Preliminary results on innocuity and immunogenicity of an inactivated vaccine against Peste des petits ruminants

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Summary
Peste des petits ruminants (PPR) virus belongs to the family Paramyxoviridae and represents a major threat to small livestock industry. In recent years, outbreaks of PPR have occurred in Turkey and North Africa. In endemic areas, disease prevention is accomplished using live‑attenuated vaccines. However, the use of live vaccines in non‑endemic regions, such as Europe, is not approved by Veterinary Authorities. In these regions inactivated vaccines are then the only viable alternative. In this study an inactivated vaccine (iPPRVac) was formulated with either a water‑in‑oil emulsion (ISA 71 VG) or with delta inulin adjuvant, alone (AFSA1) or combined with a TLR9 agonist oligonucleotide (AFSA2). These formulations were then tested for immunogenicity on rats. The iPPRV formulation with AFSA2 adjuvant induced 100% seroconversion in rats after 2 injections and was subsequently evaluated in goats. Five goats were immunised twice subcutaneously, 36 days apart with iPPRVac + AFSA2. The immunised goats all seroconverted to PPR by day 9 and remained seropositive until the end of the experimental period (133 days). These data indicate that the rat model is useful in predicting vaccine responses in goats and that inactivated vaccine, when formulated with a delta inulin adjuvant, represents a promising alternative to live attenuated vaccines for PPR vaccination campaigns in non‑endemic areas.

Keywords
Adjuvant, Goat, Inactivated vaccine, Peste des petits ruminants, Rat.

Parole chiave
Adiuvante, Capra, Peste dei piccoli ruminanti, Ratto, Vaccino inattivato.

Riassunto
Il virus della Peste dei piccoli ruminanti (PPR) appartiene alla famiglia Paramyxoviridae e rappresenta, nei paesi dove la malattia è endemica, un problema per l’allevamento ovicaprina. Negli ultimi anni l’areale di diffusione della malattia si è espanso e focolai di PPR si sono verificati nelle aree del bacino del Mediterraneo (Turchia e Paesi del Nord Africa). Nelle zone endemiche la prevenzione della diffusione della malattia viene attuata mediante la vaccinazione con vaccini vivi attenuati. L’utilizzo di questo tipo di vaccini in una regione come l’Europa, dove la malattia non è endemica, potrebbe non essere accettata dalle Autorità Veterinarie e dagli allevatori. L’uso di un vaccino inattivato, in queste regioni, potrebbe quindi rappresentare una valida alternativa. In questo studio sono state utilizzate sei formulazioni vaccinali contenenti virus inattivato. Le formulazioni differivano tra loro sia per la tipologia di adiuvante utilizzato sia per la concentrazione antigenica presente. I sieri ottenuti prelevando il sangue dalla vena caudale dei ratti, sono stati analizzati utilizzando un kit commerciale ELISA PPR. La formulazione che, tra le sei testate, ha indotto sieroconversione in tutti i ratti inoculati è stata quindi saggita nella capra. Due dosi vaccinali, da 1 ml ciascuna, sono state somministrate a distanza di 36 giorni l’una dall’altra a cinque capre sieronegative per PPR, tre capre sono state utilizzate come controllo non vaccinato. Gli animali vaccinati hanno sieroconvertito 9 giorni dopo la somministrazione della prima dose e sono rimasti positivi per tutto il periodo di osservazione della durata di 133 giorni. Il modello animale ratto e l’uso quantitativo del test ELISA si sono dimostrati due utili strumenti per un saggio preliminare nello studio
Introduction

Peste des Petits Ruminants (PPR) is a highly contagious viral disease of sheep and goats characterized by pyrexia, mucopurulent nasal and ocular discharge, necrotizing and erosive stomatitis, enteritis, and pneumonia (Abu-Elzein et al. 1990). The aetiological agent of PPR is a non-segmented RNA negative-stranded virus (PPRV), which belongs to the family Paramyxoviridae, genus Morbillivirus.

The disease can cause considerable economic losses. In severe cases, mortality and morbidity rates can be as high as 90-100%. Poor nutritional status, and concurrent parasitic and bacterial infections enhance the severity of clinical signs (Kitching 1988). It therefore represents a major threat to livestock industry in many countries in Africa and Asia (Banyard et al. 2010). The disease was first reported in Ivory Coast in West Africa in 1942 and later on it in Senegal, Central Africa, Sudan, Ethiopia, East Africa, Saudi Arabia, Jordan, India, Bangladesh, Pakistan, Nepal, and Israel (Saravanan et al. 2008). In recent years, outbreaks of PPR have occurred in European Turkey and North African countries (Albina et al. 2013). Only 1 PPRV serotype has been identified so far. However, on the basis of nucleotide sequences of genes N (nucleocapsid protein) and F (fusion protein) (Munir et al. 2011), 4 lineages have been identified. Peste des Petits Ruminants viruses belonging to lineages I and II have been isolated exclusively in West African countries. Lineage III is largely restricted to the Middle East (Yemen, Qatar, and Oman) and East Africa, although some lineage III strains have also been isolated from Southern India. Lineage IV, historically responsible for outbreaks in Asian countries (Munir et al. 2011, Shaila et al. 1996) has been recently reported to be circulating in North (Kitching 1988) and East African countries (Cosseddu et al. 2013).

Vaccines are the most effective means to control viral diseases like PPR and minimize the considerable economic losses caused by outbreaks. Four live attenuated vaccines are commercially available. The PPRV Nigeria 75/1 vaccine belongs to Lineage I and is currently used for protecting sheep and goats in Africa and the Middle East. The Sungri 96, Arasur 87, and Coimbatore 97 vaccines belong to lineage IV (Saravanan et al. 2010) and are currently used for protecting sheep and goats in African and Asian countries.

A major issue is that in the event of PPR outbreaks in Europe, the use of live attenuated vaccines is likely to be blocked by Veterinary Authorities, since live vaccines induce viraemia, with the potential for the vaccine virus to revert to virulence and infect unvaccinated animals. Hence, immunization campaigns in non-endemic regions would best be conducted with inactivated vaccines or, alternatively, with virus vectored vaccines (Diallo et al. 2002). Various recombinant PPR vaccines expressing PPRV H or F proteins have been tested, with the better performing H protein vaccine inducing neutralizing antibodies in 80% of immunised animals (Chen et al. 2010).

In this study, we hypothesised that inactivated PPRV antigen, when combined with the right adjuvant, might provide high level of herd seroprotection and could, therefore, be used to ring-fence any PPR outbreak in non-endemic countries, without running the risk of live vaccine reversion to virulence.

Our inactivated vaccine was based on a virulent strain isolated during the outbreaks occurred in Morocco in 2008. It was grown in tissue culture and inactivated with binary ethylenimine and then formulated with either a standard water in oil emulsion adjuvant, commonly used in veterinary vaccine studies, or with 1 of 2 novel adjuvant formulations (AFSA1 and AFSA2), based on delta inulin polysaccharide particles (Advax™) (Cooper and Petrovsky, 2011). The immunogenicity of these inactivated vaccine formulations were then evaluated in rats and finally in goats, as reported below.

Materials and methods

Virus production

Trypsinated VerodogSLAMtag cells, kindly provided by Dr. Yusuke Yanagi (Department of Virology, Faculty of Medicine, Kyushu University, Fukuoka 812-8582, Japan), were seeded in roller bottles at the density of 60,000 cells/cm² and simultaneously infected with seed PPRV at a 0.01 multiplicity of infection (MOI). The PPRV inoculum originated from the Moroccan strain M/08, belonging to lineage IV (Hammouchi et al. 2012), which was isolated from goats succumbing to disease during the 2008 outbreak. The infected cells were cultured using D-MEM growth medium (Gibco Gaithersburg, Maryland, USA).
Gaithersburg, Maryland, U.S.A.) supplemented with 10% (v/v) foetal calf serum (MultiCell Technologies, Woonsocket, Rhode Island, U.S.A.) and incubated at 37°C ± 1°C. Cells were observed daily under inverted microscope (20X - 40X). The growth medium was replaced with D-MEM medium in absence of foetal calf serum 48 hours after the cell monolayer reached confluence. Seven days later, when the cytopathic effect (CPE) approached 100%, the virus suspension was harvested and tested for purity, infectivity titre, and sterility for bacteria, fungi, and mycoplasma. The virus suspension was then stored at 5°C ± 3°C until further use.

**Determination of inactivation kinetics of PPR virus with binary ethyleneimine**

To determine the inactivation kinetics, 2 concentrations of binary ethyleneimine (BEI) (1mM and 3mM), prepared as described by Bhaneman (Bahneman 1975), were each added to 2 aliquots of the virus suspension. Both aliquots were incubated at 37°C ± 1°C for 5 hours under continuous low speed stirring. To monitor inactivation, aliquots were collected at hourly intervals. To neutralize BEI, sodium thiosulphate (Sigma-Aldrich St. Louis, St. Louis, Missouri, U.S.A.) was added at 2% V/V (Bahneman 1975). Virus titres (Rossiter et al. 1985) were calculated according to the Reed and Munch formula (Reed and Muench 1938) and used to define the inactivation curve. Raw data were analysed by Microsoft® Excel 2010 (14.0.6112.5000, Redmond, Washington) (Ronchi et al. 2012).

**Virus inactivation, purification and concentration**

For vaccine production, BEI at the concentration of 1mM was used. The inactivated virus suspension was centrifuged at 9,000 x g for 30’ at 5°C ± 3°C, the supernatant collected and stored at 5°C ± 3°. The pellet suspended in a small volume of phosphate saline buffer (PBS) at pH 7.2 was frozen at -80°C and thawed twice to allow for the release of any cell-associated virus. After centrifugation, the virus containing supernatant was separated and the suspension purified and concentrated 10 times, through tangential flow filtration using Millipore cassettes with a molecular cut of 300kD at 5°C ± 3°C (Ronchi et al. 2012). In order to verify the efficiency of the process, samples were collected at each step and tested for virus concentration by real-time reverse transcription-polymerase chain reaction (RT-PCR) using previously described methods (Polci et al. 2013). One sample of concentrated virus was tested for inactivation by performing 3 serial passages on VerodogSLAMtag cells. The cells were observed daily using an inverted microscope (20X - 40 X) to evaluate the presence of CPE. The supernatant obtained from the third passage was analysed by RT-PCR to exclude PPR virus presence. Sterility for bacterial, fungal, and mycoplasma contamination was tested as described in the European Pharmacopoeia (Ph. Eur. 2004).

**Vaccines formulation**

The purified inactivated PPRV (iPPRV) product was divided into 2 aliquots: 1 was kept concentrated, while the other was diluted 10 times with PBS. Each aliquot was sub-divided and used to prepare different vaccine formulations, with one of 3 adjuvants: ISA 71 VG, a water in oil emulsion adjuvant kindly provided by Seppic (Seppic, Srl, Milan, Italy), and 2 batches of Advax™ delta inulin adjuvant (AFSA1 and AFSA2), kindly provided by Vaxine (Vaxine Pty Ltd, Bedford Park, Australia). The 6 formulations, obtained by mixing the iPPRV antigen with the adjuvants according to the manufacturer instructions, were tested for bacterial, fungal, and mycoplasma contamination and then stored at 5°C ± 3°C until use.

**Ethical statement**

All described animal experiments were performed in compliance with the National Law 116/921. Prior to carrying out the experiment on rats and goats, a written communication was sent to the Ministry of Health, Department for Veterinary Public Health, Nutrition and Food Safety, Office VI.

**Vaccine immunogenicity in rats**

Six groups of 5 adult male albino rats, 1 years old (weight of 380-400 g), were immunised subcutaneously (s.c.) with 0.5 ml of each vaccine formulation, with a booster dose administered 36 days later (Groups B-G as detailed in Table I). A control group of rats was inoculated with saline solution only (Group A). Rats were bled from the caudal vein at days T₀, T₁, T₂, T₃, and T₄ after immunisation. The serum antibody response was assessed using a c-ELISA kit coated with PPRV nucleoprotein (ID Screen® PPR Competition, IDVET, Grabels, France). The results are expressed as optical density (O.D.) values. All sera were tested in duplicate.

**Vaccine immunogenicity in goats**

Because of the results obtained in the rat trial, the concentrated iPPRV antigen adjuvanted with

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AFSA2 was used for the goat trial. Five randomly chosen dwarf goats (1-2 year old) were immunised at T₀ and T₁₂ with 1ml of vaccine administered s.c. in the inner side of the thigh. Three animals were kept as controls and inoculated with saline solution. Local reactions at the inoculation site and rectal temperature were monitored daily, for 18 days, after each immunisation. The antibody response was assessed by c-ELISA 3 times a week for 133 days post-vaccination and by Serum Neutralisation (SN) (OIE 2012) at T₉, T₁₂, T₃₆ and T₁₁₀.

### Statistical analysis

Statistical comparisons were conducted by Student's t test, and a P value < 0.05 was considered significant.

### Results

#### Virus production and inactivation kinetics

The PPRV suspension had a titre of 10⁷ TCID₅₀/ml before the inactivation process. The slope equation obtained using 1 mM BEI concentration was 

\[ y = -1.3229x + 7.1571 \]

with a correlation coefficient \( R^2 = 0.9705 \) (Figure 1). Complete virus inactivation was achieved in 5 hours. The inactivated, purified, and 10x concentrated virus suspension collected at the end of the purification/concentration process had a virus concentration of 10⁻² PPRV RNA copies/ml. The results of the quality controls for each working phase demonstrated the absence of any bacterial, fungal, or mycoplasma contamination. The virus was identified as PPRV and the absence of foreign viruses confirmed.

#### Vaccine immunogenicity in rats

In rats, the iPPRV vaccine formulations induced a detectable immune response starting from T₄₉ in all vaccinated groups (B, D, E, F, G, H), with the exception of the group vaccinated with diluted antigen in the ISA 71 VG adjuvant (Group C) (Table I). There was an antigen dose effect with higher levels of seroconversion in rats receiving higher dose of iPPRV vaccine formulation.

<table>
<thead>
<tr>
<th>Vaccine formulation</th>
<th>Antigen</th>
<th>pos/tot tested</th>
<th>pos/tot tested</th>
<th>pos/tot tested</th>
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<tbody>
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<td>A</td>
<td>Saline</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
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<tr>
<td>B</td>
<td>ISA 71 VG</td>
<td>0/4 **</td>
<td>0/5</td>
<td>0/4 **</td>
<td>3/4 **</td>
</tr>
<tr>
<td>C</td>
<td>ISA 71 VG</td>
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<td>F</td>
<td>AFSA 2</td>
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<td>0/5</td>
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</tr>
<tr>
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<td>AFSA 2</td>
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<td>1/5</td>
<td>4/5</td>
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*Administration of the booster dose. **The number of animal tested in each group varied because sera were haemolytic and not useful for ELISA test.

![Figure 1. Inactivation kinetics of Peste des petits ruminants virus (PPRV) using BEI 1 mM. Sodium thiosulphate at a final concentration of 2% (v/v) was added to each sample to neutralize the BEI at the end of each incubation.](image)
Figure 2. Mean serum Peste des petits ruminants antibody levels in immunised rat groups as determined by PPR competition ELISA. Booster vaccine administration was given on day 36. Low O.D. values correspond to high PRR antibody titers. OD values below lower line on graph indicate positive seroconversion.

Figure 3. Mean rectal temperatures in goats vaccinated with iPPRV antigen + AFSA2 adjuvant versus control animals. Temperatures were monitored for 18 days after each immunisation. The mean rectal temperature taken for two consecutive days in control goats after five days acclimatization (39.3°C), was considered normal and is indicated by the lower horizontal line in the graph. The highest temperature recorded before vaccination was (39.8°C) and is indicated by the upper horizontal line in the graph.
In this study, a new monovalent inactivated and adjuvanted vaccine to protect goats against PPR was prepared using the Moroccan M/08 virus strain belonging to lineage IV (Hammouchi et al. 2012) and assessed for immunogenicity in goats, following a preliminary trial in rats. The iPPRV antigen was inactivated with BEI, at a concentration 1 mM for 5 hours. Binary ethyleneimine acts only on the nucleic acids and not on the viral capsid proteins (Bahneman 1990) and has been extensively used to inactivate other viruses including rabies (Larghi and Nebe 1980), foot-and-mouth disease (Tekerlekov et al. 1983), bluetongue (Stott et al. 1979), porcine parvovirus (Buonavoglia et al. 1988) and Newcastle disease (King 1991). The surface glycoproteins, namely, hemagglutinin (H) and fusion (F) proteins of PPRV, have previously been shown to stimulate protective immunity against lethal PPRV challenge in goats (Rojas et al. 2014).

In this study, 3 adjuvants were tested for their capacity to enhance the iPPRV vaccine response: Montanide™ ISA 71 VG is a water-in-oil emulsion adjuvant used for bovine, ovine, and poultry vaccines. AFSA1 and AFSA2 are 2 delta inulin-based adjuvant formulations designed for human and veterinary use, which have previously been shown to induce protective immunity against lethal PPRV challenge in goats (Petrovsky et al. 2015).

The concentrated iPPRV vaccine with AFSA2 adjuvant (Group F) induced the earliest seroconversion in rats, achieving 100% seroprotection after 2 doses, closely followed by the AFSA1 formulation. By contrast the Montanide™ ISA 71 VG failed to protect all animals, even at the high iPPRV, and completely failed to induce seroconversion at the low antigen level.

**Discussion**

In this study, new adjuvanted vaccines were tested for their capacity to enhance the iPPRV vaccine response: Montanide™ ISA 71 VG is a water-in-oil emulsion adjuvant used for bovine, ovine, and poultry vaccines. AFSA1 and AFSA2 are 2 delta inulin-based adjuvant formulations designed for human and veterinary use, which have previously been shown to stimulate protective immunity against lethal PPRV challenge in goats (Petrovsky et al. 2015).

Vaccine immunogenicity in goats

Five goats were vaccinated subcutaneously at T₀ and T₁₆ with 1 ml of concentrated iPPRV antigen with AFSA2 adjuvant. The vaccine was well tolerated with no rise of body temperatures (Figure 3) or adverse reactions following administration of either of the 2 vaccine doses. In response to the first immunisation, a transient seroconversion was seen with anti-PRRV antibody detected at T₀ (mean O.D. 0.315), but not at T₀₉, T₀₃₉, or T₀₂₆. The administration of the booster dose at T₀₃₆ induced a robust and persistent seroconversion in all but 1 animal from T₀₄₀ up to the end of the study, at T₁₁₀ (Figure 4). Control animals did not develop any detectable anti-PRRV antibody response. The competition ELISA results were further confirmed by a serum neutralisation (SN) assay, which showed that all goats in the iPPRV + AFSA2 group, but none in the control group, had detectable PPR neutralisation antibodies at T₁₁₀ with the SN results in all immunised goats being positive as early as T₀₁₀.

![Figure 4. Anti-PPRV antibody responses as measured by PPR competition ELISA in goats vaccinated with concentrated iPPRV antigen + AFSA2 adjuvant versus control animals. A booster dose was administered at T₀₁₆.](image-url)
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concentration. No side effects or local reactions were seen at the site of inoculation of AFSA2 or AFSA1. Although the differences between the 2 delta inulin adjuvant formulations were not significant, the AFSA2 formulation appeared to induce marginally higher antibody titers than AFSA1. Consequently, AFSA2 was selected for the goat trial.

In the goat study, iPPRV + AFSA2 vaccine induced seroconversion as early as T9, as detected by both competition ELISA and serum neutralisation assay. However the competition ELISA early antibody response attenuated over time whereas the serum neutralisation results remained consistently positive from T9 forwards, suggesting the serum neutralisation might be more sensitive than the competition ELISA. The administration of a booster dose of iPPRV + AFSA2 vaccine at T36 induced a robust and persistent recall antibody response, reaching a peak at T44, 8 days post boost. According to Rossiter and colleagues (Rossiter et al. 1985), a serum neutralizing titre above 1.8 is indicative of PPRV protection. Using this as a guide, just a single dose of iPPRV + AFSA2 vaccine was able to induce seroprotection in all goats, 9 days post the first immunisation, with all immunised goats having SN titers of 1:10 or greater. This is a major achievement, as inactivated vaccines typically need 2 or more doses to achieve seroprotection. It is however consistent with studies of Advax delta inulin adjuvants in other inactivated vaccines, including Japanese encephalitis (Larena et al. 2013), West Nile virus (Petrovsky et al. 2013), and influenza (Layton et al. 2011), where robust protection was similarly seen after just a single vaccine dose, when formulated with Advax adjuvant.

The production cost of inactivated vaccine in cell culture is typically high. As the low dose iPPRV + AFSA2 adjuvant formulation still induced seroconversion in 80% of rats after the booster dose, further investigation is needed to ascertain the minimum antigen concentration able to achieve high levels of seroprotection, to maximise the affordability of this vaccine.

Our data confirm the utility of using rats as a preliminary model to assess the immunogenicity of inactivated PPR vaccines, prior to formal testing in larger at-risk animal species, like sheep and goats. The use of rodents to test PPR vaccines is not new, as it has been recommended for assessing of nonspecific PPR vaccine toxicity (OIE 2012). They were also used by Ezeibel and colleagues (Ezeibel et al. 2010) to evaluate the immunogenicity of Nigeria 75/1 attenuated vaccine. Interestingly, in our study, the rats only became seropositive by the ELISA test after administration of the booster dose, whereas goats became seropositive at T9 after a single immunisation. If anything, the lower responsiveness of rats to PPR vaccine is an advantage in respect of their use as a screening tool, as vaccines found effective in rats should be even more effective when tested in the larger PPR host species such as sheep and goats.

The anti-PPR competition ELISA as a quantitative test is faster and more convenient than the SN method to study the antibody response to PPR vaccination (Reizenstein et al. 1995). Nevertheless, our results suggest caution with use of the competition ELISA, as it was less sensitive than the SN method and differences were observed between the 2 methods (Table II). For example, goat 5 at T12 was negative by SN test, while positive at ELISA, with the contrary being seen at T44. Furthermore, at T36, 4 goats were still seropositive by SN test while resulting negative by ELISA. Singh and colleagues also reported these differences between the 2 assays and found the SN assay to be more sensitive than the ELISA (Singh et al. 2004).

The present study has several limitations. First, our vaccine was produced from a field strain of PPR virus and thereby it does not offer the possibility to use serology to differentiate infected animals from vaccinated ones. Secondly, PPR challenges were not undertaken as part of this study to confirm actual protection of the goats against PPR, although previous studies have shown a high correlation between SN titres and protection. A commercial vaccine should provide protection for at least a year, and hence it will be important to conduct further studies to extend the current seroprotection results beyond 3 months post-immunisation. Finally, due to limited rat group sizes, statistically significant differences were not evident between AFSA1 and AFSA2 groups, such that it is not possible to

<table>
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<tr>
<th>Goat</th>
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<td>ELISA</td>
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<tr>
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<td>20</td>
<td>16%</td>
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<td>10</td>
</tr>
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<tr>
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</tr>
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<td>Goat 8</td>
<td>84%</td>
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<td>80%</td>
<td>Neg.</td>
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</table>

* Administration of the booster dose.  

Table II. Anti-PPRV antibody responses in vaccinated and control goats as assessed by c-ELISA and serum neutralization (SN) assays. C-ELISA results are expressed as percentage values (Positive ≤ 35%, 35% < Borderline(doubt) ≤ 45%, Negative > 45%). SN is expressed as the reciprocal of the neutralizing titre.
determine which might be the better adjuvant for iPPRV vaccine.

Inactivated PPR vaccine when combined with AFSA2 delta inulin adjuvant presents a promising alternative to the use of live attenuated vaccines in PPR vaccination campaigns in non-endemic areas.

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References


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Conflict of interest

Nikolai Petrovsky is affiliated with Vaxine Pty which produces the Advax™ adjuvants.


