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Enhancing the immunogenicity and protective efficacy of inactivated pigeon paramyxovirus vaccine (PMV-1) using synthetic polymeric adjuvant

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ABSTRACT

Pigeon paramyxovirus type 1 (PPMV-1) is a serious pathogen facing the pigeon industry. Thus, this study aimed to preparation and evaluation of two inactivated vaccines using Carbopol and Aluminum hydroxide-based adjuvants to control such a problem. A total of 45- four weeks old, apparently healthy pigeons were allocated into 3 groups (15 birds each) as follow: group 1 (G1) received 0.5 ml, S/C Carbopol adjuvanted vaccine, group 2 (G2), received 0.5 ml S/C of aluminum hydroxide-based vaccine, group 3 (G3) received 0.5 ml S/C sterile saline and kept as control group. Five serum samples were obtained from each bird weekly post vaccination to measure anti-PPMV-1 antibodies using HI test. At 35 days post immunization, all vaccinated pigeons were challenged with 20µl containing 10^6 EID_{50}/bird of PPMV-1 YA/14 strain by eye drop route, all pigeons were kept under observation for 10 days during the observation period any clinical signs or moralities were recorded also, cloacal swabs were collected 4-, 7- and 10-days post challenge to measure the shedding level. The potency test revealed that the prepared inactivated vaccine (PPMV-1) with synthetic polymeric adjuvants were potent and efficient. By studying the elicited serum antibody dynamics in vaccinated pigeon using HI test showed that antibody, the beak antibody titer reached by the 4th week post immunization (6 and 5 log 2) for both G1 and G2 respectively (but G1 is significantly increase than G2) and continued to the 5th week where it reaches 6.5 and 5.6 log2 for both groups in order. (G1 is significantly increase than G2), which indicated that the PPMV-1 inactivated vaccine in both G1 and G2, results in systemic adjuvant activity, including a long-lasting protective immune response after a single dose administration without any adverse reaction. G1 and G2 showed the lowest shedding level that was zero at the 10th day post challenge and the overall reduction level of both vaccinated groups was 4.9 and 4.5 for both G1 and G2, respectively. However, the prepared inactivated PPMV-1 Carbopol based vaccine is safe and could significantly poses the immune response more effectively than the aluminum hydroxide-based vaccine.

Introduction

Pigeon paramyxovirus type 1 (PPMV-1) is a highly contagious viral disease with high morbidity and mortality rates, as well as trade restrictions that have a catastrophic global economic impact. PPMV-1, also known as Newcastle disease virus (NDV) of pigeons (Ramsubeik *et al*., 2023). Clinical signs are like the nervous manifestations of ND but have fewer visible respiratory indicators. It can be transmitted at any time of year and by any age of pigeons (Ramsubeik *et al*., 2023). The most typical neurological indications that develop during infections with PPMV-1 are head and neck twists (torticollis), instability, paralysis of wings and legs, or difficulty in food intake, and infected birds may have watery or bloody diarrhea (Zhang *et al*., 2018). PPMV-1 is a negative-sense, non-segmented, and single-stranded RNA virus belonging to genus Avulavirus of the subfamily Paramyxovirinae, family Paramyxoviridae (Wang *et al*., 2021).

PPMV-1 originated in the Middle East, notably Iraq, in the late 1970s, but soon progressed into a global outbreak between 1981 and 1983, infecting Europe and beyond (Mansour *et al*., 2021). PPMV-1 is currently found in both domestic and feral pigeons around the world, including the United States and Europe. Despite the control mechanisms in Egypt, PPMV-1 maintained its enzootic status (Mansour *et al*., 2021).

Control of PPMV infection includes substantial legislative measures, including stamping out and trade restrictions, which may result in significant economic losses (Aldous *et al*., 2014). Dortmans *et al*. (2011) and Pestka *et al*. (2014) found that infected pigeons with PPMV-1 suffered clinically from sneezing, gasping, greenish diarrhea, drop in egg production accompanied with nervous manifestation including tremors, spasms, circling, paralysis, dropping in wings, torticollis (twisted of neck) and may lead to mortalities among the infected birds.

Vaccination reflected the best method for control of the PPMV-1, mainly heterologous vaccination using the lentogenic NDV strains (HB1 and Lasota strains) either live or inactivated oil adjuvanted vaccine beside the bio-safety measures (Mansour *et al*., 2021). Vaccination of pigeons is needed to be sure that the disease outbreaks are controlled, and their impact is diminished (Soliman *et al*., 2019). The use of live NDV vaccines produce strong humoral and cell mediated immune responses, which can protect vaccinated individuals from infection and decrease or even limit the viral shedding (Kapczynski *et al*., 2013). On the other hand, studying the cross-HI testing indicated that PPMV-1 had a clear antigenic difference with lentogenic vaccine strain Lasota, which results in escape of the variants viruses from the immune response followed by vaccination failure (AKhtar *et al*., 2016). Studies proven that, using a homologous inactivated vaccine with good adjuvant either S/C or IM could achieve a complete protection from lethal infection could reduce but not eliminate the shedding level (Al-Alhially *et al*., 2024).

Aluminum hydroxide-based adjuvant Al (OH)3 have been used in vaccines since the 1930, it known to discriminatory superior Th2-type immune responses by triggering the attraction and antigen uptake by antigen-presenting cells (APCs) and it enhance both Th1 as well as Th2 cellular response also activate endogenous-cellular immune responses mediated by NLRP3 and promote macrophages to secrete high-levels of pro-inflammatory factors such as IL-1β and IL-18 (Yang *et al*., 2024). However, it depends on the vaccination route. Vaccines containing aluminum hydroxide-based adjuvants are beneficial, sometimes they cause adverse reactions. Further, these vaccines cannot be stored frozen (He *et al*., 2015). Subcutaneous (S/C) inactivated PPMV-1 based on aluminum

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hydroxyl-based formula results in a higher level of humoral immune response accompanied with low level of virus shedding than the oil-based vaccine (Soliman *et al*., 2019).

Carbopol is a promising synthetic anionic polymer adjuvant can enhance the immune system through increasing the humoral immune response also it is able to attract and triggered leukocyte, initiation of pro-inflammatory cytokine secretion and antigen capture by APCs besides, direct B-cell activation, possibly acting as an antigen delivery system (Gartlan *et al*., 2016). Carbopol consider ideal adjuvant because it is safe, stable, biodegradable, inexpensive, and it can stimulate specific immune response against the target antigen and ensuring that the vaccination potency is reproducible during manufacturing, also no suggestion of local or systemic reactions were recorded (Aly *et al*., 2020). During preparation of vaccine, antigen is mixed with the Carbopol gel by gentle shaking, and the produced vaccine formula is homogenous emulsion with no separation also, Carbopol is safer for mammals and more effective than antigens alone (Zhang *et al*., 2018). It has been found to boost the duration and strength of antibody responses induced by the inactivated vaccination (Zhang *et al*., 2018). Immunogenicity is regarded a critical criterion in measuring vaccines' efficiency and is measured by the titer of antibody-induced following injection and measure the shedding level of virus in environment, so this study conducted to compare the immune response and the protection level of inactivated PPMV-1vaccine by using synthetic polymeric adjuvant (water-soluble acrylic acid- Carbopol) and inactivated PPMV-1 vaccine by using aluminum hydroxide-based adjuvant.

Materials and methods

Ethical approval

This work is ethically approved by the Institutional Animal Care and Used Committee (Vet. CU. IACUC) with code Vet CU 18042024924.

Vaccine strain and challenge viruses

Virulent local isolate PPMV-1 YA/14 (accession. # KX708505.1) was kindly provided by Prof Dr. Yousef Adel Soliman (Biotechnology Department, Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Agricultural Research Center (ARC), Egypt; and used for the preparation of vaccine and the challenge experiment.

Adjuvants

Synthetic polymeric adjuvant Carbopol was supplied by Lubrizol Co. In the form of a fluffy white powder. 0.5% aqueous stock solution was prepared in hot water, sterilized by autoclaving and stored at 4°C until further use (Aly *et al*., 2020). Alum gel was prepared and provided by Veterinary Serum and Vaccine Research institute (VSVRI) and level of 30% was used in vaccine preparation.

Embryonated eggs

Nine-day old specific pathogen free (SPF) embryonating chicken eggs (ECE) were purchased from the National Project for SPF eggs, El-Fayoum-Egypt and used for preparation of the target vaccine.

Pigeons

Four-weeks old pigeons purchased from a local loft appeared clinically healthy and free from detectable antibodies against PMV1 infection as screened by Haemagglutination inhibition (HI) test using PPMV1 YA/14 strain as antigen. Pigeons were kept in the Bio-isolators of The Central Laboratory For Evaluation of Veterinary Biologics (CLEVB) under restricted measures, and they were supplied with sterilized water and autoclaved balanced feed that meet the birds' requirements.

Preparation of the PPMV-1 vaccine

Egg inoculation

PPMV-1 was aseptically inoculated through the allantoic sac route of nine-days old SPF-ECE, the allantoic fluid harvested after 72 h and kept at -80°C till use. Following NRC (1971), a ten-fold serial dilution of the harvested virus was inoculated in 9 days old SPF -ECE (5 eggs for each dilution) to determine the virus titer and estimate the virus infectivity using the formula of Reed and Munch (1938).

Virus inactivation

The harvested titrated virus was clarified by centrifugation at 10,000 rpm for 10 min at 4°C then 0.1% formalin was used for virus inactivation, added drop wise to the virus suspension with low speed stirring for 24 h at 37°C and kept at 4°C overnight (Soliman *et al*., 2019).

Detection of complete virus inactivation

Complete virus inactivation was ensured for residual viral infectivity through three blind egg passages by inoculation of 9-day old SPF-ECE with undiluted virus through the allantoic route. Incubated for 5 days at 37°C and HA assay was applied for any outstanding live virus

Vaccine formulation

The vaccine was prepared following the standard operating procedures (SOPs) and the legislation of the Veterinary Serum and Vaccine Research Institute (VSVRI) for the formulation of inactivated vaccine. For inactivated PPMV-1 Carbopol adjuvanted vaccine an equal volume of the inactivated virus was mixed with the aqueous solution of Carbopol and then neutralized with 20% sodium hydroxide (Aly *et al*., 2020) while for the inactivated PPMV-1 Aluminum hydroxide-based adjuvant (Al OH_3), alum was added as 30 % (Soliman *et al*., 2019).

Quality control of the prepared vaccine

Safety and sterility testing of the prepared vaccine formulation were applied following the standard procedures of WOAH (2023). Prepared vaccines were cultured on aerobe and anaerobic media and observed for one week for the growth of any contaminants. Ten SPF one day old chicks were inoculated with a ten-time vaccination dose to assess the safety of the prepared vaccine.

Experimental design for evaluation of the prepared vaccines

Pigeon immunization

Forty-five 4-weeks old clinically healthy pigeons were kept in Bio-isolators and divided into 3 groups (15 birds/ each) as follow: Group 1 (G1) received 0.5 ml s/c of Carbopol adjuvanted vaccine. Group 2 (G2) received 0.5 ml s/c of aluminum hydroxide-based vaccine. Group 3 (G3) received 0.5 ml s/c of sterile saline and was kept as control group.

Five blood samples from the wing vein were obtained from each group (individually tested, not pooled) weekly post vaccination to measure anti-PPMV-1 antibodies using HI test. At the 5th week (35 days of vaccination) all vaccinated birds were challenged with 20 µl containing 10^6 EID_{50} / bird of PPMV-1 YA/14 strain by the eye drop route (Soliman *et al*., 2019). All pigeons were kept under observation for 10 days post challenge and any clinical signs of infection or moralities were recorded.

Cloacal swabs were collected 4-, 7- and 10-days PC to follow up the virus shedding.

Evaluation of the prepared vaccine potency

The prepared inactivated vaccine was evaluated according to the policy of the Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), through the detection of the level of specific PPMV-1 antibodies using HI test, measuring the shedding level of the virus after the challenge test and assessing the protection rate and of both vaccines after the challenge effect.

Detection of the level of specific PPMV-1 antibodies

Using HI test according to OIE (2021), on brief, two-fold serial dilution of the collected serum samples from all groups were incubated for 20 min with 4HAU of PMV, then 1% washed chicken RBCs was added. The antibody titer is the highest serum dilution that can prevent the HA activity of the virus.

Following up the level of virus shedding

From all living birds 4-, 7- and 10-days PC, cloacal swabs were obtained. In 1 mL sterile saline the swabs were vortexed, then centrifuged at 14000 rpm for 10 min and filtered through 20 µØ Millipore filter. To estimate the shed virus titer, tenfold serial dilutions of each swab at different point times, were inoculated in 9- day old SPF –ECE (5 eggs per dilution), incubated at 37 °C for 5 days then chilled, each egg was tested for HA activity. The virus titer was estimated by the formula of Reed and Munch (1938).

Data analysis

The statistical analysis was performed using graph pad prism version 10. HI titer of the two different vaccines were analyzed using two-way ANOVA (multiple comparison) with Tukey analysis as a post hook test.

Results

Preparation of PPMV vaccines

Inoculation of the YA/14 strain in 9-day SPF-ECE showed that all inoculated eggs died after 72 h of inoculation and the collected allantoic fluid showed an HA titer 11 log_2, and EID_{50} 10 log10. After inactivation with formaldehyde, the virus fluid showed HA titer 10 log_2. Completion virus inactivation was confirmed through three successive passages in ECE showing no embryo mortalities and no HA activity of the inactivated virus. The two prepared vaccine formulas were sterile where there was no observed microbial growth along the observation period also the prepared vaccines were safe, and birds appeared healthy and active along the inspection period.

Evaluation of the prepared PPMV vaccines formulas

Measuring the elicited humoral immune response after vaccination

The antibody titer induced in response to both vaccine formulae was shown in Figures 1 & 2. The demonstrated data showed that, both vaccinated pigeon groups exhibited detectable antibody titer by the first week post vaccination (2.8 and 1.1 log_2 for G1 and G2 respectively). The beak antibody titer reached by the 4th week post vaccination (6 and 5 log_2 for G1 and G2 respectively) and continued to the 5th week where it reached 6.5 and 5.6 log_2 for both groups in order. Statistical analysis revealed the Carbopol adjuvanted group showed a significant difference $p < 0.05$ in the HI titer at the fourth and the fifth week.

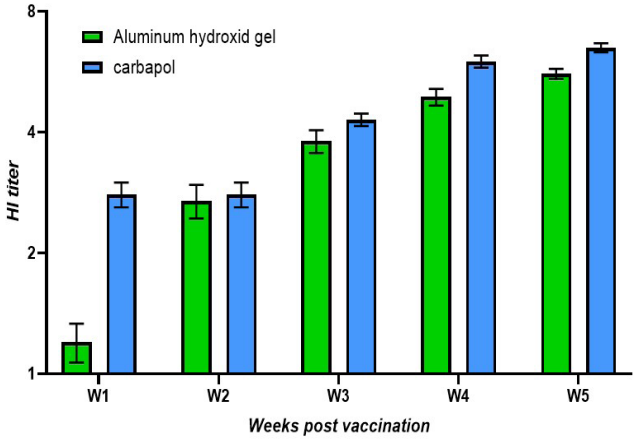

Fig. 1. Antibody titer (Mean HI titer (log_2) ±SE) of aluminum hydroxide and carbapol adjuvanted PPMV vaccine produced from different immunized groups.

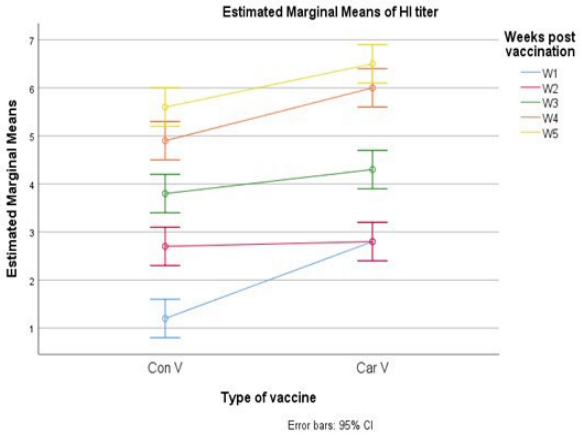

Fig. 2. The mean differences of HI Titer against aluminum hydroxide and carbopol adjuvanted vaccine.

Clinical signs and protection rate post Challenge (PC)

The control un -vaccinated birds showed characteristic clinical symptoms that manifest by the third day PC, and within 5 days PC, all birds displayed the classic signs of PMV infection, including, fine tremor of the head, lameness and Torticollis. By the 6th day PC all birds in the control group died. The postmortem examination (PM) showed a slight hemorrhage on the proventriculus, and hemorrhage in the brain. In the two immunized groups, Carbopol and aluminum hydroxide, six birds from both G1 and G2 of the challenge vaccinated birds displayed mild clinical signs (anorexia and general signs of illness), two birds in G1 and 3 birds in G2 showed greenish diarrhea, but no nervous signs were observed. There were no deaths in the Carbopol adjuvanted group, which had 100% protection, whereas just one bird died in the aluminum hydroxide group on day 6 after the challenge, with a protection of 93.3%. In the control un-vaccinated group 15 birds died (5 birds followed by another 10 birds at the 4th and 5th day PC respectively.

Monitoring of the virus shedding level

The shedding level of the virus was measured from the cloacal swabs as seen in Figure 3, G1 and G2 showed the lowest shedding level titer, and the shedding level was zero at the 10th day PC. The overall reduction level of both vaccinated groups was 4.9 and 4.5 for both G1 and G2 respectively.

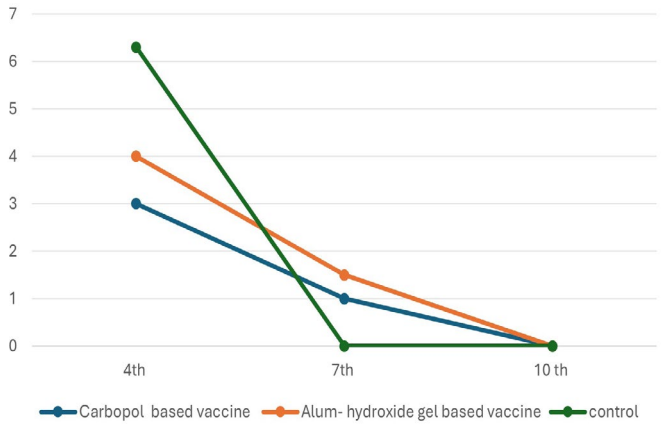

Fig. 3. Virus shedding at different time pints post challenge at different groups.

Discussion

Up to 75% of birds infected with paramyxovirus die within 3 days (Pestka *et al*., 2014), thus the main goal of vaccination side by side with application of biosecurity measures are to protect birds from viral infections that can be prevented by eliciting a robust and long-lasting immune response; to accomplish this goal, an adjuvant is added to enhance the potency of the vaccine (Sivakumar *et al*., 2011).

Vaccine adjuvants are crucial in increasing the immune response to vaccines and improving their efficacy, effective adjuvants can boost vaccine efficacy through, eliciting specific antibodies with high avidity and affinity, and boosting cytotoxic T lymphocyte (CTL) responses (Moni *et al*., 2023). The main advantage of aluminum-based vaccines is the ability to produce a higher humoral immune response even after initial immunization (Baylor *et al*., 2002). This is mostly due to the creation of a short-term depot at the injection site, which slowly releases antigens and is easily phagocytosed, thereby stimulating immune systems. However, various adverse effects have been recorded, such as granulomas and erythema, after vaccine is S/C or intra-dermal applied rather than intramuscularly (Butler *et al*., 1969).

Carbopol administration adjuvants increase the' immunogenicity of the used antigen and thus increase the efficacy of the produced vaccine (Coffman *et al*., 2010) and recently, have been evaluated as adjuvants in veterinary vaccines (Gualandi *et al*., 1988; Tollersrud *et al*., 2002; Liu *et al*., 2005; Hoogland *et al*., 2006; Mair *et al*., 2015). During this study, two vaccine formulations of PPMV were prepared using aluminum hydroxide gel and Carbopol. The mean HI titer estimated by HI test revealed that humoral antibodies produced by Carbopol based is significantly elevated than the antibodies produced by aluminum hydroxide gel-based vaccine especially at the 1st, 4th and the 5th week post immunization, this may be due to the ability of Carbopol to direct B-cell activation (Gartlan *et al*., 2016). This result in concur with Soliman *et al*. (2019) where their study has main conclusion that the aluminum hydroxyl-based vaccine can elicit a higher antibody titer with lower shedding level when compared with the oil-based vaccine. During challenge, the non-vaccinated group showed typical nervous signs of PMV infection however the two vaccinated didn't show any nervous signs, these results go with Pestka *et al*., (2014) found that infected pigeons with PPMV-1 suffered clinically from sneezing, gasping, greenish diarrhea, drop in egg production accompanied with nervous manifestation including tremors, spasms, circling, paralysis, dropping in wings, torticollis (twisted of neck)

Regarding the effect of both vaccine formulation on the protection level and the shedding after challenge, the protection rate was 100% and 93.3% for both Carbopol and aluminum hydroxyl-based vaccine respectively, on the other hand the shedding level of Carbopol was moderately lower than aluminum hydroxyl gel vaccine, this may be related to the significant differences between the antibody level produced at the 4th and the 5th week post immunization .During the study of Soliman *et al*. (2019) they discuss the ability of aluminum hydroxyl gel vaccine to stimulate and produce high interferon gamma level that was able to decrease the shedding level. Regarding immune response to Carbopol was also studied by Mair *et al*. (2015) and confirmed that Carbopol can induce early IFN-γ production stimulate and T cell differentiation to effector phenotypes thus improves cellular immunity. Even with the use of two powerful immunogenic adjuvant (Carbopol and aluminum hydroxyl gel), shedding still appears, and this is in agree with Al-Alhially *et al*. (2024) where their study confirm that, using of a homologous inactivated vaccine with good adjuvant either S/C or IM could achieve a complete protection from lethal infection could reduce but not eliminate the shedding level.

Conclusion

The prepared inactivated PPMV-1 Carbopol based vaccine is safe and could significantly poses the immune response more effectively than the aluminum hydroxide-based vaccine.

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Conflict of interest

The authors have no conflict of interest to declare.

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