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Efficacy of Prepared Oil Inactivated Pigeon Paramyxo Vaccine.

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ABSTRACT

Control of pigeon paramyxo virus infection relays on the production of a vaccine that is abl to protect the birds from the lethal virulent infection as well as reducing shedding level t prevent virus circulation in the environment. In the current study inactivated vaccine wa prepared from the recent field isolate (PPMV-1 YA/14) which is extensively characterize and identified. The vaccine was prepared in two formulas. The aluminum hydroxide gel (give S/C and I/M) and the oil adjuvanted formula. Evaluation of the 2 formulas revealed that th gel-based formula gave a higher antibody titer than the oil-based preparation during the cours of the vaccination period and after challenge. The cellular immune response was als evaluated by the qRT-PCR analysis of the IFN-γ transcripts and it was found that the oil-base formula gave a high fold change than the gel-based formula although it was not reflected i decreasing the shedding level. Studying the shedding level by the EID_{50} and qRT-PCR of th cloacal swabs after challenge revealed that the gel-based formula administrated S/C gav lesser degree of shedding than the oil-based formula.

In conclusion the gel-based formula of the vaccine prepared from the recer characterized PPMV-1 YA/14 isolate could protect pigeon from lethal infection with th virulent virus and could reduce but not eliminate the shedding level.

1. INTRODUCTION

Pigeon paramyxovirus type 1 (PPMV-1) is classified as an antigenic variant of avian paramyxovirus type 1 (APMV-1) , among Newcastle disease virus , they included in list A of the Office International des Epizooties (OIE) infection in avian species. It is caused by avian paramyxovirus serotype -1 (APMV-1) which is classified in the genus Avulavirus of the subfamily Paramyxovirinae, family Paramyxoviridae, (Abolnik et al., 2006).

Due to its high economic losses, it is paid attention of many researchers for the control of the PPMV infection. Control of PPMV depends mainly on vaccination beside the control of lateral transmission.

Vaccination trials depended mainly on the using of the heterologous strains of NDV (HB1 and Lasota strains) either live or inactivated oil adjuvanted vaccine (Viaen et al., 1984; Kaleta et al., 1985 and Amer et al., 2013).

Despite the use of such vaccine formulation, PPMV-1 is still enzootic in pigeons in some countries (Alexander 2001) for this reason the using of homologous vaccine is the most suitable solution for the control pigeon paramyxo infection.

In Egypt however, the commercially available vaccine was prepared from PPMV-1 isolate which was isolated back to the year 1983, till the current date, updating the isolate from which the vaccine was prepared was never achieved. Recently a very virulent PPMV-1 was isolated in Egypt during the year 2014

and was molecular identified (Soliman et al., 2016) this strain was designated as PPMV-1 /YA-14. This newly identified strain was used for the preparation of the current commercially available pigeon paramyxo vaccine by Vet. Serum and vaccine research institute.

Hence in this work we aim to perform in-depth investigation for the immune response either humoral or cellular and the protective efficacy as well as the shedding level after challenge of inactivated oil adjuvanted and aluminum hydroxide gel formulated PPMV vaccine prepared from the PPMV-1/YA-14 strain.

2. MATERIALS AND METHODS

2.1. Virus

Virulent PPMV local isolate PPMV-1 YA/14 (Soliman et al., 2016) was used in the current study, the virus was completely identified and genetically characterized, and the partial sequence of *f* gene was submitted in the gene bank (accession numberKX708505.1).

2.1.1. Virus purification by sucrose gradient ultracentrifugation:

The virus preparation was filtrated through 0.22µØ syringe Millipore filter before inoculation in 9 days old SPF embryonated chicken eggs (SPF-ECE) via the Allantoic route (n=7). The eggs were incubated at 37°C in a humid champers till embryo death (usually within 3-5 days). Cell debris and high molecular weight proteins were removed from the propagated virus on ECE by centrifugation at 14.000rpm/10min /4 °C, then the virus suspension was laid onto sucrose cushion (3 mL 20% and 3 mL 50% sucrose prepared in TNE buffer [20 mM Tris-HCl (pH 7.), 100 mM NaCl, 2 mM EDTA].) and centrifuged for 2h at 30000rpm in Sorvall® Surespin™ 630 swinging bucket ultracentrifuge rotor using Sorvall WX 100 ultracentrifuge (thermo Fisher scientific, USA). The layer contain the virus was aspirated, and the virus particles were sedimented at 65000rpm/3h/4 °C using sorvall. The sedimented virus was resuspended by gentle agitation in1 mL of TNE (prepared with nuclease free water) overnight at 4 °C and stored at -80 °C till used (Serdyuk et al., 2007 and Soliman et al., 2016). One hundred µl of the purified virus was titrated using EID_{50} (Reed and Meunch 1938). This purified virus was used in preparation of the vaccine and the challenge test.

2.2. Preparation of PPMV-1 vaccines.

2.2.1. Preparation of the virus seed.

Purified virus was inoculated in 9 days old SPF-ECE via allantoic route at a concentration of $2Log_{10}$ EID_{50}. Dead eggs (usually within 3 days) were opened and the virus was collected, titrated and adjusted at 10^9 EID_{50}/ 0.1 ml before formalin inactivation.

2.2.2. Virus inactivation.

The virus preparation was clarified by centrifugation at 10,000 rpm/10 min /4 °C. Formalin was added drop wise to virus suspension at a concentration of 0.1% and kept with low speed stirring for 24hrs at 37°C (Razmaraii et al., 2012). The suspensions were centrifuged as before and kept at 4°C overnight.

2.2.3. Screening for Virus Infectivity.

Inactivated virus preparations were tested for residual viral infectivity by inoculation of 9 d old SPF-ECE with undiluted and 10 folds' serial dilutions via allantoic route. Eggs were incubated for at least 5 days at 37°C. Chorioallantoic fluid was collected and tested for any residual live virus by traditional HA assay. For residue of any viral nucleic acid, viral RNA was extracted from the chorioallantoic fluid using QIAamp viral RNA mini kit (Qiagen, Germany) from the allantoic fluid, quantitative RT-PCR was done (Soliman et al., 2016).

2.2.4. Vaccine formulation.

Two vaccine formulas were prepared, the aluminum hydroxide gel adjuvanted vaccine and the oil adjuvanted vaccine using montanide ISA71-VG (Société d'exploitation pour les produits de l'industrie chimique SEPPIC cat # 36514P) according to the manufacture instructions.

2.3. Experimental design for evaluation of prepared vaccine.

2.3.1. Pigeon immunization.

Forty clinically healthy pigeons (four weeks old) were obtained from commercial lofts confirmed to be sero-negative against PPMV type 1 using HI assay (using PPMV1 YA/14 strain as antigen) were kept in Bio-isolators to prevent exogenous infection during the experiment and divided into 4 groups (10 birds each) and vaccinated as in table 1. Heparinized blood and serum samples were obtained from all ten birds (tested individually, not pooled) from all groups via wing vein route at 3rd, 5th , 7th, 15th and 21st day post vaccination for IFN-γ transcript quantitation using qRT-PCR and anti-PPMV-1 antibodies using HI assay. After 21 days of vaccination all pigeons were challenged with 20µl containing 10^5 EID_{50} / bird of PPMV1 YA/14 strain by eye drop route (Soliman et al., 2016), all pigeons were

kept under observation for 15 days. Any clinical signs of infection or mortalities were recorded. Heparinized blood and serum samples were collected at the 3rd, 5th and 7th day post challenge to evaluate the immune response. Cloacal swabs were collected 7 days post challenge to measure the virus shedding.

2.4. Measuring the level of anti-PPMV antibodies using HI test.

The level of antibodies in the sera from pigeons in each group was measured using standard HI test according to OIE 2012.

2.5. Analysis of IFN-gamma transcript using qRT-PCR assay.

Interferon gamma quantification was done according to Kaiser et al., 2003

2.5.1. Purification of paramyxovirus protein antigen.

The viral proteins of PPMV-1 YA/14 strain was purified using Trizol Reagent (Invitrogen cat No 15596026) according to the manufacture instructions.

Briefly, 300 µl of the purified virus was vortexed for 15s with equal volume of Trizol reagent till complete homogenization then 100 µl of chloroform was added and centrifuged at 12000 g/15min /4 °C. The upper aqueous phase was discarded, and the lower organic phase was collected then 500 µl of absolute ethanol was added and centrifuged as before. The protein in the supernatant was precipitated with 500 µl of absolute iso-propanol and washed twice with 500µl of 0.3 M guanidine hydrochloride in 95% ethanol. The purified protein was resuspended in 1% SDS containing CAHPS and the concentration of the protein was measured using Qubit II assay (Invitrogen Cat # Q33211) and adjusted at 5µg/ml. The protein preparation kept in aliquots in Low bind poly propelling tubes at -80 °C till used.

2.5.2. Purification of peripheral blood mononuclear cells (PBMCs) from pigeon groups.

Three milliliters of heparinized blood were diluted 1:1 with sterile PBS pH7.2 and the PBMCs were separated by density-gradient centrifugation using Ficoll-Paque (density, 1.077 g/ml; biowest cat# L0560-500) and washed twice with RPMI 1640 medium (Invitrogen, Burlington, Ontario, Canada). The PBMCs were diluted in RPMI 1640 containing 10% fetal calf serum (Invitrogen) and supplemented with penicillin G and streptomycin (100 U/ml and 100 mg/ml, respectively) and then counted using trypan blue dye exclusion assay and adjusted at $1X10^6$/ml. cells were dispensed in 96 well tissue culture plate (200 µl/ well). To induce the IFN-γ transcript, the cells were pulsed with either 10 µl of PPMV protein (specific stimulant) or 10µl of phytoheamagglutinin (PHA) at a concentration of 10µg/ml (nonspecific positive control stimulant) or with PBS (unstimulated negative control cells). Cells incubated at 37 °C/ 20 h in 5% CO2 incubator. After 20h, the cells were harvested, and subjected to RNA extraction using Trizol reagent

2.5.3. Quantification of the IFN- γ transcript.

The level of IFN-γ mRNA from cultured PBMCs pulsed with either PPMV proteins or PHA or the un-pulsed culture was measured using qPCR assay (Pete et al., 2003 and Yeong et al., 2014).

The total cytoplasmic RNA from the stimulated and unstimulated PBMCs were purified using Trizol reagent (Thermos fisher scientific Cat # 15596026) according to the manufacture instructions. All RNA samples were treated with RNase-free DNase I (Qiagen cat# 79254) to remove any traces of genomic DNA contamination. RNA was then reverse transcribed to cDNA with the Omniscript cDNA Synthesis kit (Qiagen Cat # 205111). Briefly, approximately 50ng of the purified total RNA was reverse transcribed in a final volume of 20 µl of reaction mix containing 1X RT buffer, dNTPs mix (5 mM each), ribonuclease inhibitor (10 U/ml), and 4 U of Omni script reverse transcriptase (RT) enzyme. The samples were incubated at 37 °C for 60 min. To control for genomic DNA contamination, every reaction set contained an RNA sample without Omniscript RT enzyme (negative RT reaction), which was carried forward into subsequent PCR reactions. The resulting cDNA was stored frozen (-80°C) until assayed by real-time PCR.

The real-time PCR reaction mixture contained 5µl of sample cDNA in 20 µl final reaction volume containing 200 nM of the probe and 100 nM each of forward and reverse primers (sequence shown in table 2), and1X of the brilliant II qPCR master mix (Agilent cat # 600804). The 5µl volume of cDNA sample was added to the initial reaction mixture as a 1:2 dilution of the RT reaction in DNase/RNase-free water. Each set of reactions also included negative controls (i.e., water instead of cDNA sample). the reaction was run on Agilent MX3005P instrument using the following cycling parameters: Initial activation of Taq DNA polymerase in the PCR core reagent, and denaturation of DNA, all at 95°C/10 min; and then 40 cycles of denaturation at 95°C/20 sec and annealing with primer at 50°C/20 sec and extension at 60°C/45 sec, with the

fluorescence data being collected at the end of the extension step.

Optical data obtained by real-time PCR was analyzed using the default parameters available with the MX pro 3005P software (Agilent). The PCR threshold cycle number (C_t) for each tested cDNA sample was calculated at the point where the fluorescence exceeded the threshold limit. The threshold limit was fixed along the linear logarithmic phase of the fluorescence curves at 10 standard deviations above the average background fluorescence. The average C_t for duplicate samples was calculated.

β actin genes were used as housekeeping non-regulated reference genes for normalization of target gene expression. Results were analyzed using comparative Ct method. Relative transcript abundance of the IFN-γ gene equals ΔCt values (ΔCt = C_t reference – C_t target). Relative changes in transcript ($2^{\wedge\ -\Delta\Delta Ct}$)are expressed as ΔΔCt values between the different groups (Schmittgen and Livak 2008).

2.6. Measuring the level of shedding.

Cloacal swabs were taken from all living and dead birds 7 days post challenge. The swabs were swirled in 1mL sterile saline, centrifuged at 14000rpm/10 min and filtered through 20μØ Millipore filter. The virus titer was then measured either with EID50 or by QRT-PCR (Soliman et al., 2016).

2.7. Data analysis.

The statistics (ANOVA test) was done using SPSS v21 windows platform and relative gene expression analysis was done using REST 2009 software. EID50 was calculated using Reed and Muench 1938.

Table (1) the vaccination schedule.

Group	Vaccine	Dose and the rout of vaccination
Group-1 G1	negative control group kept without vaccination	0.5 mL phosphate- buffered saline (PBS) S/C
Group-2 G2	inactivated pigeon paramyxo virus aluminum hydroxide gel adjuvanted vaccine	0.5ml S/C In the dorsal aspect of the neck.
Group-3 G3	inactivated pigeon paramyxo virus oil adjuvanted vaccine	0.5ml S/C In the dorsal aspect of the neck.
Group-4 G4	inactivated pigeon paramyxo virus aluminum hydroxide gel adjuvanted vaccine	0.5ml injected I/M In the thigh muscle.

Table 2: The sequence of both primers and probes for both, the target IFN-γ gene and β actin reference gene.

Gene		Sequence	Ref
IFN-γ	Forward primer	GTGAAGAAGGTGAAAGATATCATGGA	**Kaiser et al., 2003**
	Reverse primer	GCTTTGCGCTGGATTCTCA	
	Probe	Fam- TGGCCAAGCTCCCGATGAACGA -Tamra	
β actin	Forward primer	CTCCATCATGAAGTGTGACGTT	**Hong et al., 2006**
	Reverse primer	ATCTCCTTCTGCATCCTGTCAG	
	Probe	Fam-CAAGGACCTCTATGCCAACACAGTGCT-Tamra	

3. RESULTS.

3.1. Preparation of the PPMV vaccine.

The YA/14 strain was subjected to purification in order to obtain a high-quality virus seed to be used for the vaccine preparation. The EID_{50} of the purified PPMV-1 YA/14 strain was estimated to be $10^{9.8}$. This master seed virus was further inoculated in ECE, egg deaths were beginning at the 3rd day post inoculation and the harvested allantoic fluid gave HA titer of 14 Log_2 (Arithmetic mean) while the formalin inactivated virus gave HA titer of 12 Log_2. To test for viral infectivity, the inactivated virus was 10-fold serially diluted in inculcated in ECE for at least 3 successive times, no egg deaths was recorded and the collected allantoic fluid showed no HA activity. qRT-PCR was performed to ensure the complete inactivation and the results were negative for all tested eggs (fig 1), while the RNA extracted from the live seed virus gave a C_t of 14.49

3.2. Evaluation of the PPMV vaccine.

3.2.1. Results of Challenge test through clinical signs and mortalities

In the control non-vaccinated group, clinical signs began to appear by the 3rd day post challenge with maximum 5 days all birds showed the typical sings of paramyxo infection such as fine tremor of eyes or head, Twisting neck, turning in circles paralysis of the legs and wings with the head twisted fully upside-down (torticollis). In the P/M there was few hemorrhagic foci on the proventericulous and sever petechial hemorrhagic on the brain surface. Deaths begin with the forth days post challenge and all the birds in the control group were died by the 7th days post challenge. Concerning the G2 and G4 which receive the gel formula vaccine, clinical signs were so mild (slight wing drops but without any head twisting and slight anorexia without deaths of any bird.

The G3 have the same signs but with more sever and 3 birds (30%) were dead by the 7 days post challenge. some minor clinical signs appears on some pigeons and they ranges from loss of appetite, and fine eye tremors. no paralysis or drop of the wings have been observed. P/M examination showed some lesions like hemorrhagic spots on proventericulous but no hemorrhagic pitchy were observed on the brain

3.2.2. Measurement of the humoral immune response by HI test.

The humoral immune response against the vaccine was measured for all groups using the standard HI titer, table 3 and fig 2 showed the mean HI titer of each group.

Data obtained revealed that G2 had a relative higher titer among all groups during vaccination and after challenge, while the G4 was the least. Comparative analysis of the mean HI titer of the four groups during the experiment showed that the G2 gave a mean titer which remains higher among the 4 groups during the vaccination and after challenge while G4 with the lower except after 15 d post challenge when the mean HI titer was higher than G3.

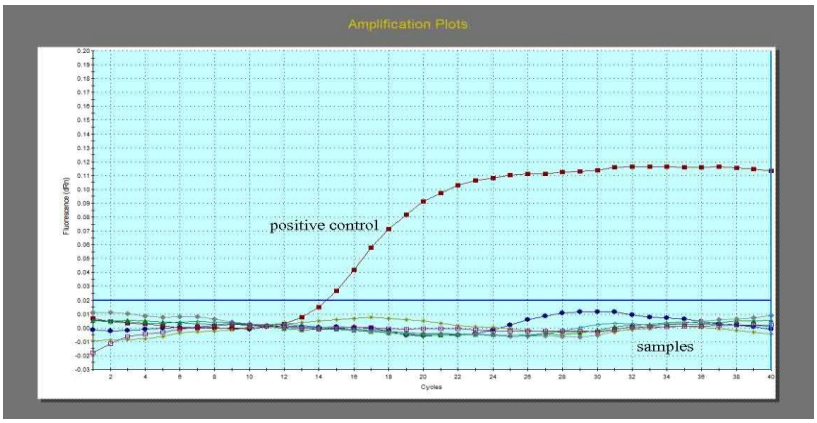

Fig (1) The qRT-PCR of the F gene of the allantoic fluid of eggs inoculated with 10-fold serially diluted inactivated PPMV-1 YA/14 isolate. Live virus was included in the assay (positive control). Note that all the tested inactivated samples were negative (no Ct) whereas the positive control gave a Ct of 14.49.

Table (3) Mean HI titer in sera of vaccinated and control pigeons in different groups determined at different time points post vaccination and challenge

groups	Arithmetic mean of HI titer ± SE								
	Days post vaccination					Days post challenge			
	3d	5d	7d	15d	21d	3d	5d	7d	15d
G1	0	0	0	0	0	0	0.2±0.13	Nd*	Nd*
G2	0	2.4±0.16	4.1±0.23	6.6±0.16	9.3±0.15	6.7±0.15	8.7±0.15	9.2±0.13	10.4±0.16
G3	0	1.3±0.21	1.8±0.13	3.5±0.16	7.8±0.24	5.7±0.15	7.1±0.17	7.3±0.15	7.6±0.3
G4	0	0	1.7±0.15	2.2±0.13	5.6±0.16	3.8±0.13	5.1±0.1	7.5±0.16	8.3±0.15

*Nd not determent as the birds dead by the 7th day in the unvaccinated (G1) group
G1: negative control group kept without vaccination
G2: group vaccinated S/C with inactivated pigeon paramyxo virus aluminum hydroxide gel adjuvanted vaccine.
G3: group vaccinated S/C with inactivated pigeon paramyxo virus oil adjuvanted vaccine
G4: group vaccinated I/M with inactivated pigeon paramyxo virus aluminum hydroxide gel adjuvanted vaccine.

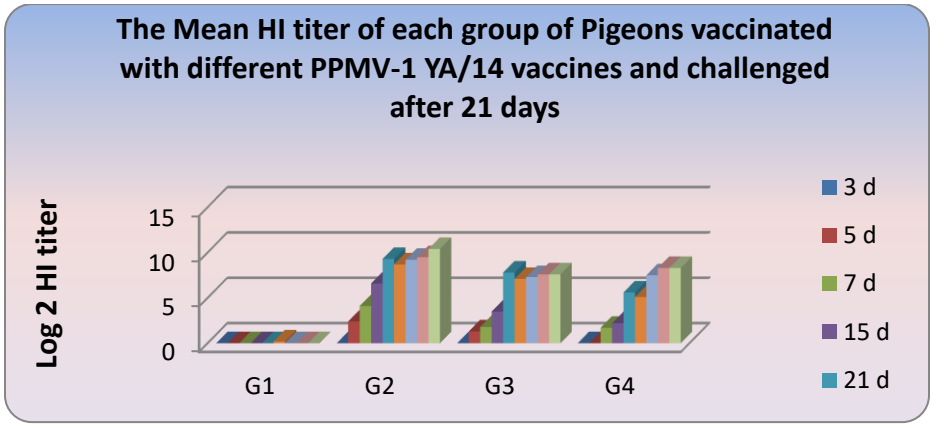

Fig (2). The Mean HI titer of each group of Pigeons vaccinated with different PPMV-1 YA/14 vaccines and challenged after 21 days during the time points of sampling

3.2.3. Measurement of the cell mediated immune response by quantitation of IFN-γ using real time PCR:

After vaccination, G2 and G3 showed slight elevation of the transcript level by the 5th day and seen till the 7th days post vaccination without any further change in the expression level till the 21th day post vaccination (table 4) both groups showed significant up regulation in the IFN-γ gene expression when compared with G1 P < .05 (table 5). The expression level of IFN-γ in the G3 with also significantly higher than G2 at day 5 till 21th day post vaccination. Comparison between G1 and G4 revealed that no detectable level during the 21 days post vaccination. However, after challenge, G1 control unvaccinated group showed increased level of IFN-γ transcript by the 3rd days post challenge which lasted till the death of all birds. G2 and G3 showed moderate increase of the transcript level by the 3rd day post challenge that lasts till the 7th days post challenge. It was noticed that G3 level of transcript was significantly higher than that of the G2 (P<0.05) (table 5) . G4 group showed slight increase in the level of the IFN-γ transcript which is far lower than G2 and G4.

Table (4). The mean of normalized C_t of target gene (IFN-γ) after vaccination and post challenge

	Mean of Normalized ΔCt of the target IFN-γ gene							
	Days post vaccination (PV)					Days post challenge (PC)		
	3d	5d	7d	15d	21d	3d	5d	7d
G1	22.173	21.973	22.102	21.973	22.073	-11.287	-8.195	22.073
G2	21.973	-16.956	-15.997	-17.292	-17.57	-8.899	-8.719	-9.135
G3	21.973	-15.287	-14.167	-14.234	-14.49	-4.862	-3.225	-8.996
G4	22.204	22.184	22.183	22.204	22.184	-17.262	-17.308	-17.164

G1: negative control group, kept without vaccination
G2: group vaccinated S/C with the inactivated pigeon paramyxo virus aluminum hydroxide gel adjuvanted vaccine
G3: group vaccinated S/C with the inactivated pigeon paramyxo virus oil adjuvanted vaccine
G4: group vaccinated I/M with the inactivated pigeon paramyxo virus aluminum hydroxide gel adjuvanted vaccine

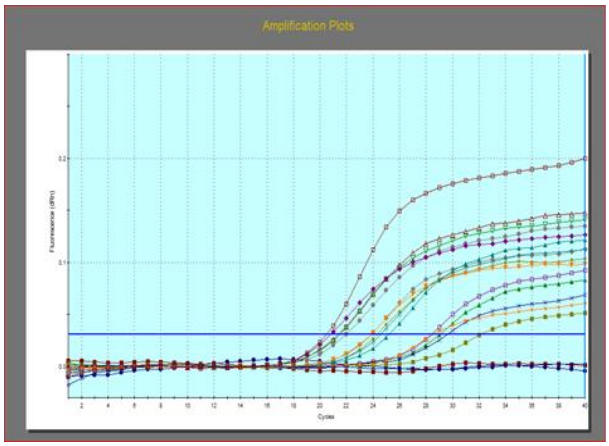

Fig 3. A representative Amplification plot of IFN-γ of stimulated PMNCs from different group 21 days' post vaccination the normalized Ct shown in table 4.

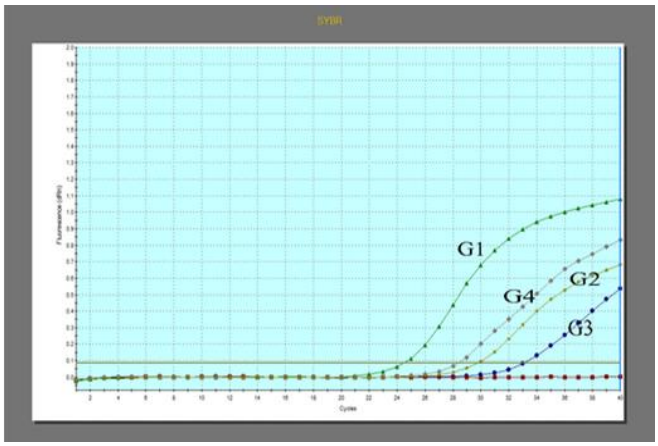

Fig 4. the qRT-PCR of the shedding expermint using F gene .

3.2.4. Shedding level.

The virus titer in the rectal swabs was measured using EID50. As seen in table (6) G2 and G3 gave the least virus titer indicating lower shedding level while G4 gave more titer. The control group nearly shed as high titer as the challenge dose .Real time PCR have been used to evaluate the shedding level in the original saline in which the swabs been swelled, as seen in fig (4) control group (G1) gave an early Ct (24.52) while both G2 and G3 gave a Ct 29.83 and 33.31respectively indicating lower shedding level and G3 is much more less titer than G2

4. DISCUSSION.

Pigeon Paramyxovirus type 1 (PPMV-1) is closely related to Newcastle Disease virus (NDV), both belonging to Paramyxoviridae family (Abolink et al., 2006), control of the PPMV-1 relays mainly on vaccination with homologous vaccines and biosafety measures, yet the only vaccine available commercially in Egypt prepares from a strain isolated in 1983. Genetically very similar, but distinct types of virus particle have been proved to be coexisted in the pigeon with different pathogenicity indices (Fuller et al., 2007). Deduced amino acid sequences of the hemagglutinin-neuraminidase (HN) and precursor fusion proteins (F0) showed single amino acid substitution (proline to serine at position 453) this substitution resulted in increase in the virulence of the virus (the intracerebral pathogenicity index become >0.7).

Table (5) the results of differential expression level of IFN-γ transcript between the groups with the expression factor and the net over all expression profile.

Duration	expression factor	P<0.05	result	
	comparison between G1 and G2			
5d pv	1.558	0.007	UP	
7d pv	3.095	0.000	UP	
15d pv	1.352	0.023	UP	
21d pv	1.195	0.136		
3d pc	5.348	0.000	UP	
5d pc	0.7	0.294		
7d pc	352.383	0	UP	
	comparison between G1 and G3			
5d pv	5.426	0.000	UP	
7d pv	10.77	0.000	UP	
15d pv	10.505	0.000	UP	
21d pv	8.61	0.000	UP	
3d pc	81.912	0.000	UP	
5d pc	29.446	0.000	UP	
7d pc	455.403	0.000	UP	
	comparison between G1 and G4			
5d pv	1.022	0.689		
7d pv	1.022	0.689		
15d pv	1.022	0.689		
21d pv	1.022	0.689		
3d pc	0.017	0.000		DOWN
5d pc	0.002	0.000		DOWN
7d pc	1.734	0.000	UP	
	comparison between G2 and G3			
5d pv	3.732	0.000	UP	
7d pv	3.73	0.000	UP	
15d pv	8.328	0.000	UP	
21d pv	7.722	0.000	UP	
3d pc	16.416	0.000	UP	
5d pc	45.067	0.000	UP	
7d pc	1.385	0.363		
	comparison between G2 and G4			
5d pv	0.642	0.006		DOWN
7d pv	0.323	0.000		DOWN
15d pv	0.74	0.025		DOWN
21d pv	0.837	0.153		
3d pc	0.003	0.000		DOWN
5d pc	0.003	0.000		DOWN
7d pc	0.005	0.000		DOWN
	comparison between G3 and G4			
5d pv	0.172	0.000		DOWN
7d pv	0.087	0.000		DOWN
15d pv	0.089	0.000		DOWN
21d pv	0.108	0.000		DOWN
3d pc	0	0.000		DOWN
5d pc	0	0.000		DOWN
7d pc	0.003	0.000		DOWN

NOTE:UP mean up regulation in gene expression
DOWN= down regulation in the IFN- expression

Table 6. The clinical sings, mortality rates, EID50 and Ct values of the virus titer from the cloacal swabs taken 7 days post challenge.

group	Clinical signs	% of mortality	EID50/100µl	Ct
G1	++	**100%**	**2.08E-06**	**24.52**
G2	-	**0%**	**1.00E-02**	**29.83**
G3	+	**30%**	**1.78E-02**	**33.31**
G4	-	**0%**	**2.08E-03**	**28.44**

G1: negative control group, kept without vaccination
G2: group vaccinated S/C with the inactivated pigeon paramyxo virus aluminum hydroxide gel adjuvanted vaccine
G3: group vaccinated S/C with the inactivated pigeon paramyxo virus oil adjuvanted vaccine
G4: group vaccinated I/M with the inactivated pigeon paramyxo virus aluminum hydroxide gel adjuvanted vaccine
++. Sever clinical signs include loss of appetite and sever nervous manifestations.
+. Moderate nervous manifestations (tremors of the eye without paralysis)
No clinical manifestation appear on the birds

In isolates that had an ICPI of 0.49 there were two nucleotide changes T/C at position 1769 in the untranslated region of the fusion gene and G/A at position 437 of the HN gene, resulting in the amino acid change G/R at position 116 in the HN protein (Sandra et al., 2014) . In China, eight PPMV isolates with different pathogenicity indices (with range of 0.6 -31.45) and different nucleotide sequences of the f gene have been reported (Guo et al., 2013). These finding suggests that sub strains may exist in the PPMV-1 which necessities the continuous update of the vaccine master seed with new field isolate to maintain high protection level. During 2014, the author have isolated and fully characterized a PPMV-1 isolated (PPMV-1 YA/14) from dead pigeons (Soliman et al., 2016). The sequence of the *f* gene has been deposited in the gene bank under the accession number KX708505.1. This isolate was used in the production of the currently commercially available pigeon paramyxo inactivated vaccine produced by veterinary serum and vaccine research institute (VSVRI).

First the master seed of the PPMV-1 YA/14 isolate was inactivated with formalin. The mechanism of formalin inactivation is that it causes covalent CH2 bonds between amino acids and DNA molecules a process known as alkylation which is time dependent. Formaldehyde interacts with protein molecules by attaching itself to the primary amide and amino groups whereas phenolic moieties bind less well to the aldehyde resulting in an intermolecular cross linkage of protein or amino groups with phenolic or indole residues. In addition to interacting with many terminal groups in viral proteins, formaldehyde can also react extensively with the amino groups of nucleic acid bases (Metz et al., 2004 ; 2006 and Thaysen-Andersen et al., 2007).

Evaluation of the inactivated paramyxo vaccine in different adjuvant formulations revealed that after challenge the aluminum hydroxide adjuvanted formulation was superior to the oil adjuvant formula both in terms of mortalities and the clinical signs. Nearly no clinical symptoms or mortalities have been observed after challenge whereas the group vaccinated with the oil adjuvanted formula showed mortalities (30%) and there were mild clinical symptoms on the living birds and those who died showed mild P/M lesions. The unvaccinated group showed the typical symptoms of pigeon paramyxo infection after challenge (Tangredi, 1985 and Soliman, et al., 2016)

Humoral immune response as measured by HI assay revealed that aluminum hydroxide adjuvanted formula gave better response when administered S/C while it was inferior when given I/M, this observation mainly due to the distribution and the type of the antigen presenting cells. Dendritic cells considered to be the professional antigen presenting cells which present in large population in the dermis and to a lesser extend in the subcutaneous tissues which also contain the macrophages (Sallusto and Lanzavecchia, 2002) , while I/M injection will deliver the virus particle to the Myocytes which are considered less efficient as antigen presenting cells. However, the oil adjuvanted formula, the mean HI titer was moderately elevated than the G4 but far beyond the

G2 that may due to that oil entangled with the virus particles and releases it over a longer period of time resulting in lower antigen mass delivered to the immune effectors cells.

Although it is well established that inactivated vaccine gave little interferon gamma production than live replicating vaccine, some inactivated vaccine recorded to be able to induce gamma interferon in a detectable yet protective level such as the inactivated hepatitis A vaccine (Hayney et al., 2003 ; Long et al., 2008 and Cardenas-Garcia et al., 2016).In our experiment we found that there was significant difference ($P< 0.05$) in IFN-γ transcripts level between the vaccinated groups when measured by quantitative RT-PCR. Relative expression analysis revealed that aluminum hydroxide gel adjuvanted vaccine administrated S/C or I/M gave less fold change than the oil-based formula crosses all the time points (table 5) and the I/M rout with further less than S/C rout. The SEPEC oil might capable of inducing interferon production. Such IFN-γ level was found to reduce but not able to eliminate the virus shedding after challenge.

Concerning the effect of different vaccine formulation on the shedding level, as seen in table 4 and fig 6, the G2 gave the lower level of shedding. Surprisingly the G3 shedding level was more than G2 although the level of interferon gamma was much higher. This could contribute to the limitation of virus replication and hence shedding with the higher humoral immune response seen in this group

In conclusion, the aluminum hydroxyl-based formula of PPMV-1 YA/14 vaccine given S/C will give a higher humoral immune response with lower shedding level than the oil-based vaccine.

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