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Potency of a combined Peste des petits ruminants and sheep pox freeze dried vaccine

Mohamed A. Saad, Magda A. Kalad, Afaf A. Abd El Wahab, Amira A. El Saied, Mohamed M. Youssef, Mohamed H. Kafafy*, Dalia A.M. Abdel-Moety, Namaa A. Mohamed, Mohamed H. Khodeir

Agricultural Research Center (ARC), Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo, Egypt.

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***Correspondence:**

Corresponding author: Mohamed H. Kafafy
E-mail address: mohamedkafafy116@gmail.com

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ABSTRACT

Pest des petits ruminants (PPR) and sheep pox (SP) viruses represent a hazard facing the sheep population. The present work dealt with the preparation of a combined live attenuated vaccine that protects sheep against the two diseases. Successfully, combined Peste des petits ruminants and sheep pox freeze-dried vaccine was proven to sterile and be free from different aerobic and anaerobic bacteria, fungi, and mycoplasma contaminants; safe (as no atypical reaction either local or systemic in mice and sheep and did not affect close contact unvaccinated sheep) and potent (providing vaccinated sheep with high protective specific immunity against both causative viruses) for 6 months (the experimental period) suggesting that such immunity will remain to not less than one year. It could be recommended the use of such a vaccine to control the two diseases using one shot of vaccination saves time, effort, and cost.

Introduction

The sheep industry represents a well-known source of the general economy where they are considered a source of wool, meat, skin, and dairy in addition to their role as experimental animals in science and medicine research. Sheep face some dangerous diseases affecting their population dramatically, especially those of a viral nature (Wendy *et al*., 2015).

Peste Des Petits Ruminants (PPR) and sheep pox are two viral diseases that affect sheep causing great economic losses that can result in considerable damage at many levels in the affected countries (Thang *et al*., 2014).

Peste des petits ruminants (PPR) is an acute highly contagious viral disease of sheep and goats. In cattle, pigs, and camels, it appears with sub-clinical manifestation. Even in some wildlife species such as Dorcas's gazelles (Gazella dorcas) the disease was reported (Asil *et al*., 2019). The symptoms appear as anorexia, fever, sores in the mouth, nasal and ocular discharges, profuse diarrhea, and pneumonia, and often end with death. Abortion in infected goats was reported in PPR with a high rate (Abubakar *et al*., 2008). Morbidity and mortality rates ranged between 90–100% and 50–100%, respectively (OIE, 2009).

The causative agent of PPR is Peste des petits ruminants virus (PPRV) or as named recently small ruminant morbillivirus (SRMV) (ICTV, 2019). It is a member of the genus Morbillivirus, family Paramyxoviridae (Amarasinghe *et al*., 2019) as it is closely related to the other members of the genus (rinderpest virus, measles virus, and canine distemper virus) (Banyard *et al*., 2010).

The causative virus is highly contagious, and easily transmitted by direct contact of healthy animals with secretions and/or excretions from infected animals, or by contact with infected fomites (Parida *et al*., 2019). PPRV is well known to have only one serotype but four genetically distinct lineages were genotyped by sequence analysis of the nucleoprotein (N) gene and the fusion protein (F) gene in different studies (Misinzo *et al*., 2011).

The first recorded outbreak of PPR in Egypt was in January (1987) among goats in a private farm at Kafr-Hakim, Embaba, Giza governorate (Ikram *et al*., 1988). PPR has reappeared in some Egyptian governorates (Mouaz *et al*., 1995; Abd El-Hakim, 2006; Abd El-Rahim *et al*., 2010; Soltan and Abd-Eldaim, 2014; Safwat, 2015; Mahmoud *et al*., 2017; Ahmed *et al*., 2021). The only way to control PPR is by vaccination. PPR homologous live attenuated vaccine is well-known as the cornerstone of disease control (Khodeir and Mouaz, 1998; OIE, 1998).

Sheep pox is a malignant disease of sheep easily recognizable by their characteristic clinical signs, a systemic disease where marked lymphadenopathy appears after viremia (Kitching, 2008).

Sheep pox virus is a member of the family *Poxviridae*, subfamily *Chordopoxviridae*, genus *Capripoxviruses* with two closely related viruses; goat pox and lumpy skin disease viruses (Bhanuprakash, 2006).

The virus tropism is keratinocytes causing the characteristic skin lesions due to hyperplasia of keratinocytes with the formation of micro-vesicles (Fenner, 2017). Sheep pox virus (SPV) is highly contagious and transmission can occur either by aerosols or by indirect contamination of skin through cuts and abrasions (Wouter Pieters, 2019).

The virus can remain viable in infected crusts and scabs for a long duration up to months. Virus shedding occurs through infected papules as well as infected secretions from the eye, nose, and mouth that cause environmental contamination (Verma *et al*. 2011).

Vaccination is still considered the main method for sheep pox disease control, as the quarantine restrictions are not adequate to control the outbreaks (Tuppurainen *et al*., 2017). No symptoms of the disease can appear in vaccinated animals, on the contrary to immune-susceptible animals because immune status controls the severity of the disease

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(Bhanuprakash *et al*., 2006). Live attenuated Capri pox virus (CPPV) strains vaccine is still the most effective and commonly used method to control sheep pox outbreaks (Petra Fay *et al*., 2022).

Sheep pox (SP) is a small ruminant transboundary disease found in Africa and the Middle East. It was concluded that the SP virus is still circulated in Egypt (Sharawi *et al*., 2017) and that SPV is considered the main cause of significant economic losses in the animal industry in Egypt (Khameis *et al*., 2018).

Combined sheep pox and Peste des Petits Ruminants (PPR) vaccine was found to be safe and potent as confirmed by seroconversion and challenge studies in sheep. The lyophilized form of the combined vaccine was prepared to provide the recommended doses of both vaccine viruses. Immunization of sheep by subcutaneous route with 1ml of live attenuated vaccine consisting of 10^3 $TCID_{50}$ each of sheep pox virus (SPV) Romanian Fanar (RF) strain and Peste des Petits Ruminants virus (PPRV-Sungari/96 strain) revealed that all the immunized animals could resist challenge with virulent SPV or PPRV on day 3 post infection, while the control group showed the characteristic clinical signs of the disease. These results confirmed that no interference between the components of the vaccine and considered as a successful economic strategy for vaccination (Chaudhary *et al*., 2009).

Previous trial was conducted against PPR and Sheep/Goat Pox (SGP) using vaccine strains: PPR Nigeria 75 strain with a titer of $10^{4.1}$ $TCID_{50}$ and Sheep Pox Romania strain with a titer of $10^{4.0}$ $TCID_{50}$. Sheep and goats were evaluated for efficacy and safety in comparison with monovalent PPR and SGP vaccines. Sheep and goats were challenged by its relevant virulent strain. The result showed a good protection levels in sheep flocks for SGP and PPR infection with good sero-conversion the same results as the protection provided with monovalent vaccines for both diseases at 14 days post vaccination (Fakri *et al*., 2015).

Another study mentioned that the immunogenicity of combined lyophilized PPR and sheep pox vaccine revealed that protection appeared from the 2nd week post vaccination and continued till 24 weeks (end of the experiment) in vaccinated sheep and it was found to be safe and potent based on sero-conversion and challenge studies with no evidence of interference between the combined vaccine components (Zeidan *et al*., 2016). Others confirmed that only single dose (2 ml) of combined vaccine of PPR and SPP viruses with titers of 103.0 TCID50/mL was capable to give efficient sheep protection from two simultaneous infections for 12 months during the observation period (Zhanat *et al*., 2021). So, combined vaccines are considered the best effective method to reduce the frequency of vaccination and the stress of needle puncture to simplify the plan of immunization (Daisy Dodo, 2003).

So, the object of the present work was to provide safe potent combined PPR/SP vaccine aiming to protect the local sheep population in addition to exportation purposes.

Materials and methods

Ethical approval

Institutional animal care and Use committee at Zagazig University, El-Sharkia governorate, Egypt, gave its approval to the study plan under number (ZU-IACUC/2/F/272/2023).

Viruses

The Nigerian PPR virus (N75/1) was attenuated through 6 passages in lamb kidney cell culture followed by 77 passages on VERO cells (AU-PANVAC) representing the master seed of PPR virus.

The Romanian sheep pox virus strain was subjected to five passages in lamb testicle cell cultures and six passages in VERO cell line.

The two viruses were obtained kindly from African Union "Pan African Veterinary Vaccine Centre (PANVAC), Debrazit, Ethiopia and maintained in the Department of Rinderpest Vaccine Research (DRVR) and Department of Pox Vaccines Research (DPVR); Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo, Egypt and used for preparation of PPR and SP and combined PPR and SP vaccines in addition to serological tests.

Vaccines

Peste des petits Ruminants and sheep pox lyophilized vaccine provided by VSVRI was used for vaccination of sheep in single dose to be compared with those immunized with the prepared combined PPR/SP vaccine. The attenuated PPR virus (N75/1) was used with a dose of 102.5 TCID50/sheep and the Romanian sheep pox virus strain was used with a dose of $10^{2.5}$ $TCID_{50}$/sheep inoculated subcutaneously.

PPRV and SPV antigen

PPRV and SPV antigens were prepared according to Chaudhary *et al*., (2009) from infected Vero cells and used in indirect ELISA to estimate PPR and SP antibodies in vaccinated sheep.

Animals

Animals used in the safety test

Twenty weaned Albino Swiss mice and six sheep of 6-8 months of age tested to be free from PPR and SP antibodies by serum neutralization test were used to check the safety of the prepared vaccine.

Animals used in the potency test

Potency of the prepared combined vaccine was assessed in comparison with that of PPR and SP vaccines separately using fifty healthy native breed sheep of 6-8 months of age free from PPR and SP antibodies.

Virus titration

Titration of PPR and SP viruses was done in Vero cell culture using the microtiter technique according to Burleson *et al*. (1997) and the virus titer was expressed as $\log_{10}$ $TCID_{50}$/ml and calculated according to Reed and Muench (1938).

Stabilizer

The used stabilizer consisted of 5% lactalbumin hydrolysate (LAH), 10% Sucrose and 1% sodium glutamate (Mousumi *et al*., 2019) and was sterilized by filtration.

Preparation of combined Peste des petits ruminants and sheep pox freeze dried vaccine

PPR virus (Nigeria 75/1) and SP virus with 0.03 MOI was co-infected separately to Vero cell flasks incubated at 37°C and the cell viability was evaluated daily by microscopic examination. When the viability became less than 15%, the suspension fluid was kept at -20°C. Freeze-thawing of the harvest was done, clarification by centrifugation at 2000 rpm for 10 min and then titrated (OIE, 2018). The harvest was kept at -70°C till use. PPR and SP were tested for bacterial, fungi, and mycoplasma contamination (FAO, 1994).

PPR ($10^{2.5}$ $TCID_{50}$/dose) and SP ($10^{2.5}$ $TCID_{50}$/dose) viruses were mixed with an equal volume of sterile and chilled stabilizer then dispensed into glass vial as 2.5ml/ vial followed by lyophilization procedures on Teflon lyophilize. The vials were introduced into precooled freeze-dryer at −60°C for quick freezing where cooling continues for 2 h then the primary drying at −32°C while the vacuum was adjusted under 10 Pa for 16 h (Wang

and Zhang, 2007; Zhou *et al*., 2007). Warming up was carried out at a rate of 0.2°C/min to 20°C and lasted for 6 h. At the end of freeze-drying, the vials were sealed and kept at room temperature for 2 h (Shao-Zhi *et al*., 2010) then kept frozen at +4°C till use.

Quality Control Tests of the prepared combined PPR/SP vaccine

Sterility test

Random samples of the lyophilized combined PPR/SP vaccine were inoculated separately into tubes of nutrient agar, thioglycolate medium, Sabouraud agar and mycoplasma medium and was examined for any extraneous viruses according to the recommendations of FAO (1994); OIE (2017) and OIE (2019).

Safety Test

Using PPR and SP susceptible sheep, the contents of randomly selected vials are pooled and used to inoculate three sheep subcutaneously, each with 100 field doses leaving the other three sheep without inoculation as control closely kept with the inoculated sheep for the following three weeks. During this period, they are subjected to a daily temperature recording and frequent clinical inspections. The vaccine is considered safe if it induces no abnormal clinical reactions and there is no evidence that the vaccine virus has been contact transmitted.

Potency test

The fifty sheep were divided into 4 groups as following:
Group 1 consisted of ten animals that was inoculated with the PPR vaccine receiving a dose of $10^{2.5}$ TCID50/animal subcutaneously (Khodeir and Mouaz, 1998).
Group 2 included ten animals that was vaccinated with SP vaccines using a dose of 102.5 TCID50/animal inoculated S/C (Sharma *et al*., 1987).
Group 3 had twenty sheep that was vaccinated with the prepared combined PPR/SP vaccine using the same doses of the single vaccines and the route of inoculation (Samir *et al*., 1999).
Group 4 included ten sheep was kept without vaccination as control.

Complete hygienic measures were followed up for all sheep groups during housing providing them balanced ration and adequate water and subjected to daily clinical examination and rectal temperatures were recorded. Serum samples were collected from all sheep groups weekly till the first month then collected monthly up to 6 months post vaccination (the experimental period) for monitoring of PPR and SP induced immunity by the tested vaccines.

Serological Tests

Serum neutralization test (SNT)

The test was performed in Vero cell culture using micro-technique method as described by Ferreira, (1976) in flat bottom tissue culture micro titer plates for monitoring of PPR and SP antibody titers in vaccinated sheep. The end point of PPR neutralizing antibody titers was expressed as the reciprocal of the final dilution of serum inhibiting the CPE (Singh *et al*., 1967) meanwhile SP antibody titer was expressed as neutralization index (Sharma *et al*., 1987).

Indirect Enzyme Linked Immune Sorbent Assay (ELISA)

It was carried out according to the combined methods of Voller *et al*. (1976) and Hubschle *et al*. (1981) and the results were interpreted by the positive antibody titer expressed as $\log_{10}$.

Results

Evaluation of prepared combined vaccine

The sterility test for the prepared combined PPR/SP vaccine proved that it is free from extraneous viruses, aerobic, anaerobic bacteria, fungi and mycoplasma contamination.

Also, the vaccine safety was assessed in sheep vaccinated subcutaneously with 100 field doses of combined vaccine in comparison with each vaccine showing that the clinical post-vaccination condition of the vaccinated sheep was within normal limits for 2 weeks and the body temperature of both vaccinated and unvaccinated control sheep stayed within the normal range (38.5–39.8°C) as shown in Fig.1 During the three weeks following vaccination, all vaccinated and contact control sheep remained healthy, without any effect on their appetite and behavior.

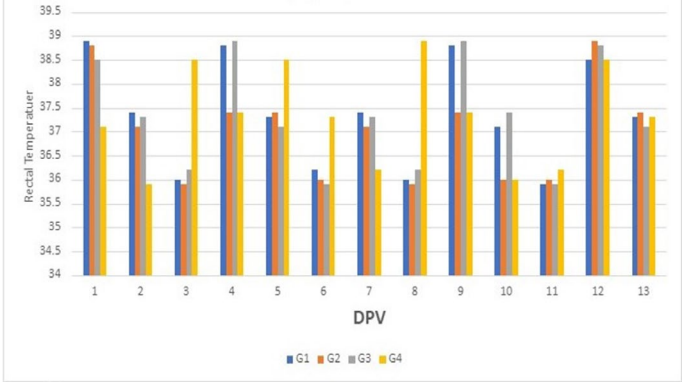

Fig. 1. Mean rectal temperature of the experimental vaccinated sheep groups.
*DPV= Days Post Vaccination; G1: vaccinated with PPR vaccine; G-2: vaccinated with SP vaccine; G-3: vaccinated with the prepared combined PPR/SP vaccine; G4: unvaccinated control

Potency of the developed combined PPR/SP vaccine in comparison to the monovalent vaccines

SNT and indirect solid phase ELISA were used for demonstration of PPR and SP antibodies. The potency of the combined vaccine to PPR virus was estimated by humeral immune response comparing with the monovalent attenuated PPR vaccine as shown in Figs. 2 and 3. Fig. 2 demonstrates the results of SNT. The data in that Figure showed that PPR antibody titer increased gradually in sheep vaccinated by the monovalent PPR vaccine (group-1) and prepared combined PPR/SP vaccine (group-3) recorded their peaks (64 and 128 respectively) by the 8th week post vaccination while unvaccinated sheep remained sera-negative. These obtained antibody titers were found to be with unchanged levels up to 24 weeks (the experimental period) post vaccination.

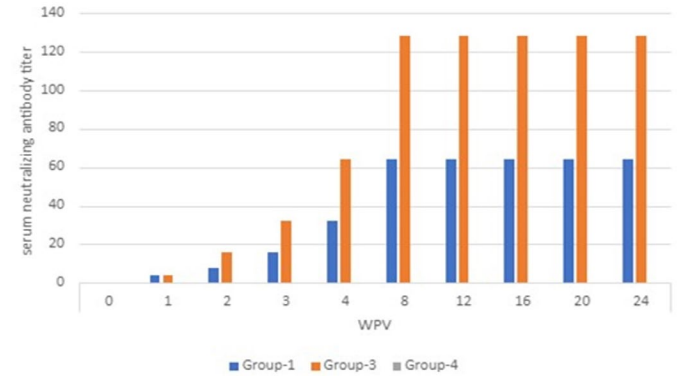

Fig. 2. PPR serum neutralizing index in experimentally vaccinated sheep groups .
*PPR antibody titer (Index) = the reciprocal of the final serum dilution which neutralized and inhibited the CPE of 100 TCID50 of PPR virus; PPR serum neutralizing antibody titer ≥8 is considered protective; **WPV= Week Post Vaccination; Group-1: vaccinated with single PPR vaccine; Group-3: vaccinated with the prepared combined PPR/SP vaccine; Group-4: unvaccinated control

The results of indirect ELISA (S/P ratio) demonstrated that S/P ratio increased gradually in groups 1 and 3 until the 8th weeks post vaccination (Fig. 3).

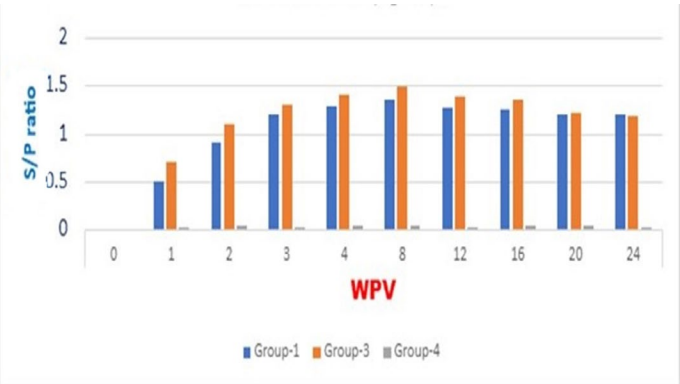

Fig. 3. PPR antibody ELISA S/P ratio in experimentally vaccinated sheep groups.
*WPV= Week Post Vaccination; Sample of S/P ratio ≥ 1 is considered protective; Group-1: vaccinated with PPR vaccine; Group-3: vaccinated with the prepared combined PPR/SP vaccine; Group-4: unvaccinated control.

The Sheep Pox serum neutralizing antibody index in vaccinated sheep with Sheep Pox vaccine (group-2) and combined PPR/SP vaccines (group-3) were 1.2 and 1.0 respectively in the 1st week (Fig. 4). The peak of SP neutralizing antibody (2.5) in group (2) and (2.8) in group (3) was obtained in the 4th week post vaccination.

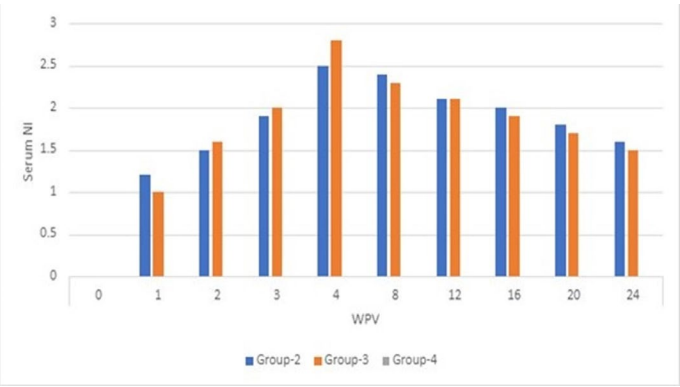

Fig. 4. Sheep Pox serum neutralizing index in experimentally vaccinated sheep groups.
*NB: SP neutralizing antibody index ≥1.5 is considered protective (OIE 2010)
**WPV= week post-vaccination; Group 2: vaccinated with SP vaccine; Group 3: vaccinated with the prepared combined PPR/SP vaccine; Group 4: unvaccinated control.

Also, the results of ELISA showed that the S/P ratio increased gradually in both sheep vaccinated groups (groups 2 and 3) by Sheep Pox vaccine and combined PPR/SP vaccine until reaching the peak (1.70 and 1.60 respectively) in 4th week post vaccination (Fig. 5). It was recommended that Seep Pox neutralizing antibody index ≥1.5 is considered protective (OIE 2010).

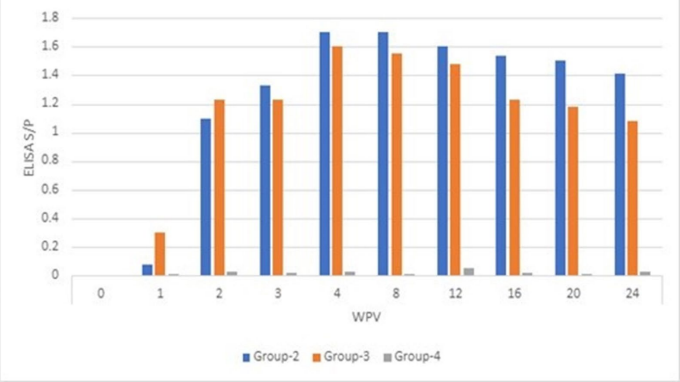

Fig. 5. Sheep Pox antibody ELISA S/P in experimentally vaccinated sheep groups
N.B.: Sample S/P ratio≥ 1 is considered protective.
*WPV= week post-vaccination; Group 2: vaccinated with SP vaccine; Group 3: vaccinated with the prepared combined PPR/SP vaccine; Group 4: unvaccinated control

Discussion

Peste des petits Ruminants (PPR) and sheep pox (SP) are highly infectious diseases of small ruminants that lead to considerable economic losses to sheep population. The epizootic situation leads to the need to introduce a new vaccine to confer full protection against both PPR and SP disease. Consequently, trials for preparation a combined vaccine for the simultaneous protection of sheep against PPR and SP were done using Nigeria 75 PPR strain and sheep pox Romania strain in lyophilized form. The use of combined vaccines is recommended due to the simple methodology, high compliance, lower vaccination costs and better disease control (Francis, 1999).

Sterility testing of the prepared combined PPR/SP vaccine proved that it is free from extraneous viruses, aerobic, anaerobic bacteria, mycoplasma and fungi contamination according to the guidelines of FAO (1994) and (OIE 2019).

Also, the vaccine safety was detected in sheep inoculated subcutaneously with 100 field doses of combined vaccine in comparison with each vaccine separately in agreement with Madhusudan *et al.* (2006) and Chaudhary *et al.* (2009) showing that the post-vaccination clinical condition of the vaccinated sheep was within normal range for 2 weeks and the body temperature of both vaccinated and unvaccinated control sheep recorded the normal body temperature (38.5–39.8°C) as shown in Fig. 1. During the three weeks post vaccination, all vaccinated and contact control sheep remained healthy, without any effect on their appetite and behavior. Similar findings were reported by Fakri *et al.* (2015).

Regarding the potency of the developed combined PPR/SP, SNT and indirect ELISA were used for detection of PPR and SP antibodies. The potency of the combined vaccine to PPR virus was shown in Figs. 2 and 3. Fig. 2 demonstrates the results of SNT where PPR antibody titer increased gradually in sheep vaccinated by the monovalent PPR vaccine (group-1) and prepared combined PPR/SP vaccine (group-3) recorded their peaks (64 and 128 respectively) by the 8th week post vaccination. Similar PPR serum neutralizing antibody titers were recorded by Khodeir and Mouaz (1998) and in this respect OIE (2013) mentioned that a PPR serum neutralizing antibody titer of at least 10 was detected 3 weeks post vaccination of susceptible sheep or goats and it was considered protective. These obtained antibody titers were at constant levels up to 24 weeks (the experimental period) post vaccination in agreement with that reported by Abeer (1997); Afaf (1998) and Khodeir and Mouaz (1998) suggesting longer duration of immunity.

On the other side, the results of indirect ELISA (S/P ratio) shown in Fig. 3 demonstrated that S/P ratio increased gradually for PPR in groups 1 and 3 until the 8th weeks post vaccination these results agree with those of Hosamani *et al.* (2004); Chaudhary *et al.* (2009); Ayalet *et al.* (2012) and Fakri *et al.* (2015). It was also clear that no interference between PPR and SP viruses' immune response was detected in the combined vaccine. The results in Figs. 2 and 3, spot the light on the immunostimulant effect of SPV when used with PPRV in combined vaccine that agree with the findings of Ghaly *et al.* (1996) and Fakri *et al.* (2015)

Also, the results of ELISA regarding SP virus showed that the S/P ratio increased gradually in both sheep vaccinated groups (groups 2 and 3) by Sheep Pox vaccine and combined PPR/SP vaccine until reaching the peak (1.70 and 1.60 respectively) in 4th week post vaccination (Fig. 5). It was recommended that SP neutralizing antibody index ≥1.5 is considered protective according to OIE (2010). In addition, the obtained results came parallel to those of Ayalet *et al.* (2012); Fakri *et al.* (2015) and Zeidan *et al.* (2016) showing serum protective (S/P) ratio indicating that the developed combined PPR/SP vaccine elicited good levels of specific immunity protection against both SP and PPR viruses.

Conclusion

The developed combined sheep pox and Peste des Petits ruminants (PPR) vaccine is safe and potent as evident from sera conversion in sheep indicating that component vaccines did not interfere each other and can be used in sheep for economic vaccination strategies saving time and effort as an alternative to the monovalent vaccines against PPR and SP.

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Conflict of interest

The authors declare that they have no conflict of interest.

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