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

Gabr F. El-Bagoury,1 Asmaa M. Mohamed,2 Mohamed A. Abo El-Kher1

1Department of Virology, Faculty of Veterinary Medicine, Benha University
2Newcastle Disease Vaccine Research Department, Veterinary Serum and Vaccine Research Institute, Abb

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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 losses in the global poultry industry. Vaccination is the most effective method for prevention and control of viral infection. Recently, nanotechnology played a special role to develop new methods for preparation of adjuvants, 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 21st day 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 Orthoanvulavirus-1 which is commonly designated as the Avian avulavirus-I 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 carps (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 anontoxic, 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 principal 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.

* Correspondence to: asmaamomohamed@yahoo.com
2. MATERIAL AND METHODS

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

2.2. ND Virus (genotype VII): Velogenic NDV (KM2886090-1-NDV-B7-CHEG-12) was kindly provided by (Central Laboratory for Evaluation of Veterinary Biologics) (CLEVB), Abbassia, Cairo, with a titer $10^{8.5}\text{EID}_50/\text{ml}$ and $9\text{ Log}_2$; HAU /25 microliter) It was used in the vaccine preparation and challenge after vaccination with a titer $10^{6.0}\text{EID}_50/\text{ml}$.

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 was 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 (Zha 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 $(10^{9.5}\text{EID}_50/\text{ml})$, 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 spectrophotometrically according to (Xu et al., 2003) depending on the following equation:

$$\text{EE}\% = \frac{W_1 - W_o}{W_0} \times 100\%$$

$W_0$ = total amount of NDV added & $W_1$ = amount of free NDV in suspension.

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 thiglycolate broth and nutrient agar searching for aerobic and anaerobic bacterial contamination after incubation at 37°C for 24 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 kept 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 chicks were divided into 3 groups (30 chicks / group). Group (1) chickens were vaccinated by I/N route with NDV-CS-NPs vaccine with dose $0.1 \text{ml / each chick with virus titer not less than } (10^{5.5} \text{EID}_50/\text{ml})$. Group (2) chickens were immunized I/N with 0.1 ml 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, 14th, and 21th 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 ficoll 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:

$$\text{Phagocytic percent } = \frac{\text{No. of phagocytes which ingest Candida / Total No. of phagocytes} \times 100}{\text{Phagocytic index } = \text{Total No. of phagocytes which ingest more than two Candida / Total No. of phagocytes ingest Candida}}$$

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^{5.3} ELDs/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.

<table>
<thead>
<tr>
<th>Exp. No</th>
<th>NDV-CS-NPs preparation</th>
<th>NDVCS ratio (mg/ml)</th>
<th>Encapsulation efficiency %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>1.4</td>
<td>48%</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>1.2</td>
<td>78%</td>
</tr>
<tr>
<td>3</td>
<td>1.5</td>
<td>1.1</td>
<td>85%</td>
</tr>
</tbody>
</table>

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).

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.

<table>
<thead>
<tr>
<th>Days post vaccination</th>
<th>NDV-CS-NPs vaccine</th>
<th>CS-NPs as blank</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>3^a</td>
<td>0.486</td>
<td>0.148</td>
<td>0.191</td>
</tr>
<tr>
<td>5^b</td>
<td>0.647</td>
<td>0.153</td>
<td>0.179</td>
</tr>
<tr>
<td>7^c</td>
<td>0.630</td>
<td>0.184</td>
<td>0.165</td>
</tr>
<tr>
<td>10^d</td>
<td>0.743</td>
<td>0.172</td>
<td>0.174</td>
</tr>
<tr>
<td>14^e</td>
<td>0.646</td>
<td>0.194</td>
<td>0.123</td>
</tr>
<tr>
<td>21^f</td>
<td>0.425</td>
<td>0.174</td>
<td>0.142</td>
</tr>
</tbody>
</table>

Table 3 Evaluation of phagocytic activity by Phagocytic percent.

<table>
<thead>
<tr>
<th>Days post vaccination</th>
<th>NDV-CS-NPs vaccine</th>
<th>Phagocytic percent</th>
<th>CS-NPs as blank</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>3^a</td>
<td>43.75%</td>
<td>32.5%</td>
<td>5.2%</td>
<td></td>
</tr>
<tr>
<td>5^b</td>
<td>54.50%</td>
<td>28.6%</td>
<td>5.4%</td>
<td></td>
</tr>
<tr>
<td>7^c</td>
<td>73.90%</td>
<td>28.2%</td>
<td>7.6%</td>
<td></td>
</tr>
<tr>
<td>10^d</td>
<td>80.00%</td>
<td>28.2%</td>
<td>7.0%</td>
<td></td>
</tr>
<tr>
<td>14^e</td>
<td>75.00%</td>
<td>33.3%</td>
<td>5.4%</td>
<td></td>
</tr>
<tr>
<td>21^f</td>
<td>53.33%</td>
<td>42.5%</td>
<td>5.4%</td>
<td></td>
</tr>
</tbody>
</table>

Table 4 Evaluation of phagocytic activity by Phagocytic index.

<table>
<thead>
<tr>
<th>Days post vaccination</th>
<th>NDV-CS-NPs Vaccine</th>
<th>Phagocytic index</th>
<th>CS-NPs as blank</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>3^a</td>
<td>0.44</td>
<td>0.25</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>5^b</td>
<td>0.45</td>
<td>0.29</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>7^c</td>
<td>0.61</td>
<td>0.14</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>10^d</td>
<td>0.75</td>
<td>0.14</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>14^e</td>
<td>0.65</td>
<td>0.17</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>21^f</td>
<td>0.30</td>
<td>0.13</td>
<td>0.10</td>
<td></td>
</tr>
</tbody>
</table>

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 logs) from the 1st week after immunization (WAI). It reached maximum value (7.3 logs) at the 5th WAI and persisted (5.3 logs) till 25th WAI as shown in (table 5).

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
Cellular immunity may be against NDV. 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 10th 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 (Kaczynski et al., 2005). In this work vaccinated chickens with inactivated 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, 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