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Specific detection of peste des petits ruminants virus antibodies in sheep and goat sera by the luciferase immunoprecipitation system

Francisco J. Berguido[a,*], Sanne Charles Bodjo[b], Angelika Loitsch[c], Adama Diallo[a]

[a] Animal Production and Health Laboratory, FAO/IAEA Agriculture and & Biotechnology Laboratory, IAEA Laboratories Seibersdorf, International Atomic Energy Agency (IAEA), Wagramer Strasse 5, P.O. Box 100, A1400, Vienna, Austria
[b] Pan African Veterinary Vaccine Center of the African Union (AU-PANVAC), P.O Box 1746, Debre-Zeit, Ethiopia
[c] Institute for Veterinary Disease Control, Austrian Agency for Health and Food Safety, Moedling, Austria (AGES)

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A B S T R A C T

Peste des petits ruminants (PPR) is a contagious and often fatal transboundary animal disease affecting mostly sheep, goats and wild small ruminants. This disease is endemic in most of Africa, the Middle, Near East, and large parts of Asia. The causal agent is peste des petits ruminants virus (PPRV), which belongs to the genus *Morbillivirus* in the family *Paramyxoviridae.* This genus also includes measles virus (MV), canine distemper virus (CDV) and rinderpest virus (RPV). All are closely related viruses with serological cross reactivity.

In this study, we have developed a Luciferase Immunoprecipitation System (LIPS) for the rapid detection of antibodies against PPRV in serum samples and for specific differentiation from antibodies against RPV.

PPR and rinderpest (RP) serum samples were assayed by PPR-LIPS and two commercially available PPR cELISA tests. The PPR-LIPS showed high sensitivity and specificity for the samples tested and showed no cross reactivity with RPV unlike the commercial PPR cELISA tests which did cross react with RPV. Based on the results shown in this study, PPR-LIPS is presented as a good candidate for the specific serosurveillance of PPR.

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1. Introduction

Peste des petits ruminants (PPR) is an increasingly alarming, highly contagious, viral disease primarily affecting sheep, goats and wild small ruminants (Libeau et al., 2014). Due to its high mortality and morbidity, PPR is responsible for severe economic losses in the countries where it is endemic (Libeau et al., 2014). This disease, which can cause 50–90% mortality in naïve populations, is widely spread throughout most of Africa, the Middle and Near East, South and Central Asia and China. Most of these areas rely on subsistence farming (Nanda et al., 1996; Libeau et al., 2014; Wang J et al., 2015). Thus, the control and global eradication of PPR, which are currently being undertaken by the World Organization for Animal Health (OIE) and the Food and Agriculture Organization of the United Nations (FAO), are seen as relevant aspects of poverty alleviation policies (Perry B.D. et al., 2002).

The causal agent of PPR, the peste des petits ruminants virus (PPRV), belongs to the genus *Morbillivirus* in the family *Paramyxoviridae* along with measles virus, canine distemper virus, rinderpest virus, phocine distemper virus, dolphin morbillivirus and feline morbillivirus (Diallo and Libeau, 2014; Gibbs et al., 1979; Libeau et al., 2014).

PPR is characterized by nasal and ocular discharges, gastroenteritis, necrotic stomatitis, pyrexia, and erosion of the pulmonary tract mucosa (Wolhsein and Saliki, 2006; Roeder and Obi, 1999). Death occurs primarily by bronco-pneumonia or extreme dehydration due to acute diarrhoea (Banyard et al., 2010). Apart from the broncho-pneumonia, symptoms of PPR are similar to those of rinderpest. Although rinderpest was officially declared eradicated in 2011, it remains imperative that PPR surveillance is conducted with laboratory assays that are specific to this disease, and do not cross-react with other pathogens. Such assays are available for nucleic acid detection by polymerase chain reaction (PCR) and antigen detection by monoclonal antibody-based immunocapture ELISA (Diallo and Libeau, 2014; Diallo et al., 1995). For detection of

Abbreviations: PPR, peste des petits ruminants; LIPS, luciferase immunoprecipitation System; RPV, Rinderpest.

* Corresponding author. Tel.: +43 1260028356; fax: +43 1260028222.
E-mail address: f.berguido@iaea.org (F.J. Berguido).

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antibodies directed against PPRV, two commercial assays are available, the PPRV haemagglutinin-protein-based cELISA (Anderson & McKay, 1994) and the PPRV nucleoprotein-based cELISA (Libeau et al., 1995). The haemagglutinin protein of PPRV is a protective antigen capable of generating neutralizing antibodies (Barrett et al., 2006). The nucleoprotein of PPRV generates a strong immune-response, most of the antibodies for morbilliviruses are directed against it, although these antibodies are not protective (Diallo et al., 1995). Proteins such as the fusion protein of PPRV (FPPRV) are not used for serological assays. FPPRV is a protective antigen and generates a strong cellular immune response, but it has a weak humoral immune-response (Barrett et al., 2006).

Both ELISA tests, the PPRV haemagglutinin-protein-based cELISA and the PPRV nucleoprotein-based cELISA, detect PPR serum antibodies in a similar fashion (Couacy-Hymann et al., 2007). However, they present some cross reactivity with rinderpest sera (Anderson and McKay, 1994; Couacy-Hymann et al., 2007).

In this paper, we describe the development and characterization of the Luciferase Immunoprecipitation System (LIPS) for the specific detection of antibodies against PPRV using a specific peptide of the PPRV nucleoprotein. This fragment showed high specificity to PPRV and adequate antibody response (Bodjo et al., 2013).

In general, LIPS functions by detecting luciferase activity from the interactions of a crude extract containing a fusion protein of luciferase and a target antigen, serum and protein A/G beads (Burbelo et al., 2009). Whenever antibodies against the target antigen are present in the test serum, they bind to the antigen portion of the fusion protein. Protein A/G beads bind to the antibodies in sera and in doing so precipitate the luciferase-target-antigen fusion protein. Luciferase substrate is then added and light is measured. The amount of light emitted is proportional to the amount of fusion protein precipitated which in turn is proportional to the amount of antibody present in the serum (Burbelo et al., 2009).

LIPS which has been used in the past for antibody profiling of Epstein-Barr virus (Sashihara et al., 2009), hepaciviruses (Burbelo et al., 2012) and Lyme disease (Burbelo et al., 2015; Burbelo et al., 2010b), was adapted in this study for the detection of antibodies against PPRV.

2. Materials and methods

2.1. Generation of plasmid constructs for the expression of renilla luciferase fused to the N protein fragment 420-525 of peste des petits ruminants virus

The cytomegalovirus (CMV) promoter-driven Renilla luciferase vector, pGL4.75 (Promega) was modified by PCR to generate a new plasmid, pRFX, in which the stop codon of the luciferase gene was eliminated (primers shown in Table 1). The nucleotide region encoding amino acids 420 to 525 of the nucleoprotein of PPRV vaccine strain (Nigeria 75/1) was amplified by RT-PCR from purified RNA extracted from virus-infected cells using the One Step RT-PCR kit (Qiagen). The primers used were NP-F and NP-R (Table 1) which contained *Xba*I and *Fse*I restriction enzyme sites. The amplified product was digested with *Xba*I and *Fse*I, purified and inserted into the plasmid pRFX, which had been previously digested with the same enzymes. The PPRV nucleoprotein gene fragment was inserted downstream of the Renilla luciferase gene in order to obtain a Renilla luciferase–NPPR 420-525 fusion gene, resulting in the plasmid pRNP420 (Fig. 1).

Table 1
Primers used in the construction of pRNP420.

Primer	Sequence and Modification
FB-1	5′Phosphate-TTCTAGAGTCGGGGCGGCCGGCCG
FB-2	5′Phosphate-TCTGCTCGTTCTTCAGCACGCGCTCC
NP-F	ATCTGCTCTAGAGCTCCAGCACAAAACGGGAGAG
NP-R	ATATACGGGCCGGCCTTAGCCGAGGAGATCCTTGT

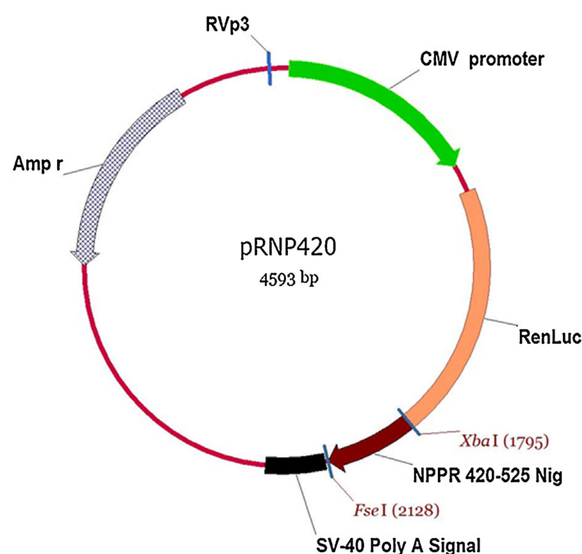

Fig. 1. Structure of the pRNP420 Mammalian Expression Vector.
Some of the features indicated are the Cytomegalovirus (CMV) promoter, the Renilla luciferase-NPPR 420-525 from Nigeria 75/1 fusion protein gene and the position of the two restrictions enzyme sites used for cloning.

2.2. Production of renilla luciferase–NPPR 420-525 fusion protein

The plasmid pRNP420 containing the gene for the fusion protein RLuc-NPPR 420-525, was purified using the HiSpeed Plasmid Midi Prep kit (Qiagen). Vero cells were grown in DMEM (Invitrogen) supplemented with 10% foetal bovine serum in 10 cm tissue culture plates. Cells were transfected with the plasmid at a ratio of 1:4 (DNA to transfectant reagent), using the standard Fugene 6 protocol (Promega). Two days after transfection, media was removed and the cells were washed with 6 ml of phosphate buffered saline (PBS), followed by the addition of 1.4 ml of cold lysis buffer [(50 mM Tris, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 50% glycerol and protease inhibitor (2 tablets of complete protease inhibitor cocktail (Roche) per 50 ml of lysis buffer)]. Cells were then harvested with a cell scraper and the lysate transferred to a 5 ml polypropylene tube on ice.

Cells were ruptured using a sonicator (Vibra-Cell VCX 750, Sonics and Materials Inc., Newtown, CT USA) set at an amplitude of 50% using four pulses of 5 s each. The samples were then centrifuged at 16,000 g for 4 min at 4 °C and the supernatants collected and stored at −80 °C until required.

2.3. Western blot

Extracts of RLuc-NPPR 420-525 fusion-protein-transfected cells (20 μl) were heated at 80 °C for 10 min in 4X LDS Sample Buffer (Invitrogen). The samples were subjected to SDS-PAGE (NUPAGE 10% (v/v) gel, Invitrogen) and transferred to a 0.2 μm PVDF membrane (Invitrogen) using standard techniques. Baculovirus expressing recombinant full length NPPR in SF21 insect cells and baculovirus alone (Bodjo et al., 2008) were used as controls. The membrane was then probed for 1 h at room temperature with anti-PPR specific monoclonal antibody (Mab) P4G5 diluted in PBS containing 0.5% (v/v) Tween and 5% (w/v) powdered milk (Sigma). P4G5 was generated and previously characterized in our laboratory (Bodjo et al., 2008). The membrane was washed three times

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Table 2
Description of PPR experimental serum samples used in this study. Thirty-five samples from 15 PPRV experimentally infected sheep and goats were tested using PPR-LIPS and the ID Vet PPR competitive ELISA test. Percentage agreement is 100% between the two methods.

Animal	PPR Challenge Virus	Lineage	Day Post Infection	Results cELISA	Results PPR-LIPS**
Goat A	CI89 Goat	1	2[a]	Neg	Neg
Goat A	CI89 Goat	1	30	Pos	Pos
Goat B	CI89 Goat	1	2[a]	Neg	Neg
Goat B	CI89 Goat	1	30	Pos	Pos
Goat C	CI89 Goat	1	2[a]	Neg	Neg
Goat C	CI89 Goat	1	30	Pos	Pos
Sheep A	CI89 Sheep	1	0	Neg	Neg
Sheep A	CI89 Sheep	1	30	Pos	Pos
Sheep B	CI89 Sheep	1	2[a]	Neg	Neg
Sheep B	CI89 Sheep	1	30	Pos	Pos
Goat D	Nigeria 75-1	2	0	Neg	Neg
Goat D	Nigeria 75-1	2	15	Pos	Pos
Sheep C	Nigeria 75-1	2	0	Neg	Neg
Sheep C	Nigeria 75-1	2	2	Neg	Neg
Sheep C	Nigeria 75-1	2	15	Pos	Pos
Sheep D	Nigeria 75-1	2	0	Neg	Neg
Sheep D	Nigeria 75-1	2	15	Pos	Pos
Sheep D	Nigeria 75-1	2	30	Pos	Pos
Sheep E	Nigeria 75-3	2	2[a]	Neg	Neg
Sheep E	Nigeria 75-3	2	15	Pos	Pos
Goat E	Dorcas	3	0	Neg	Neg
Goat E	Dorcas	3	30	Pos	Pos
Goat F	Dorcas	3	0	Neg	Neg
Goat F	Dorcas	3	15	Pos	Pos
Goat F	Dorcas	3	30	Pos	Pos
Sheep F	Dorcas	3	0	Neg	Neg
Sheep F	Dorcas	3	15	Pos	Pos
Sheep F	Dorcas	3	30	Pos	Pos
Sheep G	Dorcas	3	0	Neg	Neg
Sheep G	Dorcas	3	30	Pos	Pos
Sheep H	Dorcas	3	2[a]	Neg	Neg
Sheep H	Dorcas	3	30	Pos	Pos
Sheep I	India Calc 95	4	0	Neg	Neg
Sheep I	India Calc 95	4	15	Pos	Pos
Sheep I	India Calc 95	4	30	Pos	Pos
Goat	Neg Control			Neg	Neg
Sheep	Pos Control			Pos	Pos

[a] No Day 0 Available. Day 2 Post infection used instead.
** Mean of two to three LIPS assays in duplicate. Negative RLU values were given the value of 0.

with PBS containing 0.5% (v/v) Tween and probed with goat anti-mouse antibodies conjugated to horseradish peroxidase (Sigma). For detection, ECL substrate (GE Healthcare) was used according to the manufacturer's instructions.

2.4. Serum samples

A total of 219 serum samples were used in this study. This included 75 sheep and goat PPR positive and negative serum samples collected from both experimental and natural field infected animals in 2002-2003 and provided by Dr. Emmanuel Couacy-Hymann (Laboratoire Central de Pathologie Animale, Bingerville, Côte d'Ivoire) (Tables 2 and 3). Fourteen rinderpest samples from experimentally infected cattle, produced 17 years ago by the Kenya Agricultural Research Institute (KARI) in Nairobi, Kenya were kindly provided by Dr. Henry Wamwayi (Table 4). One hundred and thirty rinderpest field serum samples collected 20 years ago during the Global Rinderpest Eradication Programme (GREP) were kindly provided by Dr. Emmanuel Couacy-Hymann. All non-irradiated serum samples were tested in a biosafety level 3 (BSL-3) facility (AGES, Austria).

2.5. Luciferase immunoprecipitation system (LIPS)

The protocol for LIPS has been previously described (Burbelo et al., 2009). Briefly, to determine total luciferase activity, 1 μl of crude fusion protein extract was added to 100 μl of coelenterazine substrate (Promega) in a white 96 well-plate (Sterilin). Relative light units (RLU) were measured in a luminometer (Berthold Centro LB, Berthold Technologies, Bad Wildbad Germany) for 5 s and the volume of protein extract required to produce 1×10^7 RLU was determined.

Immunoprecipitation reactions were then carried out by mixing 40 μl of buffer A (50 mM Tris, pH 7.5, 100 mM $MgCl_2$, 1% Triton X-100), 10 μl of diluted serum (1 in 10 in buffer A) and 50 μl of buffer A containing enough fusion protein extract to generate 1×10^7 RLU (as calculated above) in each well of a 96-well-plate. This mixture was incubated for 1 h at room temperature with gentle shaking. The mixture was then transferred to a 96 well Multi-Screen HTS filter plate (Millipore) and incubated with 5 μl of Ultralink immobilized protein A/G beads (Pierce Biotechnology Inc) for 1 h at room temperature with gentle shaking, and then washed 8 times with buffer A and twice with PBS using a vacuum manifold. Coelenterazine substrate was added and the light emission was read for 5 s using a luminometer (Berthold Centro LB, Berthold Technologies, Bad Wildbad Germany).

2.6. Competitive ELISAs (cELISAs)

The ID Screen PPR Competition ELISA (ID Vet, France) and the BDSL PPR ELISA kit (BDSL, U.K.) were used according to the manufacturer's instructions. The ID Screen PPR Competition ELISA is based on serum antibody competition against a monoclonal antibody targeting the nucleoprotein of PPRV (Libeau et al., 1995). The BDSL PPR ELISA kit uses purified whole virus from PPR Nigeria 75/1 and it is based on serum antibody competition against a monoclonal

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Table 3
Results from forty sheep and goat PPR field serum samples. Samples were subjected to PPR-LIPS, ID Vet PPR cELISA and BDSL PPR cELISA. Some samples were also tested by the PPR virus neutralization test, as described in the 2013 OIE Manual.

Sample ID	LIPS	IDVET	BDSL	VNT
B1	Pos	Pos	Pos	Pos
B2	Pos	Pos	Pos	N/P
B3	Pos	Pos	Pos	N/P
B4	Pos	Pos	Pos	N/P
B5	Pos	Pos	Pos	N/P
B6	Pos	Pos	Pos	N/P
B7	Pos	Pos	Pos	Pos
B8	Pos	Pos	Pos	N/P
B9	Pos	Pos	Pos	N/P
B10	Pos	Pos	Pos	Pos
B11	Pos	Pos	Pos	Pos
B12	Pos	Pos	Pos	N/P
B13	Pos	Pos	Pos	N/P
B14	Pos	Pos	Pos	N/P
B15	Pos	Pos	Pos	Pos
B16	Pos	Pos	Pos	N/P
B17	Pos	Pos	Pos	Pos
B18	Pos	Pos	Pos	Pos
B19	Pos	Pos	Pos	Pos
B20	Pos	Pos	Pos	N/P
B21	Pos	Pos	Pos	N/P
B22	Pos	Pos	Pos	N/P
B23	Pos	Pos	Pos	N/P
B24	Pos	Pos	Pos	N/P
B25	Pos	Pos	Pos	N/P
B26	Pos	Pos	Pos	N/P
B27	Neg	Neg	Neg	N/P
B28	Neg	Neg	Neg	N/P
B29	Neg	Doubtful	Neg	Neg
B30	Pos	Pos	Pos	N/P
B31	Neg	Neg	Neg	N/P
B32	Pos	Pos	Pos	N/P
B33	Neg	Neg	Neg	N/P
B34	Pos	Pos	Pos	Pos
B35	Neg	Pos	Pos	Neg
B36	Neg	Neg	Neg	N/P
B37	Neg	Neg	Neg	Neg
B38	Neg	Neg	Neg	N/P
B39	Neg	Neg	Neg	N/P
B40	Neg	Neg	Neg	N/P

N/P = Not Performed.

antibody targeting the haemagglutinin protein of PPRV (Anderson and McKay, 1994).

The Rinderpest Competition ELISA kit (BDSL, U.K.) was used according to the manufacturer's instructions to test RPV field serum samples. This kit detects antibodies directed against the rinderpest haemagglutinin viral protein (Anderson et al., 1991).

2.7. PPR virus neutralization test

The PPR virus neutralization test (VNT) was performed in a BSL-3 facility (AGES, Austria), using the Nigeria 75/1 PPRV attenuated vaccine strain, and was carried out according to the specifications of the OIE (World Organization for Animal Health, 2013). Briefly, sera were heat-inactivated (56 °C for 30 min). Each serum sample was then diluted 1 in 5, and subsequently two-fold serially diluted with cell culture medium (100 μl/well) to 1 in 320 in a 96 well tissue culture plate.

One hundred microliters of virus at 1000 $TCID_{50}$/ml (100 $TCID_{50}$ final concentration/well) was added to each well containing 100 μl of diluted serum. A series of control wells were arranged as follows: 6 wells with 100 $TCID_{50}$ (100 μl) per well, 6 wells with 10 $TCID_{50}$ (100 μl) per well, 6 wells with 1 $TCID_{50}$ (100 μl) per well, 6 wells with 0.1 $TCID_{50}$ (100 μl) per well and 6 wells with 200 μl of virus-free culture medium per well. The plate was incubated for 1 h at 37 °C. After the incubation, 50 μl of Vero cell suspension (600,000 cells/ml) was added to each well. The plates were incubated at 37 °C in the presence of 5% CO_2.

Using an inverted microscope (Leitz Labovert, Germany), plates were read for cytopathic effect after 2 weeks of incubation. Any neutralising titre greater than 10 was read as positive.

2.8. Limit of detection

The limit of detection of PPR-LIPS was tested by serially diluting five PPR positive (Nigeria 75/1) serum samples. Two-fold serial dilutions of the samples were made in PBS and then tested in duplicate by both the PPR-LIPS and the ID Vet cELISA kit.

2.9. Statistical analysis

All statistical analysis was performed using SPSS version 10.0 (New York, USA).

The mean titres of PPR positive and PPR negative samples from experimental and field data sets were compared using the non-parametric Mann-Whitney U test. Mean titres were subjected to logarithmic transformation before performing a correlation analysis. Pairwise correlation among different assay methods was performed by the Spearman rank correlation test. For determination

Table 4
Test results of 14 samples from 7 Rinderpest experimentally infected animals assayed by PPR-LIPS and the ID Vet PPR competitive ELISA test. Test results are expressed in Relative Light Units (RLU) and Percentage Competition (%S/N) respectively.

			PPR IDVet cELISA		PPR-LIPS		
Animal	Virus	Days Post Infection	%S/N[a]	Results cELISA	RLU[a]	Results PPR-LIPS	Agreement cELISA-LIPS
A	Rinderpest	0	70.49	Neg	279.0	Neg	Yes
A	Rinderpest	28	16.75	Pos	517.7	Neg	No
B	Rinderpest	0	92.95	Neg	101.3	Neg	Yes
B	Rinderpest	28	15.15	Pos	103.0	Neg	No
C	Rinderpest	0	105.23	Neg	334.5	Neg	Yes
C	Rinderpest	14	38.61	Doubtful	273.8	Neg	No
D	Rinderpest	0	100.23	Neg	109.8	Neg	Yes
D	Rinderpest	28	13.61	Pos	175.7	Neg	No
E	Rinderpest	0	113.29	Neg	194.8	Neg	Yes
E	Rinderpest	28	28.07	Pos	1099.5	Neg	No
F	Rinderpest	0	93.09	Neg	98.3	Neg	Yes
F	Rinderpest	24	19.99	Pos	181.7	Neg	No
G	Rinderpest	0	96.33	Neg	27.5	Neg	Yes
G	Rinderpest	14	30.35	Pos	647.8	Neg	No
H	PPR Negative		100	Neg	25	Neg	Yes
I	PPR Positive		4.8	Pos	954477	Pos	Yes

[a] Average of two assays in duplicate.

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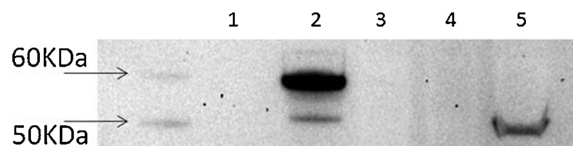

Fig. 2. Expression of pRNP420 in Vero Cells.
Western blot of Vero cell crude extracts. Primary Antibody: P4G5 anti-C -Terminus NPPR; (1) Baculovirus alone expressed in SF21 insect cells; (2) Recombinant full length NPPR Baculovirus expressed in SF21 insect cells; (3) Non-transfected Vero cell extract; (4) Vero cell extract transfected with RLuc reporter vector; (5) Vero cell extract transfected with pRNP420.

of the cut-off limits for PPR-LIPS, the average of negative samples plus 4 standard deviations was used and is indicated in Fig. 4.

3. Results

3.1. Initial Tests of the PPR-LIPS

Expression of the RLuc-NPPR 420-525 fusion protein was confirmed by Western blot (Fig. 2).

The functionality of the Renilla luciferase portion of the fusion protein was confirmed by mixing lysates from transfected Vero cells expressing the fusion protein with the Renilla luciferase substrate coelenterazine. The amount of RLU produced was then measured by a luminometer. In the presence of coelenterazine substrate, values of over 1×10^7 RLU per microliter were produced by the fusion protein, while the negative control (non-transfected Vero cell lysate) generated less than 900 RLU (background levels) (data not shown).

The functionality of PPR-LIPS was determined by testing two samples: a negative goat serum from Austria, an official PPR-free country, and a positive serum from an animal experimentally immunized with the Nigeria 75/1 PPRV attenuated vaccine strain and then challenged with the PPR India Calcutta 95 strain. Both samples had been previously tested for PPR and their results confirmed by PPR cELISA ID Vet test (data not shown).

When the Renilla luciferase-NPPRV fusion protein was precipitated with the PPR positive serum sample, it generated more than 5,000,000 RLU compared to 190 RLU generated when the same serum sample was precipitated with the lysate from cells expressing the Renilla luciferase alone (Fig. 3).

The PPR negative serum sample, when used to precipitate lysates containing either Renilla luciferase alone or the fusion protein Renilla luciferase-NPPR 420-525, produced after blank adjustment, negative light unit values. In order to present the results in a log scale graph, a value of 100 was assigned to the

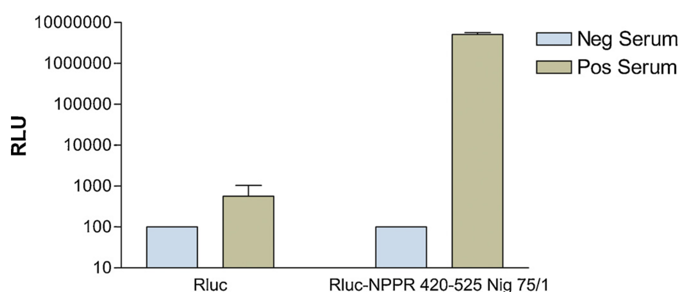

Fig. 3. Positive and Negative PPR Serum Samples Tested with PPR-LIPS.
Negative serum (light blue): serum from an Austrian goat; Positive serum (light brown): serum from PPR vaccinated sheep, challenged with the India/Calcutta 95 Strain. Precipitation of the samples was carried out using either the fusion protein Renilla luciferase-NPPR 420-525 or the Renilla luciferase protein alone (RLuc), both expressed in Vero cells. The error bars represent standard deviation from the mean value. Negative values after blank adjustment were given a value of 100.

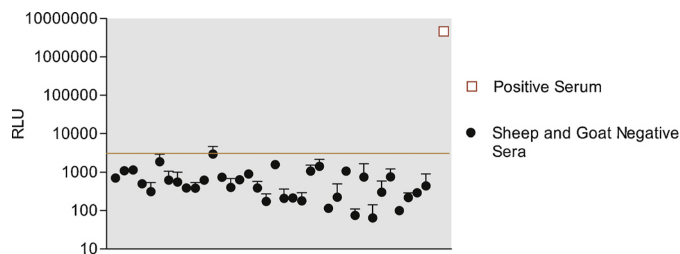

Fig. 4. Determination of Cut-Off Values for PPR-LIPS.
Thirty-six PPR negative (Austrian) serum samples from sheep and goats were subjected to PPR-LIPS in order to establish a cut-off value. Each round symbol represents a sheep or goat negative serum sample, run in duplicate. The square symbol represents a single positive serum sample from a PPRV experimentally infected animal, run in duplicate, and included for reference. The error bars represent standard deviation from the mean value. The cut-off value, based on the mean titre plus 4 standard deviations of the negative values, is shown by the long solid line. Negative values after blank adjustment were given a value of 100.

negative values (Fig. 3). The RLU value of the positive serum sample is more than 26,000 times that of the negative serum sample, which allows for a good detection range.

3.2. Cut-off determination

In order to determine a cut-off RLU value for the PPR-LIPS, negative sera from 18 sheep and 18 goats from Austria were tested. The sera, tested twice in duplicate, and following blank adjustment, ranged in value from <0 to 2995 RLU. Positive controls were above 3,900,000 RLU. The mean titre of the negative serum samples was 641 RLU (95% CI, 433 to 849). These values were used to establish a positive cut-off value, using the average plus 3 (2484 RLU) and 4 (3098 RLU) standard deviations (Fig. 4). Since average values plus higher standard deviations increased the error levels, we determined that for PPR-LIPS, any value above 3098 RLU was positive, while values below were negative.

In order to present results in a log scale, negative RLU values were given a value of 100 (Fig. 4).

3.3. Sensitivity of PPR-LIPS

Results from experimentally infected PPR serum samples tested using PPR-LIPS and the ID Vet PPR cELISA kit indicate that 35/35 samples were in agreement between the two assays (Table 2). Additionally, 40 PPR field serum samples were also tested by PPR-LIPS, ID Vet PPR cELISA and BDSL PPR cELISA kits (Table 3). Results show 38/40 samples were in agreement between PPR-LIPS and ID Vet PPR cELISA kit (95%) and BDSL PPR cELISA (97.5%). The Spearman rank test determined a highly significant correlation ($p < 0.01$) when comparing the antibody responses from the log-transformed LIPS mean titres with both ID Vet and BDSL PPR cELISA kits. All samples that were not in agreement between the three assays, were tested using the PPR virus neutralization assay (VNT), which is the gold standard assay recommended by the OIE. In all cases, the VNT and PPR-LIPS were in agreement (Table 3).

3.4. Specificity of PPR-LIPS

Experimentally infected rinderpest samples collected from cattle at days 0 and 14, 0 and 24 or 0 and 28 as indicated in Table 4, were tested using the LIPS-based PPR method and the ID Vet PPR cELISA test. All rinderpest experimental samples collected at days 14, 24 and 28, tested positive or doubtful by the PPR competitive ELISA but tested negative by PPR-LIPS. The mean titre of the rinderpest samples from days 14, 24 and 28 tested by PPR-LIPS was 296 RLU (95% CI, 130 to 463), which is markedly lower than the

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Table 5
Limit of detection of PPR-LIPS. Five different sera from sheep and goats infected with the Nigeria 75-1 (sera 1, 4, 5) or Côte d'Ivoire 89 (sera 2 and 3) PPRV strains were two-fold serially diluted. Each dilution was tested by both PPR-LIPS and the ID Vet PPR cELISA test. The highest dilution testing positive for each assay is indicated. The assays are measured either in Percentage Inhibition (PI) or Relative Light Units (RLU).

PPR Positive Serum Number		1	2	3	4	5
cELISA	Highest Dilution Testing Positive (1/X)	32	256	128	64	64
	PI	68.372	67.291	66.62	65.03	84.19
LIPS	Highest Dilution Testing Positive (1/X)	512	512	256	128	128
	RLU	5786	3541.2	4244.7	9232.8	7288.1

threshold of 3098 RLU, suggesting that the PPR-LIPS is more specific than the commercially available cELISA kit (Table 4). Samples from day 0 tested negative in both assays.

This comparison study was extended to 130 rinderpest cattle field serum samples that were collected during the Global Rinderpest Eradication Program (GREP) more than 20 years ago for rinderpest surveillance and seromonitoring. At that time, they were tested by the BDSL Rinderpest cELISA kit. We tested them again with this kit which detects no cross reactivity with antibodies against PPRV (Anderson and McKay, 1994).

Out of the 130 rinderpest field serum samples tested by the BDSL Rinderpest cELISA kit, 101 samples tested positive and 29 samples tested negative for rinderpest. The 101 rinderpest positive samples were also tested by both the ID Vet and BDSL PPR cELISA kits. With the ID Vet PPR cELISA kit, 9 rinderpest samples were also positive and one was borderline (9.9% cross reactivity). With the BDSL PPR cELISA kit, 13 rinderpest samples were either positive or borderline (12.9% cross reactivity). The cross reactivity of the PPR cELISA kits to rinderpest antibodies has previously been described (Anderson and McKay, 1994; Couacy-Hymann et al., 2007).

However, all samples tested negative by PPR-LIPS, with a mean RLU value of 268 (95% CI, 196 to 340). These results indicate the high specificity of this test (Supplementary Table 1).

3.5. PPR-LIPS limit of detection

The limit of detection (LOD) of PPR-LIPS was tested by making two-fold serial dilutions of 5 PPR positive serum samples. These dilutions were tested in duplicate by both the PPR-LIPS and ID Vet PPR cELISA kit. The average LOD for the PPR-LIPS assay was 1 in 256 where the average LOD for the commercial PPR test was 1 in 108 (Table 5).

4. Discussion

The PPR-LIPS was developed for the specific detection of PPR antibody while eliminating the cross-reactivity with like viruses. It is based on the use of the Renilla luciferase protein to which is fused the c-terminal fragment – amino acids 420-525 of the PPRV nucleoprotein. This fragment was chosen after consideration of in-house antibody mapping studies suggesting its potential to differentiate PPRV from RPV in antibody binding (Bodjo et al., 2013).

Serum samples collected from PPR infected sheep and goats, as well as non-infected animals, were tested by this new test and also by commercially available PPR cELISA kits: the ID Vet ID Screen PPR cELISA kit and the BDSL PPR cELISA kit. They are the current assays recommended for use in the 2013 OIE Manual (World Organization for Animal Health, 2013). The PPR VNT, the OIE recommended test for PPR serological diagnosis for trade issues, was used on a small sub-set of the PPR field serum samples where there was no agreement between the cELISA kits and PPR-LIPS. The results obtained indicate that PPR-LIPS is at least as sensitive as the commercially available PPR cELISA kits, requires lower sample volumes (1 μl per assay) and shows no cross reactivity between PPR and rinderpest antibodies in serum samples.

Our initial tests by PPR-LIPS using experimental PPR serum samples confirmed a sensitivity of 100% on samples that were clearly PPR positive (day 15–30 post infection). In most cases, samples at day 15 post infection produced a higher light unit value than samples at day 30 post infection. This correlates with the classical Gaussian curve of the lag, log, plateau and decline phases of antibody production in the body, where log phase starts at 5 to 7 days post infection, takes 15 days to reach the plateau after which antibody production declines (Armstrong, 2008).

While testing PPR experimental serum samples by PPR-LIPS, we observed differences of over 20,000x RLU between positive and negative samples (Fig. 3). Burbelo et al. suggested that because antibodies and target antigens interact in a liquid phase instead of being attached to a solid phase such as in ELISA, the detection is improved as is the native folding of the protein (Burbelo et al., 2010a). Moreover, this type of interaction allows for the detection of a large number of conformational epitopes (Ramanathan et al., 2008). Our results agree with this research.

In order to establish the cut-off value for the assay, we examined previous LIPS studies where the cut-off value used was the mean plus 2 standard deviations and where the cut-off value used was the mean plus 5 standard deviations (Burbelo et al., 2010b, Sashihara et al., 2009). We determined that the mean titre plus 3 to 4 standard deviations were consistent with results we obtained from the VNT. When we applied the mean titre plus 5 standard deviations we observed an increase of 2.5% in false negative results, when compared to results obtained from the VNT (data not shown). We, therefore, decided to adopt the mean plus 4 standard deviations as the threshold value for this assay. This value corresponded to 3098 RLU.

Testing serially diluted PPR positive experimental samples, gave us a way of determining not only the sensitivity of the assay, but also the LOD of PPR-LIPS and PPR cELISA from ID Vet. Our results show that each one of the 5 PPR positive sera tested could be diluted in average 1 in 256 times and still be identified as positive by both assays. Therefore, we can confidently use the indicated 1 μl serum (diluted 1 in 10) for detection of PPRV antibodies in serum using LIPS.

LIPS uses 1/10 to 1/40 of the volume required for PPR cELISA. This is particularly significant when testing wildlife samples, which are, in many cases, scarce and of poor quality (Boadella and Gortazar, 2011).

The cross reactivity of currently available PPR ELISA kits with RP positive samples is known (Anderson and McKay, 1994; Couacy-Hymann et al., 2007). This cross reactivity was confirmed by the results we obtained when testing rinderpest experimental serum samples (Table 4). It was, therefore, not surprising to find a percentage of RP positive field serum samples testing PPR positive by the ID Vet (9.9%) and BDSL (12.9%) PPR cELISA kits (Supplementary Table 1). Early results showing that PPR-LIPS was specific to PPR by failing to cross react with RP experimental serum samples, encouraged us to increase our sample size with additional RP field serum samples. Further testing of 101 RP positive field serum samples concluded a high level of specificity observed by LIPS, with all samples testing negative for PPR.

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Although this study shows the ability of PPR-LIPS to detect PPRV antibodies in sera with high sensitivity and specificity, there are several additional aspects to address in future studies. Such aspects include substrate batch variability and stability, and the need of a stable and constant source of fusion protein for the assay. All of these are currently being studied.

5. Conclusion

We have reported here preliminary results of a new assay designed to detect PPR antibodies in serum samples. This assay is highly sensitive and specific to PPR and can be performed with only 1 μl of test sample. These characteristics, to be confirmed by further studies, make the PPR-LIPS, an ideal test for seromonitoring and disease surveillance during the future PPR eradication campaign which is being prepared by FAO and OIE.

Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2015.10.008.

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