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RESEARCH ARTICLE

PRELIMINARY STUDY FOR PREPARATION OF COMBINED ATTENUATED VACCINE AGAINST SHEEP POX AND PPR VIRUSES.

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Manuscript Info *Abstract*

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The present study was performed to evaluatethe safety and immunogenicity of combined sheep pox (SP) and Peste des Petits ruminants (PPR) vaccine prepared in a lyophilized form. A sheepgroup was vaccinated with the field dose of the prepared combined vaccine subcutaneouslyand the cellular and humeral immune responses of vaccinated sheep were evaluated for six months by different serological tests and compared with the response of eachvaccine alone as a control. It was found that the vaccinated sheep became protected from the 2nd week post vaccination and keep the protective level till the end of the experiment (24 week). The combined vaccine was found to be safe and potent as evident from sero conversion as well as challenge studies in sheep. We concluded that the use of the prepared combined vaccine component did not interfere with each otherand can be used for economic vaccination strategies.

Introduction:-

Sheep pox virus is a member of genus Capri poxvirus in the family Poxviridae (Kitching, 2004). Sheep pox is a disease of sheep and goats characterized by pyrexia, generalized skin and internal pox lesions, and lymphadenopathy. Sheep pox and goat pox are ancient diseases that are cuffently endemic in the Middle East, the Indian subcontinent, and Central and Northern Africa. Kids and lambs are generally more susceptible than adults (Raox and Bandyopadhy, 2000).

Pest des petites ruminant (PPR) is an acute contagious viral disease of small ruminants characterized by fever, oculonasal discharges, stomatitis, diarrhea and pneumonia with foul offensive breath (Dhar et al, 2002; Ozkul et al, 2002; Asim et al, 2008 and 2009). PPR virus (PPRV) is a Morbillivirus belongs to the Paramyxoviridae family (Barrett et al., 2005). The disease mainly affects goats and sheep, but it is usually reported more severe in goats where it inflicts heavy losses. The morbidity and mortality rate was reach up to 100% in severe cases. (OIE, 2012). In most countries, large scale state vaccination programs are implemented against the 2 diseases, as Vaccination is the best method to prevent the disease in susceptible animals. It carried out by different type of vaccines and the most effective type was the freeze dried live attenuated vaccine (OIE, 2010 and OIE , 2012).

A combined vaccine may be defined as a vaccine that consists of two or more antigens, that is intended to protect either against more than one infectious disease or against an infectious disease caused by different types or serotypes of the same organism (WHO, 2014). The availability of combined vaccines containing protective antigens against the majority of diseases for which universal immunization is recommended due to the simplify of the

implementation, increase the acceptance, reduce the global cost of immunization programs and improve disease control, while offering the possibility of disease elimination or even pathogen eradication (Francis, 1999).

The present study was designed to systematically investigate the immunogenicity of combined PPR and SP vaccine under laboratory conditions for possible use of combined vaccine for cost efficient vaccine based diseases control strategy.

Material and method:-

Viruses:-

Attenuated pest des petites ruminant's virus (PPRV):

Attenuated strain of PPR virus (Nigerian Strain 75/1) "Diallo et al (1989)" was obtained from Rinder Pest Research Department, Veterinary Serum and Vaccine Research Institute, Abbasia Cairo, and used for vaccine preparationas well as for carrying out serological tests.

Attenuated sheep pox virus (SPV):

Sheep pox (Romanian strain) virus "Precausta et al (1978)" was obtained from Pox Vaccines Research Department, Veterinary Serum and Vaccine Research Institute, Abbasia Cairo. It was used for preparing sheep pox vaccine and serological tests.

Virulent sheep pox virus:

The sheep pox virulent strain (Egyptian strain) was obtained from Pox Vaccines Research Department, Veterinary Serum and Vaccine Research Institute, Abbasia Cairo, and used for challenging the experimental sheep.

Cell culture

African Green Monkey Kidney cell line (VERO) was supplied by VSVRI, Abbasia Cairo and used for virus propagation, virus titration and serum neutralization test.

Vaccine preparation:

Attenuated PPR Vaccine:

Virus seed (Nigerian Strain 75/1) was inoculated into VERO cells tissue culture flasks. Then they incubated at 37^{o}C until 75-80% CPE was detected at which desired flasks were exposed to 3 successive cycles of freezing and thawing. Those flasks were checked for any contaminants. The virus collected from all the flasks was titrated using the micro titer technique according to Rao and Malik (1982) and the virus titer was calculated by Reed and Muench (1938) method and mixed with stabilizer (lactalbumin hydrolysate 2.5%, sucrose 5% and sodium glutamate 1%) in 1:1 ratio. The final vaccine product was homogenized; freeze dried and stored at -20°C (Abbas et al 2011).

Attenuated sheep pox vaccine:

Preparation of sheep pox vaccine was carried out by the same steps of preparation of attenuated PPR vaccine using the Romanian strain of sheep pox virus. The infected fluid was mixed with freeze drying medium (stabilizer) (1:1 ratio). This stabilizer (pH 7.2) contains lactalbumin hydrolysate (5%) and sucrose (2.5%). The final vaccine product was homogenized; freeze dried and stored at -20°C (Anon, 1985).

Preparation of Attenuated combined SPV-PPR vaccine:

The combined vaccine was prepared by mixing one volume of PPR infected fluid with one volume of sheep pox infected fluid then Lactalbumin-Sucrose stabilizer was added to the prepared vaccine fluid in ratio 1:1 before lyophelization. The titer of each virus fluid was adjusted to vaccinate the same number of animals per volume.

Animals:

Thirty susceptible native breed sheep 6 months old were screened using serum neutralization test and found to be free from antibodies against PPRV and SPV.

Experimental design:

The experimental sheep were divided into six equal groups (5 sheep/ each). The group (1) was used for SPV titration and group (2) was used for evaluation safety of the prepared vaccine. The other four groups were treated as follows:

Group-3: was kept unvaccinated as negative control

Group-4: was vaccinated with the attenuated PPRV vaccine (control positive)

Group-5: was vaccinated with the attenuated SPV vaccine (control positive)
Group-6: was vaccinated with the prepared combined lyophilized PPRV-SPV vaccine

Evaluation of the prepared combined vaccine:
The prepared vaccine was subjected to quality control testing including:
Sterility tests: These tests include testing the freedom from bacterial; fungal and mycoplasma contaminants according to OIE (2014)
Safety test: The safety of prepared combined vaccine was tested through inoculation of 5 sheep with a dose containing 10^5 $TCID_{50}$ of each virus (100X field dose of the prepared vaccine) according to OIE (2012).

Virus titration: Both of SPV and PPR viruses was titrated before mixing and lyophelization in Vero cells according to Rao and Malik (1982) and the virus titer was calculated according to Reed and Menuch (1938) as log 10 $TCID_{50}$/ml.
SPV was titrated post lyophelization in sheep according to OIE (2010) and expressed as SID_{50}.

Potency tests: four groups of sheep , one of them was used as control negative (G-3) the other groups (G4,G5andG6) were vaccinated by inoculated subcutaneously in the ventral aspect of the tail with the field dose of PPR vaccine, SP vaccine and the prepared SP-PPR combined vaccine respectively. The animals were clinically observed daily to detect post-vaccinal reaction, and their cellular and humeral immune responses were evaluated.

For PPR: The potency of combined vaccine to PPR virus was evaluated by comparing the humeral immune responseof the vaccinated sheep with that of single attenuated PPR vaccine

For SP: Challenge test was applied according to OIE (2010); 3 weeks post vaccination on 2 sheep from each of group 3, 5 and 6 with 0.5 ml of the virulent SPV through the intradermal route. The challenged animals were kept in separate isolator for observation for 14 days and any clinical signs were recorded
Samples: -Heparinized blood samples were collected for assay of the cellular immunity from vaccinated and control animals before and after vaccination at different intervals (1, 3, 5, 7, 10, 14, 21 and 28 days), .
-Serumsamples were separated for humeral immunity assay at different intervals (1, 2, 3, 4, 6, 8, 10, 12, 16, 20 and 24 weeks).

Evaluation of immune response to the prepared vaccine:
Cellular and humeral immune responses of vaccinated sheep were evaluated for six months by different serological tests.

Evaluation of cellular immune response:
The cellular immunity was evaluated by application of Lymphocyte blastogenesis assay. It was carried out according to Rai et al (1985) and Alvero et al. (2006) using XTT cell viability assay kit (MD biosciences)

Evaluation of humeral immune response:
Serum neutralization test (SNT): It was carried out using the microtitre technique according to Rossiter et al. (1985) where PPR antibody titer was calculated as the reciprocal of the final serum dilution which neutralized and inhibited the CPE of 100 $TCID_{50}$ of PPR virus according to Singh et al (1967); while SPV antibody titer was expressed as neutralizing index according to Martin et al. (1975).
Indirect ELISA: It was performed to evaluate the humoral immune response according to the method described by Anderson et al (1982) and the results were expressed by serum protective (S/P) ratio.

Results:-

The prepared combined PPR and SP vaccine was subjected to sterility testing and found to be free from foreign contaminants.
The prepared combined vaccinewhen tested for safety was found to be safe as there was only moderate nodule at the site of inoculation 4-7 days post inoculation which disappeared gradually and there was no abnormal clinical signs related to the 2 viruses.
The titer of PPRV was $10^{5.7}$TCID50/ml while the titer of SPV was $10^{5.8}$TCID50/ml and the titer of SPV in combined vaccine after lyophelization when evaluated in sheep as 10^6 SID50/ml. but the titer of PPR in combined vaccine after lyophelization in tissue culture was $10^{5.2}$TCID50/ml.

The potency of the combined vaccine to PPR virus estimated by humeral immune response comparing with the single attenuated PPR vaccine as shown in table (III). The data in that table showed that the combined vaccine was potent.

The potency of the combined vaccine to SP was estimated by challenge test against virulent SPV, where the vaccinated sheep showed only hypersensitivity reaction at the site of inoculation which disappear within 4 days while the non-vaccinated sheep showed local and generalized pox reaction.

Assessment of cellular immune response to vaccinated sheep had been done post vaccination directly and expressed in (table I and II).

Thedata of cell mediated immune response clarified that the cell mediated immune response to PPR in sheep vaccinated with combined vaccine (G6) and the control positive vaccinated with PPR (G4) was 0.116 and 0.105 respectively on the 1st day (table 1). This cell mediated immune response reached to its peak on the 7th day in G6 and G4 recorded 0.665and 0.571 respectively (table I).While the cell mediated immune response to SP in sheep vaccinated with combined vaccine (G6) and the control positive vaccinated with SP (G5) was0.121and 0.150 respectively on the 1st day (table 2).This cell mediated immune response reached its peak on the 10th day as 0.993 in sheep vaccinated with the combined vaccine (G6) and 1.081 in case of control positive group vaccinated with SP (G5) (table II).

The results of humeral immune response obtained as soon as 1st week post vaccination as shown in table (III, IV, V and VI). The PPR serum neutralizing antibody titer in vaccinated sheepwith combined vaccine (G6) and the control positive vaccinated with PPR vaccine (G4) was2 in the 1st week (tableIII).The mean titer of PPR neutralizing antibody in vaccinated sheep with combined vaccine (G6) and vaccinated sheep with single PPR vaccine (G4) reached its peak on the 4th week recording 64 and 32 respectively (table III).The SP serum neutralizing antibody indexin vaccinated sheep with combined vaccine (G6) and the control positive vaccinated with SP vaccine (G5) was 0.9 and 1.1respectively in the 1st week by serum neutralization test (SNT) (table IV). The mean index of SP neutralizing antibody in vaccinated sheep with combined vaccine (G6) and vaccinated sheep with SP vaccine (G5) reached its peak on the 4thweek recording 2.5 and 2.8 respectively (table IV).

The data of ELISA test showed that the humeral immune response was detectable by the 1st week post vaccination as shown in table (V and VI).TheS/P ratio of ELISA reading considered protectivewhen reading of sample ≥1;So that the sheep vaccinated with combined vaccine (G6) become protected against PPR on the 2nd week and S/P ratio reached itspeak on the 4th week as 1.18 and 1.4 respectively (table V) while the control positive sheep vaccinated with PPRvaccine (G3) become protective in the 3rd week and S/P ratio reached its peak on 4thweek was 1.28 and 1.33 respectively (table V). The sheep vaccinated with combined vaccine (G6) became protected against SP on the 2nd week and reached itsspeak on the 3rd week where S/P ratio was 1.24 and 1.6 respectively (table VI) while the control positive sheep vaccinated with SP vaccine (G5) become protective on 1st weekand the S/P ratio reached itspeak on 3rdweek as 1.07 and 1.71 respectively (table VI).

Discussion:-

The objective of the present study was to develop a combined vaccine against sheep pox and PPR which is able to protect small ruminants against both PPR and sheep pox viruses**.** Many successful trials of vaccination of animals with more than one vaccine at the same time were reported between viral vaccines were conducted as Rift Valley Fever (RVF) and sheep pox virus(SPV) vaccines (Taha et al, 1990); PPR and RVF (Mouaz et al, 1998); and recently PPR and goat pox vaccines (Abd El- Razek et al, 2006). All the fore mentioned statements for simultaneous and compound vaccination programs as a way to save costs, time and efforts. In this study we prepared PPR-SPV combined vaccine.

Cell mediated immunity: Bachh et al (1997); Sinnathamby et al (2001) and Diallo et al (2007) mentioned that cell mediated immunity may be important in immuno response during the PPR vaccination and so that our result of cell mediated immunity to PPR showed gradual increasing in cellular immuno response in sheep vaccinated with PPR - SPV combined vaccine and monovalent PPR vaccine. The cellular immune response reached thepeak on the 7th day in vaccinated sheep with combined and single PPR vaccine (table I). These results appear to be confirmed by those

of Herbert et al (2014) who stated that there is increasing in the percentage of T-cells at 7 days post vaccination by monovalent PPR vaccine.

It is known that sheep pox immunity depends mainly on the cell-mediated immune response in comparison to the humeral immune response (Kalra and Sharma, 1984 and Carn, 1993). The results of cell mediated immunity showed gradual increasing in cellular immuno response to sheep pox virus where it reached its peak on the 10thday in vaccinated sheep with combined single SP vaccine (table II). These results agree with those of Bachh et al (1997) and Mohamed et al (2007).

Humeral immunity:OIE (2013) acknowledged that a PPRV-serum neutralizing antibody titre of at least 10 that is found 3 weeks post vaccination of susceptible sheep or goats is considered protective against infection. The serum neutralization test showed that PPR antibody titre increased gradually in sheep vaccinated by prepared PPR-SPV combined vaccine and monovalent vaccine of PPR until the 4th week post vaccination and corroborative by indirect ELISA as shown table III and IV. This result agrees with those of Hosamani et al. (2004); Chaudhary et al (2009); Ayalet et al (2012) and Fakria et al (2015). The immune response against SP virus was nearly the same in animal groups vaccinated with SPV alone and the combined vaccine (G5 and G6) whichmeans that there was no interference between PPR and SPviruses' immune response when use the combined vaccine. While the results in table (III and V) clarify the immunostimulant effect of SPV in combination with PPRV in combined vaccine which in agreement with those obtained by Ghaly et al (1996) and Fakria et al (2015).

The detection of sheep pox antibodies by serum neutralization test and indirect ELISA were the guide point for this assay.The results showed that SPV antibody titre increased gradually in sheep vaccinated by prepared PPR-SPV combined vaccine and monovalent vaccine of SP untilthe 4th week post vaccination by serum neutralization test and indirect ELISA (table IV and VI). These results harmonize with Ayalet et al (2012) and Fakria et al (2015) andthe serum protective (S/P) ratio indicated that the prepared combined vaccine induced protection against both SP and PPR viruses from the second week post-vaccination till the end of the experiment.

The test results of the combination of these two disease vaccines indicated that the prepared vaccine produce good immunogenicity and protection and can be used safely.

Table (I): Cell mediated immune response of sheep to PPR vaccine

Animal groups	Mean delta optical density/D P V							
	1st D P V	3rd D P V	5th D P V	7th D P V	10th D P V	14th D P V	21st D P V	28th D P V
G3	0.088	0.081	0.091	0.085	0.088	0.079	0.085	0.084
G4	0.105	0.203	0.317	0.571	0.287	0.190	0.109	0.091
G6	0.116	0.210	0.391	0.665	0.312	0.280	0.155	0.110

DPV= days post vaccination
G3= control negative group
G4= control positive group given PPR vaccine (Field dose)
G6= tested group given combined vaccine (Field dose)

Table (II): Cell mediated immune response of sheep to Sheep pox vaccine

Animal groups	Mean delta optical density/D P V							
	1st D P V	3rd D P V	5th D P V	7th D P V	10th D P V	14th D P V	21st D P V	28th D P V
G3	0.088	0.081	0.091	0.085	0.088	0.079	0.085	0.084
G5	0.150	0.373	0.523	0.910	1.081	0.865	0.763	0.610
G6	0.121	0.339	0.480	0.915	0.993	0.893	0.770	0.589

D P V = day post vaccination
G3= control negative group
G5= control positive group given sheep pox vaccine (Field dose)
G6= tested group given combined vaccine (Field dose)

Table (III): Mean PPR serum neutralizing antibody titer in vaccinated sheep

Sheep groups	Mean PPR serum neutralizing antibody titer*/WPV**								
	1WPV	2WPV	3WPV	4WPV	8WPV	12WPV	16WPV	20WPV	24WPV
G3	0	0	0	0	0	0	0	0	0
G4	2	4	16	32	32	32	32	32	32
G6	2	8	16	64	64	64	64	64	64

PPR antibody titer = the reciprocal of the final serum dilution which neutralized and inhibited the CPE of $100TCID_{50}$ of PPR virus

NB: the antibody titer ≥8 is considered protective.

WPV= week post vaccination

G3= control negative group

G4= control positive group given PPR vaccine (Field dose)

G6= tested group given combined vaccine (Field dose)

Table (IV): Mean sheep pox serum neutralizing antibody index in vaccinated sheep

Sheep groups	Mean SP serum neutralizing antibody index/WPV*								
	1WPV	2WPV	3WPV	4WPV	8WPV	12WPV	16WPV	20WPV	24WPV
G3	0	0	0	0	0	0	0	0	0
G5	1.1	1.9	2.5	2.8	2.1	1.7	1.6	1.5	1.3
G6	0.9	1.7	2.3	2.5	2.1	1.8	1.6	1.6	1.2

W P V = week post vaccination

NB: the neutralizing antibody index ≥1.5 is considered protective (OIE 2010).

G3= control negative group

G5= control positive group given sheep pox vaccine (Field dose)

G6= tested group given combined vaccine (Field dose)

Table (V): Mean PPR – ELISA S/P ratio in vaccinated sheep

Sheep groups	PPR-ELISA S/P ratio in sheep groups/WPV								
	1st W P V	2nd W P V	3rd W P V	4th W P V	8th W P V	12nd W P V	16th W P V	20th W P V	24th W P V
G3	0	0	0	0	0	0	0	0	0
G4	0.54	0.91	1.28	1.33	1.28	1.27	1.23	1.26	1.29
G6	0.810	1.18	1.32	1.4	1.38	1.35	1.26	1.29	1.30

WPV= week post vaccination

Sample S/P ratio≥ 1 is considered protective

G3= control negative group

G4= control positive group given PPR vaccine

G6= tested group given combined vaccine (Field dose)

Table (VI): Mean sheep pox –ELISA S/P ratio in vaccinated sheep

Sheep groups	SP-ELISA S/P ratio in sheep groups/WPV*								
	1st W P V	2nd W P V	3rd W P V	4th W P V	8th W P V	12nd W P V	16th W P V	20th W P V	24th W P V
G3	0	0	0	0	0	0	0	0	0
G5	1.07	1.33	1.71	1.69	1.41	1.30	1.18	1.11	1.05
G6	0.93	1.24	1.60	1.58	1.49	1.29	1.20	1.05	1.01

WPV= week post vaccination

Sample S/P ratio ≥ 1 is considered protective

G3= control negative group

G5= control positive group given sheep pox vaccine

G6= tested group given combined vaccine (Field dose)

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