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Efficacy of chitosan nanoparticles as adjuvant in development of an inactivated Newcastle Disease Virus (genotype VII) vaccine

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ABSTRACT

Newcastle disease (ND) is one of the highly infectious diseases which causes high mortality rate in the poultry flocks and extensive loss in the global poultry industry. Vaccination is the most effective method for prevention and control of viral infection. Recently, nanotechnology played special role to develop new methods for preparation of adjuvant, which play a main role in the potency and safety of vaccines. In this work an inactivated Newcastle Disease virus (NDV) genotype VII vaccine was prepared using chitosan nanoparticles (NDV-CS-NPs) by encapsulation using ionic gelation technique to enhance the potency of the NDV vaccine. NDV antigen was encapsulated in chitosan nanoparticles (CS-NPs) with high stability and good morphology which was observed using the transmission electron microscope. The mean diameter of the NDV-CS-NPs after encapsulation was 389.1 nm with an encapsulation efficiency 78% and a zeta potential 2.7 mv. The prepared vaccine was safe and sterile. Cellular immune response using lymphocyte transformation and phagocytic activity of immunized chicks showed significant progress, which persisted till 21stday post-vaccination. Serum antibody titer determined by Hemagglutination Inhibition (HI) test was increased from the 1st week after vaccination (WAV) and continued till 25th WAV against NDV. Vaccinated chickens were protected after challenge with the virulent NDV. In conclusion, the results of this study demonstrated the safety and efficacy of an inactivated NDV vaccine with chitosan nanoparticles that save cost and time for vaccine production.

1. INTRODUCTION

Newcastle disease (ND) is a highly devastating viral disease of poultry which cause severe impacts on the poultry industries worldwide. The viral genome consists of a negative non-segment single stranded RNA about 15 - 16 kb in length which encodes 2 nonstructural proteins (V&W) and 6 structural proteins (NP, P, M, F, HN, L) (Murulitharan et al., 2013). Newcastle Disease virus (NDV) is a member of the *Paramyxoviridae* family of the genus *Orthoavulavirus-1* which is commonly designated as the *Avian avulavirus-1* and known as Avian *paramyxovirus* (Dimitrov et al., 2019). Genetically it has been classified into class I and class II virus. Class II viruses are divided into 18 genotypes according to the analysis of the F gene nucleotide sequence. Genotypes V and VII are the most common in the world (Alexander et al., 2008). Many viral infections occur via mucosal surfaces, so mucosal immunity is frequently important for controlling primary infections. Recently, various techniques have been established to enter the virus into cells for production more effective and low-cost vaccine. The linear polymer of chitosan contains repeating units of 2-amino-2-deoxy-*d*-glucopyranose, was obtained from chitin which is assembled in sills of shrimps or carbs (Fan et al., 1999). Chitosan was confirmed to be non-toxic in humans (Aspden et al., 1997) and experimental animals (Aouada et al., 2008), as anon toxic, polyatomic, biocompatible, biodegradable, and natural polymer (Li et al., 2013). A wide range of bioactive vehicle can be encapsulated in chitosan which act as mediators of plasmid DNA or protein antigen (Newman et al., 2002). Experimental results demonstrated the capability of chitosan nanoparticles (CS-NPs) to enhance the activation of macrophages and dendritic cells (Koppolu et al., 2013). Also, it evoke a balanced Th1-Th2 response and induce a strong potential to enhance cellular and humoral immune responses (Wen et al., 2011). The CS-NPs can be formed by molecular crosslinking between the positive charged chitosan and the negative charged sodium tripolyphosphate (TPP) depending on the principle of ionic crosslinking (Zhao et al., 2012). The amino and carboxyl groups in chitosan are attracted to glycoprotein in mucus to form a hydrogen bond and prolong the in vivo retention and release time of drugs (Wang et al., 2011). This work aimed to prepare and evaluate the potency of an inactivated NDV (genotype VII) vaccine encapsulated in chitosan nanoparticles by an ionic cross-linking technique for improvement of NDV vaccine production

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2. MATERIAL AND METHODS

2.1. This experiment was ethically approved under the following number BUFVTM02-07-22.

2.2. ND Virus (genotype VII):
Veloginic NDV (KM2886090-1-NDV-B7-CHEG-12) was kindly provided by (Central Laboratory for Evaluation of Veterinary Biologics) (CLEVB), Abbasia, Cairo, with a titer $10^{10}EID_{50}$ /ml and 9 Log_2 HAU /25 microliter) It was used in the vaccine preparation and challenge after vaccination with a titer $10^{6.5}EID_{50}$ /ml.

2.3. Adjuvant: Chitosan nanoparticles (CS-NPs):
It was proved to be a polyatomic, biodegradable, biocompatible and non-toxic natural polymer which can encapsulate bioactive agents as peptides and proteins, supplied from (Primex® Company).

2.4. Embryonated Chicken eggs (ECEs):
ECEs were obtained from the project of specific pathogen free (SPF) eggs, El-Fayoum governorate, Kom Oshim.They were used for propagation and titration of the NDV which used for vaccine preparation and testing the safety of virus suspension after inactivation.

2.5. Experimental chicks:
One hundred and twenty (120), one week old SPF chicks were obtained from the project of SPF poultry, El-Fayoum governorate, Kom Oshim.They were kept under strict hygienic condition and used for vaccine evaluation.

2.6. Vaccine Formulation
Velogenic ND virus (genotype VII) was propagated in the allantoic cavity of (SPF-ECEs) at 37°C according to (Dawson and Allan, 1973). Allantoic fluid of all eggs was collected and titrated according to (Reed and Muench, 1938). Formalin 0.1% at final concentration was used for virus inactivation. OIE Manual, (2009).

2.6.1. Chitosan solutions preparation:
Chitosan solution was prepared by slowly dissolving of chitosan in acetic acid aqueous solution 4% with sonication until the solution was transparent, then it was diluted with deionized water and filtered by a 0.22 µm filter paper (Zhao et al., 2012).

2.6.2. Preparation of NDV vaccine with chitosan nanoparticles (NDV-CS-NPs)
The ionic cross-linking method was used for preparation of NDV-CS-NPs. The NDV with a titer ($10^{9.5}EID_{50}$ /ml), a volume of 2.5 ml of NDV were added drop by drop under magnetic stirring to 5 ml of chitosan solution then, 2.5 ml of tripolyphosphat (TPP) solution was added to the above solution at room temperature. NDV-CS-NPs was separated by centrifugation for 30 min at 10,000 g/min and the supernatant was discarded (Zhao et al., 2012).

2.6.3. Optimization and characterization of NDV-CS-NPs:
The NDV-CS-NPs morphological characteristics were investigated by high resolution transmission electron microscopy (HR-TEM) - (JEM 1010, Jeol, Japan, Tokyo). The hydrodynamic diameter of the particles in the freshly prepared dispersions was determined using a Zetasizer® Nano-ZS (Malvern instruments, UK) by dynamic light scattering technique. This was performed using a scattering angle of 173° at 25 °C (Zhao et al., 2012). The encapsulation efficiency (EE%) was measured spectrophotomerically according to (Xu et al.,2003) depending on the following equation:
$EE\% = (W_0 - W_1)/ W_0 \times 100\%$
W_0 = total amount of NDV added & $W_{1=}$ amount of free NDV in susbention.

2.7. Vaccine Quality Control:
2.7.1. Sterility Test:
The prepared vaccine was examined for purity and free from any fungal or bacterial contaminants according to the Federal Regulation USA, by culturing on specific media thioglycolate broth and nutrient agar searching for aerobic and anaerobic bacterial contamination after incubation at 37 °C for 72 hours. Also, Sabaroud maltose agar searching for fungus contamination after incubation at 25°C for 14 days.

2.7.2. Safety test:
Three groups (10 chicks / each group), 2 weeks old. Group (1) was inoculated by intranasal (I/N) route with two field doses (0.2 ml) of NDV-CS-NPs. Group (2) was inoculated with 0.2 ml (CS-NPs) as blank by I/N route. Third group of chicks were left as control (non-inoculated). Chicks were observed for 2 weeks for any local reaction or appearance of any clinical signs.

2.8. Vaccination of SPF-chicks:
Ninety (90), 21-day-old SPF chickens were divided into 3 groups (30 chicks / group), Group (1) chickens were vaccinated by I/N route with NDV-CS-NPS vaccine with dose 0.1 ml / each chick with virus titer not less than ($10^{8.5}$ EID_{50}/ml). Group (2) chickens were immunized I/N with 0.1ml of CS-NPS as blank. Group (3) as negative control and chickens in group (4) as nonvaccinated challenged chicks as positive control.

2.9. Evaluation of cell mediated immune response:
2.9.1. Evaluation of Lymphocyte transformation:
Heparinized blood samples from immunized chickens at the 3rd, 5th, 7th, 10th, 14 th, and 21st day post-vaccination for evaluation of lymphocyte transformation and phagocytic activity. Lymphocyte proliferation of the vaccinated birds was estimated using XTT kit. The test was performed according to (Scudiero et al., 1988).

2.9.2. Evaluation of phagocytic activity using Candida Albicans:
Separation by ficol hypaque and cultivation of mononuclear cells was performed according to Ansley and Hazen, (1988).
Phagocytic percentage was done according to Harmon and Glisson, (1989), which was modified by El-Enbawy, (1990*)* depending on the next equation:
Phagocytic percent = No. of phagocytes which ingest Candida / Total No. of phagocytes ×100
Phagocytic index was performed according to Richardson and Smith, (1981) depending on the next equation:
Phagocytic index = Total No. of phagocytes which ingest more than two Candida / Total No. of phagocytes ingest Candida.

2.10. Evaluation of the Humoral Immune Response:
Serum samples were collected from 1st week post vaccination (WPV) till 25th WPV for detection of serum antibody using hemagglutination inhibition (HI) test. The test was carried out according to OIE-Manual, (2009).

2.11. Evaluation of protective efficacy:

Vaccinated chickens were selected for challenge test (20 chicks/ each group) at 28-day post vaccination. The challenge dose of the virulent NDV (0.1 ml) with a titer ($10^{6.5}$ ELD_{50}/ml) for each bird via intramuscular route and observed for 2 week after challenge to measure the protection % (Zhao et al., 2012).

Protection % against VVNDV = $No.\ of\ survival/total\ No.\ of\ challenge\ of\ birds\ X10$.

2.12. Real time -polymerase chain reaction (RT-PCR):

Tracheal swabs from immunized chickens were collected at 2nd, 4th and 6th days' after challenge to estimate the shedding of virus using RT-PCR—to detect relatively quantify the viral RNA in the collected tracheal swabs. Extraction of viral RNA by using Qiamp viral RNA mini kit (QIAGEN) catalogue No.52904. Preparation of PCR master mix according to Quanti-Tect probe (RT-PCR) kit while thermal cycling condition for gene-specific Probe and Primer sets was carried out according to Wise et al. (2004).

3. RESULTS

3.1. Sterility and safety test:

The prepared vaccine was confirmed to be sterile and no evidence of any bacterial or fungal contamination. The virus was completely inactivated as indicated by absence of any pathological lesions. The vaccine was safe and it didn't induce any abnormal clinical signs and no local reaction in chickens.

3.2. Optimization of NDV-CS-NPs:

The optimal encapsulation condition for NDV-CS-NPs was NDV/CS ratio 1:2, chitosan concentration 1 mg/ml, and a sodium tripolyphosphate (TPP) concentration 0.5 mg/ml with an encapsulation rate of 78% (table1).

Table 1 Optimization of NDV-CS-NPs preparation condition.

	NDV-CS-NPs preparation			
Exp. No	Chitosan density (mg/ml)	TPP concentration (mg/ml)	NDV/CS ratio (ml/ml)	Encapsulation efficiency %
1	0.5	0.5	1:4	48%
2	1.0	0.5	1:2	78%
3	1.5	0.5	1:1	65%

3.3. Characterization of NDV-CS-NPs:

The morphology of NDV-CS-NPs showed a spherical and polydisperse nature with good scattering by high resolution transmission electron microscopy, as showed in (figure 1). The mean diameter of the particle after encapsulation was about 389.1 nm with a Zeta potential 2.7 mV and an encapsulation efficiency 78% (figure 2).

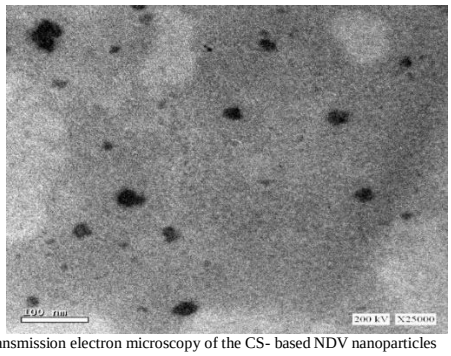

Fig.1. Transmission electron microscopy of the CS- based NDV nanoparticles

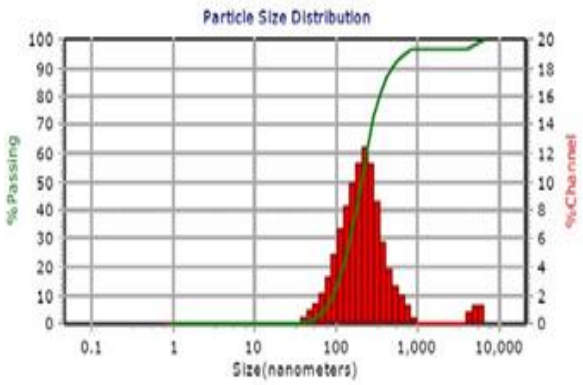

Fig.2. Particle size distribution and zeta potential of the CS-based NDV nanoparticles under the optimized condition

3.4. Cell mediated immune response:

Lymphocyte blastogenesis test showed that cell proliferation started to increase (0.486) from the 3rd day post vaccination (DPV) for NDV-CS-NPs vaccine. It reached maximum value (0.743) at the 10th DPV and (0.646) at the 14th DPV. It persisted in high values (0.425) till 21st DPV (table2).

The phagocytic % started to increase (43.75%) from the 3rd DPV for vaccines. It reached maximum value (80%) at the 10th DPV for NDV-CS-NPs vaccine. It persisted in high values (53.33%) till 21st DPV (table 3).

The phagocytic index started to increase (0.44) from the 3rd DPV for vaccine. It reached maximum value (0.75) at the 10th DPV for vaccines. It persisted in high values (0.50) till 21st DPV (table 4).

Table 2 Lymphocyte blastogenesis using XTT reagent for chicks vaccinated with (NDV-CS-NPs) vaccine.

	Cell proliferation expressed by optical density		
Days post vaccination	NDV-CS-NPs vaccine	CS-NPs as blank	Control
3rd	0.486	0.148	0.191
5th	0.647	0.153	0.179
7th	0.630	0.184	0.165
10th	0.743	0.172	0.174
14th	0.646	0.194	0.123
21st	0.425	0.174	0.142

Table 3 Evaluation of phagocytic activity by Phagocytic percent.

	Phagocytic percent		
Days post vaccination	NDV-CS-NPs vaccine	CS-NPs as blank	Control
3rd	43.75%	12.5%	5.2%
5th	54.50%	28.6%	5.4%
7th	73.90%	28.2%	7.6%
10th	80.00%	28.2%	7.0%
14th	75.00%	33.3.%	5.4 %
21st	53.33%	12.5%	5.4 %

Table 4 Evaluation of phagocytic activity by Phagocytic index.

	Phagocytic index		
Days post vaccination	NDV-CS-NPs Vaccine	CS-NPs as blank	Control
3rd	0.44	0.25	0.04
5th	0.45	0.29	0.04
7th	0.61	0.14	0.10
10th	0.75	0.14	0.06
14th	0.65	0.17	0.06
21st	0.50	0.13	0.10

3.5. Humoral immune response:

Immunized chickens with NDV-CS-NPs had significantly increase in serum antibody titers against NDV compared with chickens immunized with CS-NPs as blank and control group. Serum HI antibody titer against NDV (genotype VII) was increased (4.0 log_2) from the 1st week after immunization (WAI). It reached maximum value (7.3 log_2) at the 5th WAI and persisted (5.3 log_2) till 25th WAI as shown in (table 5).

Table 5 Mean log2 serum antibody titers against NDV in vaccinated chicks using HI test:

Weeks post vaccination	Mean log_2 HI serum antibody titer for NDV /ml		
	NDV-CS-NPs Vaccine	CS-NPs as blank	Control
1	4.0	1.4	0
2	4.3	1.6	0
3	6.3	1.5	0
4	6.7	1.9	0
5	7.3	1.6	0
6	7.0	1.0	0
7	7.0	1.5	0
8	6.7	1.0	0
9	6.3	1.7	0
10	5.7	1.4	0
12	5.3	1.9	0
14	5.0	1.6	0
17	5.3	1.6	0
19	4.7	1.4	0
21	4.3	1.5	0
25	3.6	1.6	0

3.6. Protective efficacy of the prepared vaccine upon challenge:

Challenge of vaccinated chicks with the corresponding virulent NDV virus showed that the vaccine gave 90% protection percent (table 6). Detection of NDV using real time RT-PCR in tracheal swabs of vaccinated chicks after their challenge showed that only one bird showed shedding of NDV at 2nd day post challenge DPC and only one bird at 4th DPC (table 7).

Table 6 Protection percentage in chicks vaccinated with inactivated NDV-CS-NPs vaccines after challenge with virulent NDV:

Groups	Chicks challenged with virulent NDV		
	Challenged	Dead	Protection%
G1: NDV-CS-NPs vaccine	20	2	90%
G2: CS-NPs (As blank)	20	18	10%
Control non-vaccinated	20	20	0%

Table 7 Detection of shed of NDV using real time RT-PCR from chicks vaccinated with inactivated NDV-CS-NPs vaccine after challenge with vNDV:

Days after challenge	Detection of NDV using real time RT-PCR		
	NDV-CS-NPs vaccine	CS-NPs As blank	Control
2nd	1/5	5/5	5/5
4th	1/5	5/5	5/5
6th	0/5	5/5	5/5

4. DISCUSSION

Mucosal immunization could be a successful method for protection of poultry flocks against viral infection (Jang et al., 2011). Polymeric nanoparticles have adequate adhesive effects, enhance drug stability, target specific organs, and are simply absorbed by cells, so they are widely used for drug and vaccine delivery (Löbenberg et al.,1997). The nanoparticles as immune enhancer can increase the efficacy of mucosal vaccination against viral diseases (Corbanie et al., 2006). This work demonstrates the efficacy of chitosan nanoparticles on immune response of immunized chickens. Chitosan nanoparticles are potent vectors for the drugs and vaccines delivery via nasal administration (Kang et al., 2008 and Varshosaz et al., 2004). The chitosan positive charge which is generated under physiological conditions is responsible for its enhanced bioadhesivity and site-specific applications in delivery systems which be controlled (Aksungur et al., 2004 and Senel et al., 2000). The ionic cross-linking technique was used in this work to prepare inactivated ND virus (genotype VII) encapsulated in chitosan nanoparticles as recorded by Zhao et al., (2012) and Mohammadi et al., (2021) who stated that encapsulated vaccine antigens have been designed as an effective method to control the restriction in mucosal immunization. Cellular and humoral immune responses play significant roles in protection of birds against NDV infection (Marino and Hanson et al., 1987). Cellular immunity may be necessary for virus evacuation (Russell and Ezeifeka; 1997). The lymphocyte blastogenesis test result in this study showed that cell proliferation started to increase from 3rd day post vaccination and the highest stimulation of lymphocyte proliferation was induced at 10 th day post vaccination. This result comes in agreement with Ghumman et al., (1976). Also, these results indicated positive effect of chitosan on cellular immune response as recorded by Zhao et al., (2012). Systemic antibodies in high levels are associated with protection against NDV (Kapczynski et al., 2005). In this work vaccinated chickens with in activated NDV with chitosan induced the highest HI titers at 5th weeks post immunization. The route of inoculation for the challenge post vaccination is essential factor in the vaccine evaluation(OIE, 2012). Our result showed 90% protection percent after challenge with highly virulent NDV by intramuscular route.

5. CONCLUSION

In conclusion, the prepared inactivated NDV vaccine encapsulated in chitosan nanoparticles (NDV-CS-NPs) confirmed to be safe, sterile , gave good protection after challenge, enhance humoral immune response and promotes the developments of cellular immunity, that demonstrate the efficacy of chitosan nanoparticles for improvement an inactivated NDV vaccine that save cost and time for vaccine production and vaccination.

5. REFERENCES

1. Aksungur P, Sungur A, Unal S, Iskit AB, Squier CA, et al. (2004) Chitosan delivery systems for the treatment of oral mucositis: In vitroandin vivostudies.;98(2):269-79.
2. Alexander, D.; Senne, D., Dufour-Zavala, L., Swayne, D.E., Pearson, J.E., Reed, W.M., Jackwood, M.W., Woolcock, P., Rglission, J.R., Eds.(2008) Newcastle disease and other paramyxoviruses. In A Laboratory Manual for the Isolation Identification and Characterization of Avian Pathogens, 5th ed.; OmniPress, Inc.: Norristown, PA, USA; pp. 135–141.
3. Antley, P.P., Hazen, K.C. 1988. Role of yeast cell growth temperature on Candida albica virulence in mice. Immunol, 56; 2884-2890.
4. Aouada FA, Moura MR, Mattoso LH (2008) Preparation of chitosan nanoparticles using methacrylic acid. J Colloid Interface Sci 321(2): 477–483.
5. Aspden TJ, Mason JD, Jones NS, Lowe J, Skaugrud O, et al. (1997) Chitosan as a nasal delivery system: the effect of chitosan solutions onin vitroandin vivo mucociliary transport rates in human turbinates and volunteers. J Pharm Sci 86(4): 509–513.
6. Corbanie, E.A., Matthijs, M.G., van Eck, J.H., Remon, J.P., Landman, W.J. and Vervaet, C. (2006) Deposition of differently sized airborne microspheres in the respiratory tract of chickens. *Avian Pathol.*, 35: 475-485.
7. Dawson, P.S. and Allan, W.H., (1973): Newcastle disease. Proc. Eur poult. Conf. London. (8): 303–317
8. Dimitrov KM, Abolnik C, Afonso CL, Albina E, Bahl J, Berg M, Briand FX, Brown IH, Choi KS, Chvala I, Diel DG, Durr PA, Ferreira HL, Fusaro A, Gil P, Goujgoulova GV, Grund C, Hicks JT, Joannis TM, Wong FYK (2019). Updated unified phylogenetic classification system and revised nomenclature for Newcastle disease virus. Infect. Genet. Evol (6):1567-1348.
9. El-Enbawy M.I. 1990. Some studies on Candida albicans. Ph.D. thesis (microbiology), Faculty of Veterinary Medicine, Cairo University, Cairo, Egypt.
10. Fan H, Lin Q, Morrissey GR, Khavari PA (1999). Immunization via hair follicles by topical application of naked DNA to normal skin. Nat Biotechnol 17(9): 870– 872.
11. Ghumman JS, Bankowski E (1976) In vitro DNA synthesis in lymphocytes from turkeys vaccinated with La Sota, TC and inactivated Newcastle disease vaccines. Avian Dis 20: 18–31.

12. Harmon, B.G., Glisson, J.R. 1989. In vitro microbial activity of avian peritoneal macrophages. Avian Dis., 33:177-181.
13. Jang, S.I., Lillehoj, H.S., Lee, S.H., Lee, K.W., Lillehoj, E.P., Bertrand, F., Dupuis, L. and Deville, S (2011) Montanide™ IMS 1313 N VG PR nanoparticle adjuvant enhances anti- gen-specific immune responses to profilin following muco- sal vaccination against Eimeria acervulina. Vet. Parasitol., 182: 163-170.
14. Kang ML, Kang SG, Jiang HL, Guo DD, Lee DY, et al. (2008) Chitosan microspheres containing Bordetella bronchiseptica antigens as novel vaccine against atrophic rhinitis in pigs. J Microbiol Biotechnol (6): 1179–1185.
15. Kapczynski DR, King DJ (2005) Protection of chickens against overt clinical disease and determination of viral shedding following vaccination with commercially available Newcastle disease virus vaccines upon challenge with highly virulent virus from the California 2002 exotic Newcastle disease outbreak. Vaccine 23: 3424–3433.
16. Koppolu B, Zaharoff DA. 2013. Effect of antigen encapsulation in chitosan particles on uptake, activation and presentation by antigen presenting cells. Biomaterials. 2013;34(9):2359–2369.
17. Li X, Min M, Du N, Gu Y, Hode T, Naylor M, Chen D, Nordquist RE, Chen WR. Chitin, chitosan, and glycated chitosan regulate immune responses: the novel adjuvants for cancer vaccine. Clin Dev Immunol. 2013; 2013:387023.
18. Löbenberg R, Araujo L, Kreuter J. 1979. Body distribution of azidothymidine bound to nanoparticles after oral administration. Eur J Pharm Biopharm. 1997;44(2):127–132.
19. Marino OC, Hanson RP (1987) Cellular and humoral response of in ovo- bursectomized chickens to experimental challenge with velogenic disease virus. Avian Dis 31: 293–301.
20. Mohammadi AR1,2, Zamani Moghaddam A1, Shahsavandi S (2021) Encapsulation of Inactivated Newcastle Disease Virus Onto the Chitosan Nanoparticles for Use in Mucosal Immunity. Iranian Journal of Virology, Volume 15, Number 1, 2021.
21. Murulitharan K, Yusoff K, Omar A. R., and Molouki A, (2013)"Characterization of Malaysian velogenic NDV strain AF2240- I genomic sequence: A comparative study," Virus Genes, 46 (1) :431–440.
22. Newman KD, Elamanchili P, Kwon GS, Samuel J (2002) Uptake of poly (D, L- lactic-co-glycolic acid) microspheres by antigen presenting cellsin vivo. J Biomed Mater Res 60(3): 480–486.
23. OIE Manual, (2009): Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Part 2 Section 2.1. Chapter 2.1.14.
24. OIE. Newcastle disease. Manual of diagnostic tests and vaccines for terrestrial animals 2012, Chapter 23 14. 2012:576-89.
25. Reed, L.J., Muench, H. 1938. "Simple method of estimating 50 per cent end point". Am. J. Hyg, 27: 493-499.
26. Richardson, M.D., Smith, H. 1981. Resistance of virulent and attenuated strains of Candida albicans to intracellular killing by human and mouse phagocytes. J. Infect. Dis.; 144:557-565.
27. Russell PH, Ezeifeka GO (1995) The Hitchner B1 strain of Newcastle disease virus induces high levels of IgA, IgG and IgM in newly hatched chicks. Vaccine 13(1): 61–66.
28. Scudiero, D. A.; Shoemaker,R. H.;Paull, K. D.;Monks, A.; Tierney, S.;Nofziger, T.H.; Currens, M. J.;Seniff, D. and Boyd, M. R. (1988): Evaluationa of soluble tetrazolium / Formazan Assay for cell growth and drug sensitivity in culture using human and other tumer cell lines. Cancer Res.; 48:4827-4833.
29. Senel S, Kremer MJ, Kas S, Wertz PW, Hincal AA, et al. (2000) Enhancing effect of chitosan on peptide drug delivery across buccal mucosa. Biomaterials 21: 2067–2071.
30. Varshosaz J, Sadrai H, Alinagari R (2004) Nasal delivery of insulin using chitosan microspheres. J Microencapsul 21: 761–774.
31. Wang JJ, Zeng ZW, Xiao RZ, Xie T, Zhou GL, et al. (2011) Recent advances of chitosan nanoparticles as drug carriers. Int J Nanomedicine 6: 765–774.
32. Wen ZS, Xu YL, Zou XT, Xu ZR.(2011)Chitosan nano-particles act as an adjuvant to promote both Th1 and Th2 immune responses induced by ovalbumin in mice. Mar Drugs 9: 1038–1055.
33. Wise MG, Wise MG, Suarez DL, Suarez DL, Seal BS, Seal BS, Pedersen JC, Pedersen, JC, Senne DA, Senne DA, King DJ, King DJ, Kapczynski DR, Kapczynski DR, Spackman E, Spackman E (2004). Development of a real-time reverse- transcription PCR for detection of Newcastle disease virus RNA in clinical samples. J. Microbiology, Vol. 42, No. 1, p. 329-338.
34. Xu YM, Du YM, Huang RH, Gao LP (2003) Preparation and modification of N-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride nanoparticle as a protein carrier. Biomaterials 24: 5015–5022.
35. Zhao K, Chen G, Shi X-m, Gao T-t, Li W, et al. (2012) Preparation and Efficacy of a Live Newcastle Disease Virus Vaccine Encapsulated in Chitosan Nanoparticles. PLoS ONE 7(12): e53314