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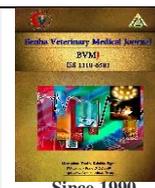
Original Paper

Preparation of a house ELISA kit for detecting Peste des petits ruminants Virus (PPRV) antibodies

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ABSTRACT

A Peste des petits ruminant (PPR) is a viral disease that affects small ruminants characterized by fever, highly contagious, and economically significant. For diagnosis of the disease, effective diagnostic techniques are essential. The immunological diagnostic procedure enzyme linked immunosorbent assay (ELISA) is used to identify antibodies or antigens. Using semi-purified antigen prepared by Peste des petits ruminant virus (PPRV) Egypt/77 grown in Vero cell culture, an indirect ELISA based on polyclonal antibodies was developed to detect PPR antibodies in goat and sheep serum samples. When using 1:40 serum dilution and 1:80 antigen dilutions, the antibody detection effect is better. Depended on the presence of known PPR antibody-negative control serum samples in the test, a cut-off value twice the average value of the negative control was obtained. Indirect ELISA and competitive ELISA were used to test 130 serum samples from sheep and goats (105 sheep samples and 25 goat samples). Indirect ELISA revealed 71 positive samples and c-ELISA revealed 79 positive samples out of 130 serum samples analyzed. When compared to the competitive ELISA, the indirect ELISA shows 83 percent specificity and 97.1 percent sensitivity. These findings show that indirect ELISA can be used instead of competitive ELISA for PPR antibody serological surveys in small ruminants.

1. INTRODUCTION

A Peste des petits ruminant (PPR) is a highly contagious disease that affects sheep and goats all over the world. Fever, stomatitis, gastroenteritis, conjunctivitis, and pneumonia are main symptoms of PPR, which affects transboundary sheep and goats (Taylor, 1984). *Peste des petits ruminant virus (PPRV)* is a single-stranded negative-sense RNA virus that belongs to the *Morbillivirus* genus, *Orthoparamyxovirinae* subfamily, and *Paramyxoviridae* family. This virus is closely related to other *Morbilli* viruses of mammals and aquatic mammals (Barrett et al., 1993, Prajapati et al., 2020). Fusion (F), nucleocapsid (N), phosphoprotein (P), large (L), hemagglutinin (H), and matrix (M) proteins are among the eight proteins encoded (Bailey, et al.2005). By altering the fusion of the viral and host cell membranes, the virus fusion protein (F gene) allows PPRV to pierce the cell membrane and enter the cytoplasm. The fusion protein has been shown to be essential for eliciting a protective humoral immune response (Berhe, et al. 2003). Because it is a key viral protein that is highly immunogenic and conserved among structural proteins, the nucleocapsid (N) protein of PPR virus (PPRV) is an interesting candidate antigen for creating specialized diagnostics, (Basagoudanavar, et al. 2018). The virus circulating in Africa and Middle East, and Goats are more susceptible than sheep (Nanda et al., 1996). Cattle, buffaloes, camels, and pigs can get a subclinical infection, but they don't play a role in virus excretion (Khan, et al., 2008, Prajapati, et al., 2020). *PPRV* has also spread to atypical hosts like gazelles, wild goats and pigs,

making disease eradication difficult (Parida et al., 2016). The disease's morbidity and mortality rates can be as high as 100% and 90%, respectively (Wohlsein and Singh, 2015).

PPRV was initially discovered in 1942 in West Africa in Cote d'Ivoire (Pope et al., 2013). The disease has now spread throughout Africa, the Middle East, Turkey, China, India, and Nepal, with instances reported in Morocco (Gargadennec et al.,1942; Baazizi, 2017), and Georgia (Sevik and Sait, 2015; Donduashvili et al., 2018). If the virus is allowed to spread unrestrained, many farmers and herders will suffer substantial damage and hardship. Annual global economic losses are expected to be in the range of USD 2.1 billion (OIE and FAO, 2015).

PPR initially appeared in Egypt in January 1987 among goats in the districts of Kafr Hakim, Embaba, and Giza. *PPRV* was isolated on Vero cells and subsequently detected using the direct fluorescent antibody technique (DFAT) (Abdelkarim et al., 1988). PPR Giza 94 was identified as a virus isolated from afflicted animals in the Giza governorate Mouaz (1995). An outbreak in Aswan province in 2006 highlighted the capacity of infected goats to be asymptomatic at times, while others acquire severe clinical disease (El-Hakim, 2006). Between 2008 and 2009, re-emergence outbreaks were documented in some Egyptian governorates (Abdel-Hamid et al., 2010). Seroprevalence of antibodies against foot and mouth disease (FMD), Peste des Petits Ruminants (PPR), and bluetongue (BT) in sheep and goats was studied and it was found that the governorates of Giza and Beni-Suef are endemic for these viruses in sheep and goats (Mahmoud et

al., 2017). *PPRV* is still circulating in various Egyptian governorates, Sharkia, Kafr El-sheikh, Marsa-Matrouh, Giza, Sharkiya, and the Red Sea (Maged et al., 2018), generating epidemics in its major host, small ruminants. PPR, FMD, and BT can be brought into Egypt through the illicit importation of sheep and goats from neighbouring countries, (Hosny et al., 2020). The emerging PPRV belongs to the IV lineage in Egypt's small ruminant animals (Ahmed et al., 2021).

Application of effective diagnostic techniques is critical for detection of diseases. PPR was initially diagnosed by methods depended on antigen detection like the agar gel precipitation test (AGPT), enzyme spot immunoassay, and differential immunohistochemistry staining of tissue sections, but these approaches have low sensitivity and lack confidence in routine diagnosis (FAO, 2018). Advanced techniques such as reverse transcriptase polymerase chain reaction (RT-PCR) have been developed as molecular biology has progressed (Kinimi et al., 2020). Appropriate diagnostic procedures and prompt immunisation of susceptible animals are required for disease prevention, management, and eradication.

The immunological diagnostic procedure ELISA is used to identify antigens or antibodies. ELISA approaches for PPR diagnosis with different sensitivity and specificity that are specifically designed to detect antibodies against the N or H protein. Because commercial ELISA kits are so expensive, many laboratories and researchers developed their ELISA methods in their labs i.e. house ELISA. The goal of this research is to develop a house ELISA kit for diagnosing of PPR disease. In addition, the results of the created kit were compared to those of a commercial ELISA kit.

2. MATERIAL AND METHODS

2.1. Samples
A total of 75 sheep and 25 goat serum samples were collected from clinically diseased and apparently healthy animals across Egypt's governorates. These samples were kept at –20°C until they were utilized in an ELISA test to detect PPR antibodies.

2.2. Reference serum samples
A total of 30 reference control sheep serum samples were sent by Cirad laboratory for proficiency test (PT) of PPR antibodies ELISA test. These samples were used for comparison between routine commercial ELISA and prepared house ELISA kits.

2.3. Reference *PPRV*
The strain Egypt/77 was kindly supplied by ELISA research and virus strains bank unit, Animal Health Research Institute. Dokki, Giza, and used for preparation of PPR antigen for house ELISA.

2.4. Cell line
African green monkey kidney (Vero) cells, kindly supplied from the African horse sickness vaccine research department, Veterinary Sera and Vaccine Research Institute, Abbasia (VSVRI). Cells were grown and sub-cultured according to (El-Dakhly, et al. 2016).

2.5. Preparation of indirect house ELISA Antigen
The *PPRV* antigen was prepared according to (Singh et al., 2000, 2004). Briefly *PPRV* was propagated on Vero cells for three passages till CPE reach more than 80%. Vero cells were harvested after freezing and thawing for three times. After centrifugation at 1000 rpm for 10 minutes the supernatant was taken and precipitated with PEG 6000 at 8% (w/v) in the presence of sodium chloride at 2.3 % (w/v). After overnight incubation at 4°C, the mixture was centrifuged at 8500 rpm for 30 minutes. Dissolve the precipitate in TNE buffer (Tris 10 mmol / L, NaCl 150 mmol / L, EDTA 1 mmol / L, pH 7.4). After reconstitution in bicarbonate carbonate buffer pH 9.6, the semi-purified antigen was stored at -20°C and used for indirect house ELISA.

2.6. Checkerboard titration
It was done depending on the method of (Singh et al., 2000, 2004). It was used to determine the working dilution of positive, negative serum and viral antigen. The antigen and reference serum samples were examined in two fold dilutions starting at 1:10 and 1:5, respectively. The antigen and serum dilution that produce the maximum absorbance difference at 450 nm between the positive and negative serum samples (P/N) are selected for testing of larger samples. Before testing field samples, the optimized indirect ELISA was validated by detecting antibodies in known positive and negative serum samples.

2.7. Screening of PPR antibodies in serum samples by indirect house ELISA:
It was done according to the method described by Sharma, et al. (2015). Briefly, 100µl of prepared antigen (1/80 according to check board titration) in coating buffer (carbonate bicarbonate buffer, (pH9.6) was added per well in the ELISA plates. The plates were incubated for one hour at 37°C, then overnight at 4°C, before being rinsed three times with washing buffer (phosphate buffer saline containing 0.05 percent Tween-20). The blocked dried wells were incubated at 37°C for two hours after being blocked with blocking solution (5 percent skimmed milk powder dissolved in PBS containing 0.05 percent Tween-20). A volume 100µl of each of tested serum, Positive and negative control serum (diluted 1/40 in blocking buffer, according to check board titration) in two wells for each were added, incubated at 37°C in a shaker for two hour, and then washed 3 times with washing buffer. 100µl of anti-sheep IgG Horse raddish peroxidase conjugate (Sigma) (for sheep samples) or anti-goat IgG Horse raddish peroxidase conjugate (Sigma) (for goat samples) were added to each well at a dilution of 1/500 in blocking buffer at 37^0c in a shaker for one hour. The conjugate was decanted and the plates were washed 3 times with washing buffer. A volume 100µl /well of the substrate (TMB substrate solution) were added. The reaction was stopped by adding 100ul of stopping solution to the plate after it had been incubated at room temperature for 15-20 minutes. Using a titertek multiskan ELISA reader, the absorbance values were obtained at 450 nm. The ELISA results were expressed as optical density (OD) and calculated as a mean OD for each sample. The cut-off point between positive and negative serum values was determined by taking twice the means of the optical density values of negative control serum.

2.8. Screening of PPR antibodies by competitive ELISA Kit:
A total number of 100 serum samples were collected from clinically infected and apparently healthy sheep and goats, and 30 reference control serum samples were examined for PPRV antibodies by a commercial competitive ELISA kit (ID Screen® PPR Competition, ID Vet Innovative

diagnostics, France) according to the manufacturer's instructions. The optical density (OD) measurements were converted to S/N percent using the formula: S/N percent = 100(OD sample/OD negative control). The manufacturer's recommended cut-off for sero-positivity was 50% (ID Screen®PPR Competition, ID vet, Innovative diagnostic, France).

2.9. C-ELISA vs. VNT effectiveness comparison:
The effectiveness of C-ELISA commercial kit was compared to VNT to validate it using method described by Libeau, et al. (1995). This validation was done by the commercial company.

2.10. Indirect house ELISA vs. c-ELISA effectiveness comparison:
The effectiveness of the developed indirect house ELISA was compared to that of a competitive ELISA, which is currently being used in our lab for PPR sero surveillance across the country. According to the procedures outlined by Singh et al., (2004), the sensitivity and specificity of the prepared house ELISA were estimated using different known positive and negative (reference serum control) and collected serum samples in comparison to competitive ELISA. The sensitivity and specificity of the assay were determined by counting the number of positive and negative samples detected out of the known actual positive and negative samples.

3. RESULTS

The results of titration of the prepared PPR viral antigen against positive and negative standard antisera are shown in Fig (1). It shows the result of antigen dilution against positive and negative standard antiserum diluted 1:40. The mean optical density (OD) reading of the prepared antigen against positive serum is 1.58, while the mean optical density reading of the prepared antigen against negative serum is 0.16, and the best working dilution of the prepared antigen for ELISA is 1:80 for PPR antigen. The binding ratio (mean optical density of positive control serum / mean optical density of negative control serum) exceeds 5.
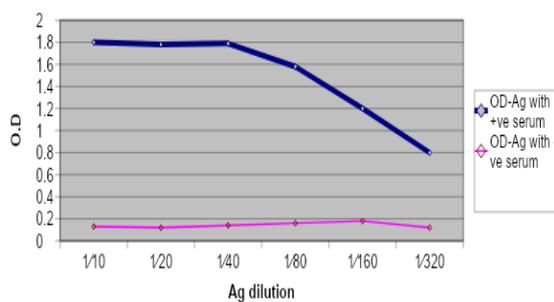

Fig. (1). Titration of the prepared PPR antigen against positive and negative reference sera (diluted 1:40) by check board titration

As showing in table (1), 71 samples give positive results for detection of PPR antibodies (54.6%) by indirect house ELISA. But 79 samples give positive results for detection of PPR antibodies (60.7%) by commercial ELISA kit. Regarding to sensitivity and specificity of indirect house ELISA, they were 97.1% and 83% respectively (table (2)). Sensitivity and specificity of competitive ELISA were 94.5% and 99.4 % respectively (Libeau, et al. (1995))

Table 1:- Showing results of detection of PPR antibodies by house ELISA and commercial ELISA Kit

Type of sera	No.	House ELISA		Commercial ELISA kit	
		Number of +ve samples (%)	Number of -ve samples (%)	Number of +ve samples (%)	Number of -ve samples (%)
Reference	30	22(73.3%)	8(26.7%)	24(80%)	6(20%)
Collected	100	49(49%)	51(51%)	55(55%)	45(45%)
total	130	71(54.6%)	59(45.4%)	79(60.7%)	51(39.3%)

Table 2: Determination of sensitivity, specificity and correlation percentage

C.ELISA	Indirect houseELISA		Total
	Positive	Negative	
Positive	69[a]	10[d]	79
Negative	2 [b]	49[c]	51
Total	71	59	

a= true positive   b=False positive c=True negative d=False negative
Sensitivity= a/a+bx100= 69/71x 100 =97.1%
Specificity= c/d+cx100= 49/59x100=83%
Correlation=a+c/a+b+c+dx100= 118/130x100=90.7%

4. DISCUSSION

In Africa and Asia, the PPR illness has been able to cause significant economic losses in the livestock industry. PPR is one of the major limitations influencing small ruminant productivity in Egypt. Rapid and precise detection of infection, as well as the adoption of effective control measures, aid in the disease's eradication. Because of the disease's economic importance, effective and alternative methods for detecting PPR infection early are required. Restricted requirements are required to ensure that diagnostic tests utilized in the laboratory fulfill the minimum threshold of diagnostic performance (Murphy et al., 1999; Wright, 1998). Large-scale virus neutralization test (VNT) based epidemiological surveys of serum samples take a long time and need a lot of effort. For open bench work, it is self-evident that developing a quick appropriate serological test is critical. The majority of antibody-based detection relied on the use of C-ELISA, which was either created in-house (Choi et al., 2005) or purchased commercially (Madboli and Ali, 2012). In-house development necessitates the creation of monoclonal antibodies (Singh et al., 2004) and the conjugation of detector antibodies, both of which are highly technical. Commercial kits are quite expensive, and they frequently expire within a year of manufacture, resulting in very low component quality. The main cause is lower power availability and variability; inappropriate storage has also been implicated (Mccullough et al., 1986).

The indirect ELISA described here may be a useful alternative to C-ELISA to detect antibodies against PPRV and can be successfully used for PPR sero-epidemiological studies (Balamurugan, et al., 2007). Figure 1 illustrates the results of the prepared antigen dilution. It shows that the prepared antigen's mean optical density reading against positive serum (diluted 1:40) is 1.58, while the prepared antigen's mean optical density reading against negative serum (diluted 1:40) is 0.16, and the best working dilution of the prepared antigen for ELISA is 1:80 for PPR antigen. The binding ratio (mean optical density of positive control serum / mean optical density of negative control serum) exceeds 5.

Standard controls (conjugate, strong positive, weak positive, and negative controls) are included in the prepared kit to assess kit quality, verify precision, and eliminate plate discrepancies (Jacobson, 1998 and Balamurugan et al., 2007). All of the controls used in this investigation had OD values that did not differ significantly between plates, indicating that the technique was accurate. Using a two-sided contingency table, the performance of indirect ELISA

was compared with that of C-ELISA in terms of relative sensitivity and specificity. Out of the 130 serum samples tested, 71 were positive by indirect ELISA which performed extremely well in comparison to C-ELISA (table. 1). With 83 percent specificity and 97.1 percent sensitivity (table.2), that is agreeing with (Jacobson, 1998). Using ELISA can quickly and cheaply analyse a large number of serum samples. For seroepidemiological studies of antibodies to PPR virus in small ruminants, the indirect ELISA reported here is an excellent instead of C-ELISA.

5. CONCULOSION

Commercial kits are expensive, and component quality is poor, but an in-house ELISA was shown to be equally specific as commercial C-ELISA, with few false negative findings. The application of a house IELISA as a final control technique for the PPR outbreak proved to be effective.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest for current data

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