Preparation and evaluation of inactivated avian Metapneumovirus vaccine from recently isolated Egyptian strain

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Primary Audience: Poultry Veterinarians, Poultry Producers, Public Health Officials, Vaccine Producers, Poultry Health Researchers

SUMMARY

Inactivated avian Metapneumovirus (aMPV) or turkey rhinotracheitis (TRT) virus vaccine was prepared from an Egyptian strain (Giza TRT-4). The virus was propagated in Vero cells and inactivated by binary ethyleneimine. The inactivated virus solution was tested for its sterility, purity, and safety. Then, it was mixed with Nigella sativa oil as nonspecific immune-stimulant adjuvant. Physical characterization of oil prepared vaccine like viscosity and emulsion stability was investigated. An experiment was designed to evaluate the locally prepared aMPV vaccine in a comparison to commercial vaccines either inactivated or live attenuated. The obtained results showed that the locally prepared aMPV vaccine gave significantly higher humoral immune response when measured by ELISA and significantly higher cell mediated immunity by evaluating phagocytic activity of inoculated turkey poults with higher protection rate reached up to 100% after challenge with wild-type virus.

Key words: Avian Metapneumovirus, inactivated vaccine, Egyptian strain, Nigella sativa oil adjuvant, locally prepared aMPV vaccine, humoral immune response, cell mediated immunity

DESCRIPTION OF PROBLEM

Avian Metapneumovirus (aMPV) causes a serious respiratory disease in chicken and turkeys and heavy economic losses to poultry industry [1]. The diseases result from aMPV infections of turkeys or chickens that have been termed turkey rhinotracheitis (TRT), swollen head syndrome, and avian rhinotracheitis. The aMPV are members of the subfamily Pneumovirinae, belonging to the family Paramyxoviridae. The subfamily consists of two genera; pneumovirus that is consisting of mammalian respiratory syncytial viruses and mouse pneumovirus and Metapneumovirus in which avian pneumoviruses are placed [2]. In Egypt, prophylactic vaccination has been adopted using imported inactivated and live attenuated vaccines for chickens and turkeys against aMPV infection. Live vaccines have been reported to be unstable and their efficacy is not fully proven. There are some suspicions about their contribution to the spread of the disease. Imported

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inactivated vaccines produce a moderate level of humoral immunity, however they are producing a low level of cell mediated immunity. Recently in Egypt, the main problem of aMPV in turkey farms occurs in the vaccinated flocks. Taking in consideration the disadvantages of imported vaccines, it become an important alternative to introduce a new inactivated vaccine prepared from recently circulating strain, in addition to the use of Nigella sativa (NS) oil as a nonspecific immune stimulant adjuvant added more advantage to increase the efficacy of the newly developed vaccine.

**MATERIALS AND METHODS**

*Preparation of Inactivated aMPV*

In 25-mL capacity flask of confluent Vero121 cells, Eagle’s minimum essential medium (Flow laboratories, United Kingdom) with Earle’s balanced salt solution containing 10% fetal calf serum (FCS) was decanted. A volume of 0.2 mL virus suspension (TRT-Giza-4) [3], of titer10⁶ tissue culture infective dose (TCID) 50/mL, was inoculated on the Vero121 cells. After adsorption for 60 min at 37°C a maintenance medium consisting of E199 plus 1% FCS (Gibco, United States) was added. The culture was examined daily for 5 d to observe developed cytopathic effect (CPE) then the virus solution was harvested and titrated on Vero121 cells [4]. The end point was determined and the virus infectivity titer was calculated [5], aMPV suspension of a titer 10⁶ TCID 50/mL was inactivated with binary ethyleneimine (BEI) working solution at a final concentration 1,200 μg/mL as previously described [6, 7]. The aMPV–BEI mixture was incubated at room temperature with continuous stirring for 30 h. Samples of aMPV–BEI mixture were taken every 3 h with immediate addition of sodium thiosulphate 20% as stopping reagent for BEI. Collected samples were stored at 4°C until used to determine suitable time for inactivation of aMPV.

*Quality Control of the Prepared Inactivated aMPV*

Safety of inactivated aMPV in tissue culture and young turkey poults were tested. Collected sample of aMPV–BEI mixture was inoculated in confluent sheet of Vero121 cells. The flasks were incubated at 37°C in 5% CO₂ incubator and examined daily for the presence of CPE. Also, the inactivated virus suspension was inoculated intratracheally into 21-days-old young poults, which were then observed daily for 10 d for detecting any respiratory manifestation or death.

Sterility test was done through microbiological examination for detection of any bacterial and/or fungal contaminants. Plates of brain heart infusion agar, MacConkey agar, and Sabouraud’s dextrose agar were prepared [8] and inoculated with 1 mL inactivated aMPV solution. The inoculated plates were incubated at 37°C for 72 h for detection of bacterial colonies and at 25°C for 7 d for detection of fungal growth. The prepared aMPV suspension was examined by agar gel precipitation test (AGPT) for detection of purity from other viruses, such as Newcastle disease virus, avian reovirus, avian influenza virus, and avian leukosis virus, using specific hyper immune serum for each one of them.

*Preparation of Oil Phase Nigella sativa Adjuvant*

*Nigella sativa* oil was purchased from herbal and medicinal plants shop and was prepared by cold pressing of *Nigella sativa* seeds, it was mixed with Arlacel A (Sigma, United States) in a ratio of 9:1 oil:Arlacel A, with thoroughly mixing before sterilization by filtration through a Seitz filter 0.45 μL. The mixture oil:Arlacel A was stored at room temperature and used within a few weeks of preparation [9].

*Preparation of Aqueous Phase of aMPV Inactivated Vaccine*

The aqueous phase of inactivated aMPV solution was prepared by mixing 96% inactivated aMPV with 4% Tween 80 (Sigma, United States).

*Preparation of aMPV Inactivated Vaccine*

Stable emulsion was prepared by thorough mixing of prepared aqueous phase and oil phase in a ratio 1:4, where one part of aqueous phase was mixed with 4 parts of oil phase with continuous mixing until production of stable emulsion [10].
Physical Characterization of Oil Prepared Vaccine

The prepared oil vaccine was subjected to the following tests.

Drop test. To determine the emulsion type, drop of prepared emulsion was allowed to fall onto the surface of water where the drops should remained discrete over the surface of water in case of water in oil emulsion [11].

Emulsion stability. It was expressed as months of storage time during which the oil and aqueous phase did not separate from emulsion of the vaccine, where separation of oil and aqueous phase indicate bad emulsion and vice versa [11].

Viscosity. Vaccine viscosity was determined as a rate of discharge of the emulsified vaccine at 24°C, discharge of 0.4 mL vaccine emulsion from 1-mL pipette in relation to glycerol follow time [12].

Experimental Design

The experiment was conducted in a manner that avoided unnecessary discomfort to the birds by the use of proper management and laboratory techniques according to Beni-Suif University guidelines. A total of one hundred 21-days-old turkey poults, where their sera proved to be free from aMPV antibodies by AGPT were divided into 4 groups in separate place (25 poults/group). Group 1 received locally prepared inactivated aMPV vaccine (by a dose 0.3 mL/bird by i.m. route), Group 2 received commercial inactivated aMPV vaccine (by a dose 0.3 mL/bird by i.m. route), Group 3 received commercial live attenuated aMPV vaccine (by a dose 102.5 TCID50/bird orally), and Group 4 was kept as control negative nonvaccinated group. Sera and blood samples were collected from all groups to evaluate the humoral and cell mediated immune response.

Evaluation of Humoral Immune Response

Serum samples were collected from each group at 0, 3, 7, 10, and 14 days postvaccination to evaluate cell mediated immune response. The circulating mononuclear cells from collected blood were separated by using Ficol–Hypaque [15]. Finally the sediment of washed cells was suspended in 1 mL RPMI (Roswer Park Memorial Institute) 1640 (Sigma, United States) medium containing 10% fetal calf serum.

Collected spleen of poults under test was harvested under complete aseptic conditions and the spleen cells were dispersed and placed in sterile plastic tube for 10 min to allow precipitating of coarse particles, then supernatant was centrifuged in conical plastic tube for 10 min at...
905 × g and the cell sediment was washed 3 times using RPMI 1640 medium for 10 min. Then the cell sediment was suspended in 1 mL RPMI 1640 medium containing 10% fetal calf serum.

The mononuclear cell concentration was determined [16]: briefly, 100 μL cell suspension was mixed with 100 μL trypan blue 0.4% and counted by haemocytometer. The numbers of viable cells per milliliter were calculated according to the following equation: 

\[ \text{No. of cells} = \frac{\text{No. of counted cells per triple square} \times 25 \times 2 \times 10^4}{\text{No. of counted square}} \].

The concentration of live cells was adjusted to final concentration 1 × 10^7 cells/mL and suspended in RPMI 1640 medium containing 10% fetal calf serum, where for each well in microtiter plate received 1 × 10^6 cells/0.1 mL.

**Evaluation of Phagocytic Activity**

One mL of washed standardized mononuclear cells was suspended in RPMI 1640 medium with 10% FCS and placed in cell culture staining chamber after incubation for 1 h in CO2 incubator at 37°C, the monolayer cell sheet was washed 3 times with RPMI 1640 medium to remove the nonadherent cells, then the adhered cells were covered with 1 mL RPMI 1640 medium containing 10% FCS and incubate for 24 h in CO2 incubator at 37°C. After 24-h incubation, the supernatant medium was discarded and 1 mL previously prepared C. albicans was added and incubated for 1 h in CO2 incubator at 37°C. At the end of incubation the monolayer cells were washed 3 times with cold RPMI 1640 medium and fixed by methyl alcohol for 10 min and stained by freshly prepared Giemsa solution for 30 min and examined under a microscope. The phagocytic percentage and phagocytic index were calculated from the following equation:

- **Phagocytic percentage**
  \[ \text{Phagocytic percentage} = \frac{\text{No. of phagocytes with ingested blastospores} \times 100}{\text{Total No. of phagocytes}} \]

- **Phagocytic index**
  \[ \text{Phagocytic index} = \frac{\text{Total No. of phagocytes ingested more than 3 blastospores}}{\text{Total No. of phagocyte ingested blastospores}} \]

**Statistical Analysis**

F-test and least significant differences were used to determine statistically significant differences between means among tested groups [18].

**Challenge and Protection Test**

At 21 d postvaccination, 10 poults from each vaccinated and control groups were challenged with field aMPV intratracheally by a dose of 100 μL virus of a titer 10^6 TCID₅₀ [19]. Challenged poults were observed twice daily for clinical signs as ocular discharge, tracheal rales, and death in a comparison between control and challenged poultes for a period of 15 d postchallenge. Trials for virus re-isolation were conducted on collected tracheal swabs from all groups at the end of the study or from dead birds postchallenge through Vero cell culture passage [14]. The culture was examined daily for 5 d to observe CPE then the virus suspension was harvested and confirmed for aMPV isolation using AGPT with specific hyperimmune serum.

**RESULTS AND DISCUSSION**

Pneumovirus infection of poultry is associated with serious economic and animal welfare problems, particularly in commercial turkey flocks, even in countries where vaccination has become routine practice [20]. In Egypt, the main problem of aMPV in turkey farms lies in the appearance of the disease in vaccinated flocks. To control the disease, inactivated and live attenuated vaccines are used routinely.

In spite of the greatest safety of inactivated vaccines (no clinical signs, no virus shedding and no back mutation), they still show some disadvantages like poor primary cell mediated response, requirement for second and third booster doses, and addition of adjuvants for enhancement of the cell mediated immune response. Many different types of companies are known to improve vaccine efficacy, but most of the commercially available products are still supplemented with classical adjuvants including mineral oil emulsions [21]. Tissue reactions in chickens injected with mineral oil emulsion vaccine remain a source of poultry condemnations and are of financial concern to the poultry industry as
well as it persists for months and that may cause undesirable tissue reactions and is considered to have carcinogenic potential for consumers [22]. The use of alum hydroxide gel and mineral oil as adjuvants in the majority of prepared inactivated vaccines caused many troubles. The former may become toxic if the injectable dose is not carefully calculated and the second has the ability to induce local granuloma at the site of injection [23].

Taking in consideration the disadvantages of inactivated vaccines with alum gel and/or mineral oil adjuvant, it has become urgent to find a new alternative by aMPV inactivated vaccine from recently isolated Egyptian strain mixed with a natural vegetable oil of *Nigella sativa* as nonspecific immune-stimulant. All mentioned disadvantages of traditionally inactivated vaccines potentiate the selection of natural oils as adjuvants. The *Nigella sativa* oil can be easily prepared by pressing the *Nigella sativa* seeds (cultivated in large quantities especially in upper Egypt) and the remained debris can be used in a concentration of 1–2% as feed additives due to its enrichment in proteins, oil, and trace minerals.

The aMPV of the isolated strain from Egypt (TRT-Giza-4) on Vero121 cells was completely inactivated by BEI after 18 h. Therefore, the preferred time of inactivation for preparing the vaccine should be after 24 h at 37°C of treatment with BIE. The inactivated solution of aMPV had been inoculated in Vero cells and young turkey poult's for testing its safety. The inactivated virus was safe as it did not induce any CPE on the Vero121 and no death or respiratory signs in young turkey poult's.

Moreover, the inactivated aMPV was subjected for testing its sterility, by cultivation on nutrient agar, MacConky, and Sabaroud’s broth. The obtained results revealed that the prepared inactivated aMPV was sterile as it was free from any bacterial and fungal contaminates; no colonies or turbidity were seen on the agar medium or broth used for this purpose. The purity of prepared inactivated aMPV solution on Vero cell culture was noticed as it was neutralized only with the aMPV hyper immune serum but did not react with any other used hyper immune sera and induced CPE on inoculated Vero cells.

*Nigella sativa* oil was selected for preparing the oil phase according to [9] for enhancing the cell mediated immune response nonspecifically with production of high antibodies standing for long time. The physical characters of locally prepared inactivated aMPV vaccine adjuvant with *Nigella sativa* oil were investigated. The prepared vaccine was stable for 4 mo at 4°C, of low viscosity and water in oil emulsion. The lower viscosity is an important characteristic for oil emulsion vaccines because it facilitates the vaccination process by lowering fatigue of the workers and saving time of work when large number of birds will be vaccinated. Locally prepared inactivated aMPV vaccine adjuvant with *Nigella sativa* oil is easily flowed through a 1 mL pipette. Addition of hydrophilic emulsifier as Tween 80 and Arlacel A was associated with increased emulsion stability, decreased emulsion viscosity and increased serological response. Reddy et al. [24] found that the type of emulsion obtained appeared to be influenced by method of emulsification or by aqueous oil ratio. *Nigella sativa* oil is highly nonspecific immune-stimulant, it increased production of IL-1, IL2α that activate macrophage and T-Helper lymphocytes [25] besides other different desirable effects such as antimicrobial [26] and anthelmintic effects [27]. It activates cell mediated immunity, contains no carcinogenic substances or hydrocarbons as in mineral oil or mentanoid oil, and it is cheap (it is used in a concentration 25% of the vaccine bulk) when compared with other adjuvants [28]. Some studies were planned to avoid the undesirable effect of the mineral oils by replacing them with suitable animal, vegetable, or synthetic oil as has been described by [28, 29].

For evaluation of humeral immune response of vaccinated turkey poult's, their collected serum samples were tested by ELISA. The obtained results (Table 1) showed that the prepared vaccine produced a significantly higher increase in ELISA OD 0.731 (*P* < 0.05) than imported and commercial aMPV vaccines (0.606 and 0.675, respectively) at 21 d postinoculation.

Evaluation of cell mediated immune response data presented in Tables 2 and 3 showed highly significant increases (*P* < 0.05) in phagocytic activity represented by higher phagocytic percentage and higher phagocytic indices for poult's inoculated with locally prepared aMPV.
### Table 1. ELISA optical densities of serum samples collected from turkey poults vaccinated with locally prepared and commercial aMPV vaccines.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. poults</th>
<th>Vaccine</th>
<th>ROI1</th>
<th>OD tested sera/days postvaccination3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>25</td>
<td>NS–aMPV2</td>
<td>i.m.</td>
<td>0.180</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>±0.03</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>Commercial. Inactivated</td>
<td>i.m.</td>
<td>0.180</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>±0.03</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>Commercial live attenuated</td>
<td>Orally</td>
<td>0.180</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>±0.03</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>Control nonvaccinated</td>
<td>i.m.</td>
<td>0.180</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>±0.03</td>
</tr>
</tbody>
</table>

1ROI = Route of inoculation.
2NS–aMPV = *Nigella sativa* oil aMPV inactivated vaccine.
3OD = Optical density.
5+ = Significant increase in humoral immune responses \((P < 0.05)\).

### Table 2. Phagocytic percentage of macrophage collected from turkey poults vaccinated with locally prepared and commercial aMPV vaccines.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. poults</th>
<th>Vaccine</th>
<th>ROI1</th>
<th>Phagocytic percentage/days postvaccination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>25</td>
<td>NS–aMPV2</td>
<td>i.m.</td>
<td>3.5 ± 0.33</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>Commercial. Inactivated</td>
<td>i.m.</td>
<td>3.5 ± 0.33</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>Commercial live attenuated</td>
<td>Orally</td>
<td>3.5 ± 0.33</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>Control nonvaccinated</td>
<td>i.m.</td>
<td>3.5 ± 0.33</td>
</tr>
</tbody>
</table>

1ROI = Route of inoculation.
2NS–aMPV = *Nigella sativa* oil aMPV inactivated vaccine.
5+ = Significant increase in cell-mediated immune response \((P < 0.05)\).
### Table 3. Phagocytic indices of macrophage collected from turkey poults vaccinated with locally prepared and commercial aMPV vaccines.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. poults</th>
<th>Vaccine</th>
<th>ROI(^1)</th>
<th>Phagocytic indices/days postvaccination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>25</td>
<td>NS–aMPV(^2)</td>
<td>i.m.</td>
<td>0.133 ± 0.002</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>Commercial, inactivated</td>
<td>i.m.</td>
<td>0.133 ± 0.002</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>Commercial live attenuated</td>
<td>orally</td>
<td>0.133 ± 0.002</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>Control nonvaccinated</td>
<td>i.m.</td>
<td>0.133 ± 0.002</td>
</tr>
</tbody>
</table>

\(^1\)ROI = Route of inoculation.
\(^2\)NS–aMPV = *Nigella sativa* oil aMPV inactivated vaccine.
\(^{++}\) = Significant increase in cell-mediated immune responses (*P* < 0.05).

### Table 4. Intratracheal challenge of turkey poults vaccinated with locally prepared and commercial aMPV vaccines.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. poults</th>
<th>Vaccine</th>
<th>ROI(^1)</th>
<th>Clinical signs</th>
<th>Postmortem lesions</th>
<th>Protection (%)</th>
<th>Virus reisolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>NS–aMPV(^2)</td>
<td>i.m.</td>
<td>Absent</td>
<td>Absent</td>
<td>100</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>Commercial inactivated</td>
<td>i.m.</td>
<td>Ocular discharge 2/10 poults</td>
<td>Mild tracheitis (2/10)</td>
<td>80</td>
<td>Yes from 2 poults</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>Commercial live attenuated</td>
<td>orally</td>
<td>Ocular discharge 1/10 poults</td>
<td>Mild tracheitis (1/10)</td>
<td>90</td>
<td>Yes from 1 poult</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>Control nonvaccinated</td>
<td>i.m.</td>
<td>Clear TRT signs(^3) 10/10</td>
<td>Clear TRT postmortem lesions (10/10)</td>
<td>0</td>
<td>Yes from 10 poults</td>
</tr>
</tbody>
</table>

\(^1\)ROI = Route of inoculation.
\(^2\)NS–aMPV = *Nigella sativa* oil aMPV inactivated vaccine.
\(^3\)Clear TRT clinical signs represented by sneezing, nasal discharge, foamy conjunctivitis, and swelling of infra-orbital sinuses.
vaccine than turkey poults inoculated with imported vaccines. The phagocytic percentage was 11.75% for locally prepared aMPV vaccines versus 8.25 and 10.25% for commercial aMPV vaccines at 14 d postinoculation and the phagocytic indices was 0.270 and for locally prepared aMPV vaccines versus 0.2 and 0.214 for commercial aMPV vaccines.

Intratracheal challenge of turkey poults at 21 d postvaccination by a dose of 100 μL field aMPV revealed 100% protection in poults that received NS –aMPV vaccine while those received commercial inactivated and commercial live attenuated aMPV vaccines showed 80 and 90% protection, respectively (Table 4). On the other hand, a typical picture of aMPV infection was seen in control nonvaccinated poults. That indicates higher efficacy of the newly prepared inactivated aMPV –NS oil vaccine than the commercial inactivated and live attenuated vaccines used in this study.

CONCLUSIONS AND APPLICATIONS

1. The results obtained from this study revealed the superiority and efficacy of the newly prepared inactivated aMPV–NS oil vaccine over the imported ones.
2. The prepared inactivated aMPV–NS oil adjuvant vaccine is highly potent, of low viscosity, long shelf life, produced minimal tissue reactivity, homogenous in appearance, cost-effective, and has the ability to activate cellular immunity to compensate the effect induced by live attenuated vaccines. So, it will be compatible with mass production.
3. The prepared aMPV–NS oil adjuvant vaccine from recent locally isolated strain will be a good alternative for currently used vaccines and that will help to control the disease in Egypt.

REFERENCES AND NOTES


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