VacciMonitor 2024;33:e103324
www.vaccimonitor.finlay.edu.cu
Original Article

Preparation and evaluation of a bivalent peste des petits ruminants and *Brucella* Rev-1 vaccine for sheep

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Sheep industry is of great importance in the economy. Infectious diseases of ruminants are an economic threat and can cause massive damage globally. Peste des petits ruminants and brucellosis are two diseases that affect sheep and cause great economic losses. The live attenuated peste des petits ruminants vaccine induces strong immunity and high protection against this disease. On the other hand, the live attenuated *Brucella melitensis* Rev-1 vaccine represents one of the best choices for controlling *Brucella* diseases. In the present study, the newly prepared bivalent vaccine against peste des petits ruminants and *Brucella* Rev-1 from local isolates was studied in comparison with the monovalent vaccines against each disease. Enzyme-linked immunosorbent assay and seroneutralization test were used for serological evaluation of the immune response in the vaccinated sheep groups; the prepared bivalent vaccine induced higher antibody titers than the monovalent vaccines and the protective antibody titer was detected 3-4 weeks after vaccination. The bivalent vaccine can provide excellent protection to sheep against peste des petits ruminants and *Brucella melitensis* compared to monovalent vaccines.

Keywords: peste des petits ruminants; *Brucella*; bivalent vaccine; neutralization test; ELISA.

Introduction

Sheep industry is important in the economy, since it is a source of wool, meat, skin, dairy and has a role in science and medical research as experimental animals.(1) There are some dangerous diseases affecting sheep population. Peste des petits ruminants (PPR), named as 'goat plague', is a transboundary and contagious viral disease of small ruminants that causes increased morbidity and mortality with a high loss of productivity of small ruminants over a wide region. PPR disease has a significant economic influence in the world; currently, the goal is to eradicate it by 2030 through the implementation of a Global Control and Eradication Strategy.(1) PPR virus belongs to *Morbillivirus* genus, *Paramyxoviridae* family. It replicates in the epithelial tissue of the gastrointestinal tract, lymphoid tissue and respiratory tract, where it produces its characteristic lesions.(2) It is a highly contagious disease, transmitted by direct contact with the infected animals or infected secretions. Clinically the disease is characterized by necrotic stomatitis, fever, gastroenteritis and pneumonia and even death. PPR virus infection is very dangerous for the small ruminant industry in Egypt, causing heavy losses.(3)

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Vaccination is considered the best way to control this disease, as quarantine or limitation of animal movement are not enough to control outbreaks. Vaccination with live attenuated PPR virus strains is an effective and widely used strategy to control PPR outbreaks.[3] Food and agricultural organization (FAO) and World Organization for Animal Health (WOAH) have established a regime for the eradication of PPR. The loss of PPR-related clinical symptoms, the lack of transmission between the positive in-contact goats and the negative by the pen-side test, are considered the safety of live attenuated PPRV vaccine.[4] The first local vaccination regime held in Egypt was at 2019. The initial national vaccine was manufactured from the Egyptian PPR virus master seed at the Veterinary Serum and Vaccine Research Institute (VSVRI), subsequently, the national PPR vaccination campaign started in 10/2022, under the authorization of the General Organization of Veterinary Services (GOVS).[5]

Brucellosis is one of the zoonotic diseases affecting humans and animals worldwide. It is considered one of the most dangerous zoonotic diseases in Middle East countries and causes considerable and frugal losses in the animal industry.[6] The causative agent of disease brucellosis belongs to the genus *Brucella*.[7] Farm animals are the main hosts for *B. abortus* and *B. melitensis* and transmission of other *Brucella* spp. was reported. Ruminants are considered the main source of brucellosis, a disease that has a wide distribution.[8] Brucellosis serotypes have been notified in humans and sheep in Egypt, and therefore, control programs should be implemented.[9]

The World Health Organization (WHO) and the WOAH had developed a plan for eradication of brucellosis, unfortunately there are few brucellosis-free countries. The greatest distribution of brucellosis is recorded in the Mediterranean region and the Middle East.[10] Vaccination with *Brucella* Rev-1 should be performed to reduce financial losses in the ruminant industry, and has an effect on the cellular immunity in sheep and goats.[11] In general, Rev-1 was considered to be effective in protecting goats and sheep from natural *Brucella* infections.

Multivalent vaccines offer opportunistic and economic control against infections in the animal industry.[12] Therefore, the objective of the present research is to develop a bivalent vaccine against both PPR and *Brucella* for sheep. This vaccine aims to simplify immunization by offering dual protection with a single injection, thereby reducing stress on both animals and owners. Additionally, the study will investigate the immunological responses elicited by this new bivalent vaccine.

Materials and Methods

Ethical approval

This work was approved by the Animal Ethics Committee of VSVRI, Abasia, Cairo and Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Abasia, Cairo. All experiments agree with the VSVRI and CLEVB guidelines for animal research.

Vaccinal strains

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

Brucella Rev-1 strain was supplied kindly by Department of Antigens and Sera Research, VSVRI.

Monovalent vaccine preparations

Live attenuated PPR vaccine was prepared in a lyophilized form[13] in a titer of $10^{5.5}$ tissue culture infectious dose $(TCID)_{50}$/mL and used for the experimental vaccine preparations. This titer is also used for serum neutralization test (SNT).

Monovalent *Brucella* Rev-1 live attenuated vaccine was prepared according to[14] in a colony count of $1X10^9$ colony forming unit (CFU)/dose.

Bivalent PPR and *Brucella* Rev-1 vaccines

The two prepared monovalent vaccine fluids (sterile and chilled) were mixed in equal amounts and adjusted to the field dose ($10^{5.5}$ TCID/mL for PPR and $1x10^9$ CFU/dose for Rev-1) and an equal volume of stabilizer consisting of 2.5% lactalbumin hydrolysate (LAH), 5% sucrose and 1% sodium glutamate was added,[15] sterilized by filtration and then dispensed as 2mL/glass vial to the lyophilization process on Teflon lyophilize

apparatus.[16] After freeze-drying, the vials were sealed and kept at room temperature for 2 h, then kept at 4-8°C until subjected to experimental work.

Sheep

Fifteen native breed female sheep, 6-8 months old, free from PPR and *Brucella* antibodies, were used for evaluation of the prepared vaccines; three of them were used in safety test, nine for potency test and three as control sheep.

All sheep groups were kept under hygienic measures receiving balanced ration and water and observed daily. The study was approved by the Institutional Animal Care and Use Committee (ARC-IACUC) Agricultural Research Center and the IACUC protocol number is ARC: VSIVRI 35 24.

Evaluation of the prepared PPR/*Brucella* vaccine[17]

It was carried out according to Egyptian Standard for Evaluation of Veterinary Biologics (2009)[17] by implementing sterility test, safety test and potency test.

Sterility test[17]

The lyophilized combined PPR/*Brucella* vaccine was inoculated into tubes of thioglycolate medium, tryptone soya broth, nutrient agar, brain heart infusion agar, MacConkey agar and mycoplasma medium. Also, the lyophilized vaccine was examined for any extraneous viruses.

Safety test[17]

The content of randomly selected vial was used to inoculate three sheep subcutaneously (S/C), each with 100 field doses (each field dose contains $10^{2.5}$TCID$_{50}$ of PPR virus/sheep[13] and 1x10^9CFU of *Brucella* Rev-1[14] leaving the other three sheep without inoculation as control closely kept with the inoculated sheep for the following three weeks. During this period, they were subjected to a daily temperature recording and frequent clinical inspections. The vaccine was considered safe if there was no induction of abnormal clinical reactions and there was no evidence that the vaccine virus had been contact transmitted.

Potency test[17]

Nine sheep were divided into three groups in the following manner:

Group 1: vaccinated with monovalent PPR vaccine

Group 2: vaccinated with monovalent *Brucella* vaccine

Group 3: vaccinated with the prepared combined PPR/ *Brucella* vaccine

There was a fourth group kept without any inoculation as control.

Each sheep was inoculated S/C in the neck side and received a field dose. Serum samples were obtained from all sheep groups at weekly intervals for 4 weeks and then at monthly intervals for up to 6 months post vaccination to monitor the induced PPR and *Brucella* immune levels.

Serological tests

Serum neutralization test[18]

The test was made in Vero cell culture using the micro-technique method in flat-bottom tissue culture microtiter plates to monitor PPR antibody titers in vaccinated sheep. The endpoint of PPR neutralizing antibody titers was expressed as the reciprocal of the final dilution of serum inhibiting the cytopathic effect (CPE). PPR antibody titer was considered as the reciprocal of the serum dilution that neutralized and inhibited the CPE of 100 TCID$_{50}$ of PPR virus; PPR serum neutralizing titer ≥ 8 was considered protective.

Indirect enzyme-linked immunosorbent assay (ELISA) to detect antibodies against PPR[19, 20]

It was carried out to follow up PPR antibody levels in vaccinated sheep; the results were interpreted as positive for an average optical density (O.D.) of PPR antibodies by ELISA > 0.5.

Enzyme-linked immunosorbent assay (ELISA) to detect antibodies against *Brucella*[19]

It was carried out to follow up *Brucella* antibody levels in vaccinated sheep and the results were interpreted by the positive antibody titer expressed as $\log_{10}$. The results were recorded as the mean absorbance values.

The obtained results were made as:

S/P= Sample OD - Control negative OD/ Control positive OD - Control negative OD

Log_{10} Titre = 1.09 (log_{10} S/P) + 3.63

Titre = Anti-log of log_{10} Titre

The cut off line 120 was considered positive for *Brucella*.

Results

Evaluation of prepared bivalent vaccine

Sterility testing of the prepared monovalent PPR and *Brucella* vaccines and the bivalent PPR/*Brucella* vaccine showed no evidence of any bacteriological growth (aerobic or anaerobic contaminant) or fungal growth after culturing on the different media used in the sterility test.

The safety test result showed that the bivalent PPR/ *Brucella* vaccine is safe.

Potency of the bivalent PPR/*Brucella* vaccine in comparison to monovalent vaccines

The SNT showed protective PPR antibody titers by the second week post vaccination (16 and 8) in sheep vaccinated with the monovalent vaccine and the bivalent one, respectively; peaks (128 and 64) were recorded in the second month and remained stable up to 6 months later (Table 1).

Indirect ELISA test showed protective PPR antibody titers by the third week post vaccination in sheep vaccinated with the monovalent vaccine and the bivalent one, respectively, recording their peaks by the third month and remaining stable up to 6 months later (Table 2).

The ELISA test showed protective *Brucella* antibody titers by the third week post vaccination in sheep vaccinated with the monovalent vaccine and by the second week post vaccination for the bivalent vaccine, the peaks (198 and 255) were recorded towards the fourth week post vaccination and remained stable up to 6 months later (Table 3).

Discussion

Sheep industry faces many infectious diseases and the vaccination is the first control strategy to defeat them. It is difficult to vaccinate sheep with different vaccines in a specific period in the field, due to lack of the labor and finance.[19]

The aim of the present study was to prepare and evaluate a bivalent PPR and *Brucella* vaccine for sheep. Our experimental results showed that all of the prepared monovalent and the bivalent PPR/*Brucella* vaccines are

Table 1. PPR serum neutralizing antibody titers in different vaccinated sheep groups.

Sheep groups	PPR serum neutralizing antibody titer $TCID_{50}$*									
	Prev.	1WPV	2WPV	3WPV	4WPV	2MPV	3MPV	4MPV	5MPV	6MPV
	0	4	16	32	64	128	128	128	128	128
G1	0	4	16	32	64	128	128	128	128	128
	0	2	16	32	64	128	128	128	128	128
Mean	0	3.33	16	32	64			←128→		
	0	2	8	16	32	64	64	64	64	64
G3	0	2	8	16	32	64	64	64	64	64
	0	4	8	16	32	64	64	64	64	64
Mean	0	2.66	8	16	32			←64→		
G4	All sheep in this group remain seronegative all over the experimental period									

*PPR antibody titer: the reciprocal of the serum dilution that neutralized and inhibited the CPE of 100 $TCID_{50}$ of PPR virus; PPR serum neutralizing titer≥ 8 was considered protective. Prev: pre-vaccination. WPV: week post vaccination. MPV: month post vaccination. Group 1: vaccinated with monovalent PPR vaccine. Group 3: vaccinated with the prepared bivalent PPR/*Brucella* vaccine. Group-4: unvaccinated control group.

Table 2. PPR antibody titer by indirect ELISA in vaccinated sheep.

Sheep groups	ELISA Optical densities (OD) for PPR antibodies									
	Prev.	1WPV	2WPV	3WPV	4WPV	2MPV	3MPV	4MPV	5MPV	6MPV
G1 Mean	0.073	0.077	0.233	0.555	0.644	0.647	1.850	1.630	1.533	1.522
G3 Mean	0.067	0.083	0.292	0.544	0.683	0.866	1.856	1.655	1.634	1.541
G4 Mean	0.079	0.079	0.066	0.073	0.077	0.070	0.073	0.078	0.070	0.076

Prev.: pre-vaccination. WPV: week post vaccination. MPV: month post vaccination. Group 1: vaccinated with monovalent PPR vaccine. Group 3: vaccinated with the prepared bivalent PPR/*Brucella* vaccine. Group 4: unvaccinated control group.

Table 3. *Brucella* ELISA antibody titer in vaccinated sheep.

Sheep groups	Mean *Brucella* antibody titer by ELISA									
	Prev.	1WPV	2WPV	3WPV	4WPV	2MPV	3MPV	4MPV	5MPV	6MPV
G2 Mean	100	130	138	170	198	198	198	198	198	198
G3 Mean	100	105	127	163	255	255	255	255	255	255
G4 Mean	100	95	96	99	99	99	99	99	99	99

Prev: pre-vaccination. WPV: week post vaccination. MPV: month post vaccination. Group 2: vaccinated with monovalent *Brucella* vaccine. Group 3: vaccinated with the prepared bivalent PPR/*Brucella* vaccine. Group 4: unvaccinated control group. Cutt off line: 120.

free from aerobic and anaerobic bacteria, other than *Brucella* growth, fungi and mycoplasma or any contamination.

The vaccine safety tests confirmed that monovalent vaccines and combine PPR and *Brucella* vaccine were safe for sheep that showed no local or systemic abnormal post inoculation signs, which is in agreement with authors who confirmed the safety of live attenuated PPR vaccine and concluded that the live attenuated PPR vaccine is safe for immunization of sheep and goats. Moreover, live *Brucella* Rev-1 vaccine showed high safety with no remarkable gross lesion or histopathological changes, even any marks of colonization in the dam and fetuses.[21,22]

The present study used the SNT to monitor neutralizing antibody titers against PPR in vaccinated sheep. Monovalent and bivalent PPR vaccines effectively induced protective antibody titers against PPR. The protective antibody titer in vaccinated sheep was detected in the 2nd week post-vaccination and the highest antibody titer was detected at 2nd month post-vaccination. Notably, unvaccinated sheep remained serologically negative for PPR antibodies. These findings align with observations reported by other researchers[23] who documented similar PPR antibody titers using SNT. Additionally, the safety of the vaccine was confirmed, with no evidence of immunosuppression in vaccinated animals.[23] Importantly, a PPR SNT titer of ≥8 is considered protective.[21]

Vaccinated groups developed protective antibody levels; sheep receiving the monovalent PPR vaccine exhibited higher peak titers compared to those vaccinated with the bivalent vaccine. This finding contradicts previous studies[24] suggesting that live *Brucella* vaccines enhance the immune response against PPR vaccines. Further investigation, including analysis of indirect ELISA data for PPR antibodies, is warranted to elucidate the underlying mechanisms and reconcile these observations.

The *Brucella* Rev-1 vaccine had been used widely for the protection against brucellosis in small ruminants.[8] Live-attenuated vaccines are the most protective and widely used vaccines to control animal brucellosis due to their superior efficacy compared to inactivated one.[9] Furthermore, live attenuated vaccines are considered more affordable and much effective and can induce a high protective antibody response throughout activating humoral and cell-mediated pathways.[8,9] In the present research, two groups were immunized by administrating a single dose of $1x10^9$ CFU of *Brucella* Rev-1 monovalent vaccine and *Brucella* Rev-1 and PPR bivalent live vaccine, respectively and a third group was kept as a negative control. In the present research, the antibody titers (by ELISA) in the first week revealed a low detectable titer, reflecting a coincidence with other authors,[24] this is probably due to insufficient time to produce a high titer.

Furthermore, the recorded antibody titers during second week were above the cut off line for the monovalent *Brucella* vaccinated group compared to the bivalent vaccinated group; it was previously stated that there was a delay in the immune response against brucellosis after PPR vaccination.[21]

Regarding our findings, in the third and fourth weeks post-vaccination, monovalent and bivalent *Brucella* vaccinated groups achieved protective antibody levels. However, the bivalent vaccine group exhibited a little increase in antibody titers compared to the monovalent *Brucella* group; suggesting that the difference in antibody levels between the two groups wasn't highly.[21,24]

This study suggests that the bivalent PPR-*Brucella* Rev-1 vaccine might provide effective protection against PPR and brucellosis in sheep. Although our findings indicate strong immune responses as early as 3-4 weeks post-vaccination, it should be considered that the peak of antibody titer for both diseases might occur later.

Conflict of interest

The authors declare that there is no conflict of interest.

Author's contributions

Mohamed Mahmoud-Youssef: owner of main idea and experimental design, applied the experiments and followed up the practical work and critically reviewed the manuscript.

Lamees A. El-Tantawy: supervisor and participated in designing and followed up of the practical work and critically reviewed the manuscript.

Gehad A. Yousef: performed and followed up the practical work, wrote the manuscript, writing-original draft preparation, writing review, editing the article and critically reviewed the manuscript.

Adel M. El- Kattan: followed up the practical work and critically reviewed the manuscript.

All authors have read and agreed to the published version of the manuscript.

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Preparación y evaluación de una vacuna bivalente contra la peste de pequeños rumiantes y *Brucella* Rev-1 para ovejas

Resumen

La industria ovina tiene una gran importancia en la economía. Las enfermedades infecciosas de los rumiantes son una amenaza a la economía y pueden causar daños masivos en todo el mundo. La peste de pequeños rumiantes y la brucelosis son dos enfermedades que afectan al ganado ovino y causan grandes pérdidas económicas. La vacuna viva atenuada contra la peste de pequeños rumiantes induce una fuerte inmunidad y una elevada protección contra esta enfermedad. Por otra parte, la vacuna viva atenuada contra *Brucella melitensis* Rev-1 representa una de las mejores opciones para controlar las enfermedades causadas por *Brucella*. En el presente trabajo se estudió la vacuna bivalente preparada contra la peste de pequeños rumiantes y *Brucella* Rev-1 a partir de aislados locales, en comparación con las vacunas monovalentes contra cada enfermedad. Para la evaluación serológica de la respuesta inmunitaria en los grupos de ovejas vacunadas se utilizaron el ensayo inmunoenzimático y la prueba de seroneutralización; la vacuna bivalente preparada indujo títulos de anticuerpos más elevados que las vacunas monovalentes y el título de anticuerpos protectores se detectó a las 3-4 semanas después de la vacunación. La vacuna bivalente puede proporcionar una excelente protección a los ovinos contra la peste de pequeños rumiantes y *Brucella melitensis* en comparación con las vacunas monovalentes.

Palabras clave: peste de los pequeños rumiantes; *Brucella*; vacuna bivalente; pruebas de neutralización; ELISA.

Recibido: 2 de mayo del 2024 *Aceptado: 27 de agosto del 2024*