Efficacy of Preliminary Prepared Mucosal Vaccine of Infectious Coryza in Chicken

Sayed RH¹, Shasha FA¹, Noura M Khalaf², Sahar M Saber¹ and Lamees A El- Tantawy²

¹Central Laboratory for Evaluation of Veterinary Biologics, Abbasia, Cairo, Egypt
²Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt

*Corresponding author: rafikhamed1010@hotmail.com

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ABSTRACT

Infectious Coryza (IC) is an acute respiratory disease of chicken that caused by Avibacterium paragallinarum types A, B and C. Protection of chicken flocks against IC using inactivated vaccine may not totally protect chicks because of lake of vascularity in the infraorbital sinus that permit low amount of antibodies against AP to reach this region. So, in this work poly D, L lactide-co-glycolic acid (PLGA) nano emulsion was used for preparation of mucosal IC vaccine. The immunizing purified hemagglutinin antigen of the three serotypes was prepared and emulsified with PLGA nano emulsion. About 270 SPF chicks (5weeks ago) were divided into three groups. The first group was dropped intranasally with mucosal IC vaccine; second group was injected with traditional oil adjuvant IC inactivated vaccine while the third group was left as unvaccinated control group. Serum sample were collected 3 weeks post vaccination. ELISA antibody titers for mucosal and inactivated vaccine respectively. Protection rates of challenge test were 95% & 87% for mucosal and inactivated vaccine respectively.

INTRODUCTION

Haemophilus paragallinarum is known to be the causative agent of infectious coryza (IC) (Delaplane et al., 1934; Elliot and Lewis, 1934) which has been described in the early literature as roup, contagious or infectious catarrh and uncomplicated coryza (Yamamoto, 1991). It is observed that there is a unique group within the bacterial family Pasteurellaceae associated with avian hosts and these bacteria are rarely isolated from any other host species, this causative agent is known as Avibacterium paragallinarum (Blackall et al., 2005).

The economic impact of the disease attributes to increase culling rate in meat chicken and 10-40% reduction in egg production in laying and breeding hens causing severe economic losses in egg industry in many parts of the world (Yamamoto, 1984; Blackall et al., 1990; Yamamoto, 1991 and Yamaguchi et al., 1993).

In recent years, Nano vaccine is a novel approach to the methodology of vaccination. Nanomaterials are delivered in the form of microspheres, nanobeads or micro-nano projections. Painless, effective and safe needle-free routes such as intranasal or oral route or patches of micro projections to the skin are some of the approaches which are in the experimental stage at present but may have a great future ahead in nano vaccination (Nandedkar, 2009).

PLGA reported as one of the most widely studied polymers of interest in the vaccine field. Since PLGA polymers can offer long-term release of their contents in a recurring, pulsatile manner, the primary focus of past studies has been in using them to replace the multiple immune boosting administrations typically required to induce protective immunity, the using nano microparticles prepared from biodegradable and bio-compatible polymers to induce both humoral and cellular immune responses (Scheerlinck et al., 2007).

PLGA polymers can potentially deliver antigens or adjuvants to a desired location at predetermined rates and durations effectively regulating the immune response over a period of time (Zhao and Leong, 1996).

Recent studies show that co-encapsulation of both antigen and adjuvant in nanoparticulate carriers have synergistic effects in augmenting immunity by targeting both antigen and adjuvant to the endosomal compartment of APCs (Kasturi et al., 2011).

So, the aim of this work was to investigate the effect of mucosal vaccine (nano vaccine), namely PLGA entrapping HA protein of *Avibacterium paragallinarum*, as an adjuvating immuno stimulant agent with nasal dropping administration to induce both humoral and cellular immune responses in the low lake vascularity region.

**MATERIALS AND METHODS**

*Avibacterium paragallinarum* bacterial strains: AP strains serotypes A, B & C were obtained from the Anaerobic Bacterial Vaccine Department, Vet. Ser. Vac. Res. Institute. Abbassia, Cairo.

Molecular identification using multiplex PCR (Patil et al., 2017): The DNA was extracted from the three serotype strains by using easy pure bacterial genomic DNA extraction kit (Trans, China). Briefly, the PCR reactions were carried out for a total volume of 20 µl containing 10 micro 1X PCR master mix solution (iNIRON), 2 µl template DNA, 1µl forward primer, 1µl each for reverse primer and 6 µl distilled water. The primers sequence was mentioned in Table 1. The amplification step was as follows: 98°C for 1 min, 30 cycles for 98°C for 10 sec, 56°C for 10 sec and 72°C for 2 min; and final extension 72°C for 7 mins. The PCR product sizes were determined using agarose gel electrophoresis 1%.

Preparation of *Avibacterium paragallinarum* hemagglutinin antigen (HA) (Yamaguchi et al., 1990): The three serotypes of *Avibacterium paragallinarum* strains were grown in Kato’s broth medium containing 1% sterile chicken serum and 2.5 µg/ ml NADH at 37°C in 10% CO₂ for 18 hours. The bacterial cells were collected by centrifugation at 8000 rpm for 20 minutes and suspended in 0.02 M phosphate buffered saline (pH 7.2). The cells were collected and washed three times in PBS (pH 7.0), then re-suspended in 0.5 M KSCN/0.425M NaCl pH 6.3 and adjusted to an optical density of 1.6 at 650 nm. The suspensions were put in 4ºC for 2 hrs with agitation and sonicated (30sec, 60% pulsed output, power output 5). The sonicated antigens were washed three times in PBS with 0.01% (w/v) thimerosal and adjusted to optical density of 1.6 at 650 nm.

Protein precipitation (Lovrien and Matulis, 2001): Protein precipitation was performed through ammonium sulfate saturation technique. The protein concentration was determined through spectrophotometer.

Preparation of mucosal nano vaccine

Preparation of PLGA nanospheres (Zaman et al., 2013): PLGA nano vaccine was prepared using a W/O/W emulsion and solvent technique. One mg from HA antigen of the three serotypes was dissolved in 150 µl water for injection. The antigen solutions were mixed and emulsified in PLGA (sigma) solution (200mg PLGA in 600µl Dichloromethane (sigma) by probe sonication for 80 sec. The resulting W/O emulsion was then emulsified in 8 ml polyvinyl alcohol (PVA) solution (5% W/V). This secondary emulsion was then added to 32ml of 5% W/V PVA solution and stirred for 3 hrs. to evaporate the organic solvent. Nanospheres were collected by centrifugation (15000rpm for 45mins) and washed twice with distilled water.

Characterization of PLGA nanospheres: Determination of surface properties of PLGA nanospheres was carried out using Scanning Electron Microscopy (SEM). The SEM photographs were taken at different magnification levels with a size bar imprinted on the micrographs.

Determination of (HA) encapsulation efficiency (loading level) in PLGA nanospheres: The dose of HA protein entrapped in was calculated based on actual entrapment of the antigen in the nanospheres. The determination was carried out by Qubit fluorometer. Ten µl samples of PLGA nanospheres was transferred in 1.5 ml Eppendorf tube. One ml of 0.2N NaOH 1% sodium dodecyl sulfate (SDS) solution was added to vials. The vials were placed on a constant shaker at room temperature. The PLGA- HA nanospheres were allowed to hydrolyze overnight, and then the hydrolyzed sample was subjected to protein estimation.

Determination of in vitro release of HA proteins from PLGA nanospheres along with checking of their stability: Ten samples of PLGA-HA nanospheres suspension of 110 µl each were added to 2 ml of PBS (pH 7.4) and incubated at 25 and 41°C on a constant shaking mixer. One volume was withdrawn at each time-point of the *in vitro* estimation, at days 1, 3, 7, 14, 21, 28, 32, 36, 40 and 49. The PLGA- HA nanosphere suspensions were centrifuged at 5000 rpm for 10 min and the supernatant was collected. The supernatant was used for protein estimation at various time-points in the release kinetics, and percent cumulative release of the antigen versus time was plotted.

Experimental design: A total of 270, five week old SPF chickens were divided into 3 groups. The first group (90 chickens) was vaccinated with the mucosal nano vaccine (one drop intranasal) and second group (90chickens) was vaccinated with imported oil adjuvant vaccine (0.5ml S/C), while the third group (90 chickens) used as control unvaccinated chickens. Blood samples were collected before immunization and at one week interval post immunization to evaluate the developed immune response to the vaccine. Sera were separated and collected separately in sterile tubes and kept at -20°C for testing by ELISA.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>A, B, C Forward primer</td>
<td>GCCTACAGCTTTATGCAACGAA</td>
<td>800 bp</td>
</tr>
<tr>
<td>A Reverse primer</td>
<td>CGCGGGATTGTTGATTTGTG</td>
<td>1100 bp</td>
</tr>
<tr>
<td>B Reverse primer</td>
<td>GGTGAATTTCACCACACCAC</td>
<td>1600 bp</td>
</tr>
<tr>
<td>C Reverse primer</td>
<td>TAATTTTCCTTATCCCGACATCAATACCAT</td>
<td>182-186.</td>
</tr>
</tbody>
</table>
Protective efficacy was determined according to (Wambura, 2010). Fifty chickens from each vaccinated groups were challenged intranasal with 0.2 mL (10^6 CFU) viable local *Avibacterium paragallinarum* cells for each serotype. After challenge, the chickens were examined daily for clinical signs of infectious coryza for fifteen days.

**Re-isolation of bacterial strains after challenge test according to** (Wambura, 2010): The chickens in all groups were euthanized at 2 week post challenge. Intraorbital sinus swabs were taken to determine the evidence of *Avibacterium paragallinarum* shedding using multiplex PCR.

**Serological tests:** Enzyme-Linked immunosorbent assay (ELISA) was carried out as outlined by (Myers, 1995)

**RESULTS**

Fig 1 Illustrated results of multiplex PCR for the three serotypes of AP, specific bands were shown at different molecular weights.

Fig 2 showed PLGA nanospheres antigen as examined by transmission electron microscope. PLGA appeared as layered spherical and semi-spherical particles with average size range from 400 nm to 950 nm.

The intermittent tracing of released protein in supernatant could give a clear picture about the kinetics of antigen release from PLGA nanospheres at 25°C and 40°C increased gradually till reached 5.21 and 7.4 respectively as shown in Table 2.

**ELISA test:** In mucosal vaccine the ELISA antibody titer against *Avibacterium paragallinarum* reached 3272 at 7th week post vaccination while in inactivated vaccine reached 1324 at the same time as shown in Table 3.

**Challenge test:** The protection rate of mucosal vaccine was 95% at 3rd week post vaccination while it was 87% in inactivated vaccine as shown in Table 4.

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**Table 2:** In vitro release of hemagglutinin antigen from PLGA nanoparticles at 25°C and 40°C.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Protein concentration (µg) / 110 µl estimated in different times (in days) for PLGA nanoparticles</th>
</tr>
</thead>
<tbody>
<tr>
<td>at 25 °C</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>at 40 °C</td>
<td>0.002</td>
</tr>
</tbody>
</table>

**Table 3:** ELISA antibody titers in vaccinated chicken groups.

<table>
<thead>
<tr>
<th>Weeks post vaccination</th>
<th>Inactivated vaccine</th>
<th>Mucosal vaccine</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt;</td>
<td>419</td>
<td>567</td>
<td>258</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>561</td>
<td>1782</td>
<td>301</td>
</tr>
<tr>
<td>3&lt;sup&gt;th&lt;/sup&gt;</td>
<td>750</td>
<td>2398</td>
<td>289</td>
</tr>
<tr>
<td>4&lt;sup&gt;th&lt;/sup&gt;</td>
<td>982</td>
<td>2780</td>
<td>322</td>
</tr>
<tr>
<td>5&lt;sup&gt;th&lt;/sup&gt;</td>
<td>1009</td>
<td>3012</td>
<td>278</td>
</tr>
<tr>
<td>6&lt;sup&gt;th&lt;/sup&gt;</td>
<td>1298</td>
<td>3210</td>
<td>256</td>
</tr>
<tr>
<td>7&lt;sup&gt;th&lt;/sup&gt;</td>
<td>1324</td>
<td>3272</td>
<td>309</td>
</tr>
</tbody>
</table>

ELISA antibody titer was 328 in all chicken before vaccination.
The use of biodegradable polymeric nanoparticles (PLGA) with entrapped antigen such as protein, peptides or DNA represents an exciting approach for controlling immune response via selective targeting of the antigen to Ag-presenting cells (Akagi et al., 2012) PLGA encapsulated immunogens are slowly and continuously released resulting in repeated stimulation of lymphocytes including antigen presenting cells (Newman et al., 2012).

Inactivated vaccines are preferable than live vaccines due to a possible genetic transmutation of live strains of bacteria into more pathogenic serovars (Deshmukh et al., 2015). Also (Chukiatari et al., 2009) stated that a new produced vaccine for infectious coryza should contain more than one strain of Av. Paragallinarum and local strains of Av. Paragallinarum.

Protection level in the challenged birds were evaluated based on the exhibited clinical signs of infectious coryza and bacterial isolation of Av. Paragallinarum strains, concerning the results of challenge test as shown in Table 4. It was cleared that chickens in immunized groups were protected in relative to unimmunized control group. The group vaccinated with mucosal vaccine was the most protected for chicken and provided the highest protection percentage as it was 95 %, while it was 87% in group vaccinated with inactivated vaccine as shown in Table 4. These results agree with (Deville et al., 2012) who used nanoparticles adjuvant intra nasal vaccine against IB virus, intranasal vaccine was able to trigger a significantly stronger humoral immune response and by challenge conferred a significantly enhanced protection. Also, these results agree with (Heba El Naggar et al., 2017) who used nanoparticles mucosal vaccine against ND and confirmed that mucosal vaccine induce superior protection against viruses that infect mucosal surfaces. Also (Abd El-Bakey, 2018) confirmed the use of PLGA nanoparticles adjuvant for enterotoxaemia bloat vaccine of Clostridium perfringens type A as it induced prolonged immunity compared with gel vaccine.

The dynamics of humoral immune response were analyzed in the two immunized groups, using one serological test (ELISA), for determination of antibody titer, pre-vaccination, 1th, 2nd week, 3th, 4th, 5th, 6th and 7th weeks post vaccination for mucosal vaccine and inactivated vaccine as well as control group.

The antibody levels estimated by ELISA continued high with higher significant differences among two vaccinated groups. This finding of ELISA supported that of challenge test in mucosal and Inactivated vaccine which reached 3272,1324 at 7 weeks post vaccination with mucosal and Inactivated vaccines as shown in Table 3 respectively.

By multiplex PCR as shown in Table 5 the evidence of Avibacterium paragallinarum shedding 2 weeks post challenge in mucosal vaccine was 2 infected samples for

\[ \text{Protection} = \frac{\text{Protection Level}}{\text{Control Protection Level}} \times 100 \]

**Table 4:** The protective efficacy of the mucosal vaccine and Inactivated vaccine using challenge test 3 weeks post vaccination.

<table>
<thead>
<tr>
<th>Vaccinated group</th>
<th>No of challenged birds</th>
<th>Daily observation for one week post challenge</th>
<th>Infection</th>
<th>Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucosal vaccine</td>
<td>60</td>
<td>1st  2nd  3rd  4th  5th  6th  7th  Total</td>
<td>3%</td>
<td>95%</td>
</tr>
<tr>
<td>Inactivated vaccine</td>
<td>60</td>
<td>0  2  3  1  0  0  0</td>
<td>8%</td>
<td>87%</td>
</tr>
<tr>
<td>Control</td>
<td>60</td>
<td>0  10  9  1  5  7  3  37</td>
<td>13%</td>
<td>87%</td>
</tr>
</tbody>
</table>

**Table 5:** The evidence of Avibacterium paragallinarum shedding 2 weeks post challenge using multiplex PCR.

<table>
<thead>
<tr>
<th>Vaccinated group</th>
<th>No. of swabs</th>
<th>Positive AP samples used</th>
<th>Multiplex PCR 2 week post challenge</th>
<th>Total infected samples*</th>
<th>Shedding %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucosal vaccine</td>
<td>60</td>
<td>Serotype A*  Serotype B*  Serotype C*</td>
<td>2  4  1</td>
<td>7</td>
<td>3.9%</td>
</tr>
<tr>
<td>Inactivated vaccine</td>
<td>60</td>
<td>Serotype A*  Serotype B*  Serotype C*</td>
<td>10  15  7</td>
<td>32</td>
<td>17.8%</td>
</tr>
<tr>
<td>Control</td>
<td>60</td>
<td>Serotype A*  Serotype B*  Serotype C*</td>
<td>50  51  45</td>
<td>146</td>
<td>81.1%</td>
</tr>
</tbody>
</table>

Shedding determination: Two weeks post challenge samples from infraorbital sinuses were examined by multiplex PCR for determining the evidence of Ap shedding. The shedding percentage at mucosal and inactivated vaccine was 3.9% and 17.8% respectively while in the control group it was 81.1% as shown in Table 5.

**DISCUSSION**

Infectious coryza is an acute respiratory disease of chickens caused by Avibacterium paragallinarum. The greatest economic losses associated with infectious coryza results from poor growth performance in growing birds and marked reduction (10-40%) in egg production in layers (Blackall and Matsumoto, 2003). PLGA Nanosphere formulations have been considered as efficient adjuvants in the recent years. Intranasal administration of antigens is used to induce potent mucosal, humoral and cellular immune responses. Combinations of the above mentioned approaches are predicted to enhance immunoregulatory responses. In this study, the PLGA nanosphere formulations containing HA antigens of Avibacterium paragallinarum A, B and C serotypes were prepared and characterized.

The size of PLGA Nanosphere is 400 nm to 950 nm and it plays an important role in immunoadjuvant properties of the particles. Due to this fact, increasing of the PVA concentration might be lead to decrease size of nanoparticles. The nano-sized particles are more potent for inducing mucosal immune responses.

This study showed that nanoparticles vaccine adjuvant can enhance the efficacy of avian mucosal vaccination against Infectious Coryza. It could be demonstrated that individual intranasal delivery of adjuvanted formulations containing nanoparticles based adjuvant technologies were able to improve the immune response and protection of infectious Coryza challenge.

The use of bio degradable polymeric nanoparticles (PLGA) with entrapped antigen such as protein, peptides or DNA represents an exciting approach for controlling the release of vaccine antigens and optimizing the desired immune response via selective targeting of the antigen to Ag-presenting cells (Akagi et al., 2012) PLGA encapsulated immunogens are slowly and continuously released resulting in repeated stimulation of lymphocytes including antigen presenting cells (Newman et al., 2012).
serotype A, 4 infected samples for serotype B, 1 infected samples for serotype C, with total infected samples 7, while in Inactivated vaccine, shedding of infected samples was 10 samples for serotype A, 15 samples for serotype B, 7 samples for serotype C with total infected samples 32. So, it could be concluded from results of this study that nano PLGA vaccine was better immunologically and induced high protection rate reached 95% compared with Inactivated vaccine.

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