

Hilbert AK, Fritzsche U & Kissel T. 1999. Biodegradable microsphere containing influenza A vaccine: immune response in mice. Vaccine, 17: 1065-1073.

Jayawardane GWL & Spradbrow PB. 1995. Mucosal immunity in chickens vaccinated with the V4 strain of Newcastle disease virus. Veterinary Microbiology, 46: 69 –76.

Kato Y, Onishi H & Machida Y. 2003. Application of chitin and chitosan derivatives in the pharmaceutical field. Current Pharmaceutical Biotechnology, 4: 303-309.

Kirubaharan JJ, Priyadarshini MLM, Suresh Babu S, Singh RK & Palaniswami KS. 2008. Single serum dilution ELISA for quantification of antibodies in chickens exposed to Newcastle disease virus. Indian Veterinary Journal, 85: 1143–1146.

Leong KW, Mao HQ, Truong-Le VL, Roy K, Walsh SM & August JT. 1998. DNA-polycation nano spheres as non-viral gene delivery vehicles. Journal of Controlled Release, 53: 183–193.

Nixon DF, Hioe C, Chen PD, Bian Z, Kuebler P & Li ML. 1996. Synthetic peptides entrapped in micro particles can elicit cytotoxic T cell activity. Vaccine, 14: 1523–1530.

OIE (Office International des Epizooties). 2004. Newcastle disease. Manual of standards for diagnostic test and vaccines. 4th Ed. Paris. OIE Chapter 2.1.15

Praveen E, Diwan M, Cao M & Samuel J. 2004. Characterization of poly (D,L-lactic-co-glycolic acid) based nanoparticulate system for enhanced delivery of antigen to dendritic cells. Vaccine, 22: 2406-2412.

Reed LJ, & Muench H, 1938. A simple method for estimating 50 percent end points. American Journal of Hygiene, 27: 493-497.

Santander-Ortega MJ, Csaba N, Alonso MJ, Ortega-Vinuesa JL & Bastos-Gonzalez D. 2007. Stability and physiochemical characteristics of PLGA: poloxamine blend nano particles a comparative study. Colloids and surfaces A, 296: 132-140.

SPSS (Statistical Packages for the Social Sciences). 1996. SPSS for Windows Release 10.01. SPSS Inc. Chicago.