COMPARISON OF DIAGNOSTIC EFFICACY OF BLOCKING ELISA WITH COMPETITIVE ELISA FOR THE DETECTION OF PESTE DES PETITS RUMINANTS VIRUS ANTIBODIES IN RUMINANTS AND CAMELS SERA IN ETHIOPIA

MVSc Thesis

By

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June, 2020

Bishoftu, Ethiopia

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A Thesis submitted to College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of the Master of Veterinary Science in Veterinary Microbiology

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As members of the Examining Board of the final MVSc open defense, we certify that we have read and evaluated the Thesis prepared by: **Kumela Lelisa** entitled: **Comparison of diagnostic efficacy of blocking ELISA with competitive ELISA for the detection of peste des petits ruminants virus antibodies in ruminants and camels sera in Ethiopia** and recommend that it be accepted as fulfilling the thesis requirement for the degree of Master of Science in Veterinary Microbiology

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LIST OF ABBREVIATIONS

AGID	Agar Gel Immunodiffusion
AU-PANVAC	African Union- Pan-African Veterinary Vaccine Centre
b-ELISA	Blocking-Enzyme Linked Immunosorbent Assay
c-ELISA	Competitive- Enzyme Linked Immunosorbent Assay
CD4	Cluster of differentiation 4
CIE	Counter Immunoelectrophoresis
CIRAD-EMVT	French Agricultural Research Centre for International Development-Institute of Tropical Breeding and Veterinary Medicine
CPE	Cytopathic Effect
CSA	Central statistical Agency
DIVA	Differentiation of Infected and Vaccinated Animals
DNA	Deoxyribonucleic Acid
ELISA	Enzyme Linked Immunosorbent Assay
FAO	Food and Agricultural Organization
LAMP	Loop Mediated Isothermal Amplification
LIPS	Luciferase Immunoprecipitation System
NAHDIC	National Animal Health Diagnostic and Investigation Center
NCM	Nitrocellulose Membrane
OD	Optical Density
OIE	World Organization for Animal Health

LIST OF ABBREVIATIONS Continued

PCR	Polymerase Chain Reaction
PI	Percentage of Inhibition
PPR	Peste des petits ruminants
PPRV	Peste des petits ruminants virus
RNA	Ribonucleic acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SE	Standard Error
SLAM	Signaling Lymphocyte Activation Molecule
SRMV	*Small ruminant morbillivirus*
TCID	Tissue Culture Infectious Dose
TMB	Tetra-Methyl-Benzidine
VNT	Virus Neutralization Test

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ABSTRACT

A *Peste des petits ruminant* (PPR) is one of the top hindrance to small ruminants production. To facilitate the global effort to eradicate PPR, sufficient, reliable, fast and cost effective screening tests are important. This study compares the diagnostic performance of two anti-PPRV antibodies detection methods namely: ID Screen® PPR Competition ELISA (ID Screen® PPR c-ELISA) and Haemagglutinin based PPR blocking ELISA (HPPR b-ELISA®) kits using 480 sera collected from goats, sheep, cattle and camels. The results of the two tests were validated against virus neutralization test (VNT). The agreements between the tests were determined using Cohen's Kappa statistics and two-way contingency table. The diagnostic sensitivity and specificity of HPPR b-ELISA® test were 79.55 and 99.74%, respectively relative to the ID Screen® PPR c-ELISA with almost perfect agreement (κ=0.86) between the two tests. Conversely, the diagnostic sensitivity and specificity of ID Screen® PPR c-ELISA relative to HPPR b-ELISA® were 98.59 and 95.60%, respectively. There was almost perfect agreement between the two tests in goats (κ=0.82) and sheep (κ=0.98), while the agreement was substantial in cattle (κ=0.78). However, there was no agreement between HPPR b-ELISA® and ID Screen® PPR c-

ELISA in detecting antibodies against PPRV in camels' sera (κ=0.00). On the other hand, the results of the two tests were validated against VNT. The HPPR b-ELISA® test had a diagnostic sensitivity and specificity of 80 and 96.36%, respectively compared to VNT with substantial agreement. The agreements were almost perfect in goats (κ=0.83) and sheep (κ=0.89), moderate in cattle (κ=0.50) and none in camels (κ=0.00). The sensitivity and specificity of ID Screen® PPR c-ELISA relative to VNT were 92.00 and 76.36%, respectively. The ID Screen® PPR c-ELISA test had substantial agreement in goats (κ=0.69) and sheep (κ=0.78), fair agreement in cattle (κ=0.30) and no agreement in camels (κ=0.00) in detecting specific antibodies directed against PPRV relative to VNT. The HPPR b-ELISA® test was shown to be a good screening test to be used alone or in combination with ID Screen® PPR c-ELISA test for PPR serological surveys or monitoring in ruminants. Based on these results it can be concluded that the serology based on both tests represents a reliable and valid method for detection of anti-PPRV antibodies in ruminants, however, the use of ID Screen® PPR c-ELISA and HPPR b-ELISA® tests in detection of anti-PPRV antibodies in camels' sera requires further investigation. Findings suggest that the newly developed HPPR b-ELISA® is suitable for screening of antibodies against PPRV in ruminants.

Keywords: - *Antibodies, Camels, Comparison*, ID Screen® PPR c-ELISA, HPPR b-ELISA®, *Peste des petits ruminants, Ruminants*

1. INTRODUCTION

There are about 30.2 million goats and 30.7 million sheep in Ethiopia (CSA, 2017). Despite the large population of goats and sheep, their productivity is very low due to poor nutrition, poor husbandry system and prevailing animal diseases (Gizaw *et al*., 2010). Among viral diseases of goats and sheep, *peste des petits ruminant* (PPR) is one of the top constraints to small ruminants production in the country (Tembely, 1998).

PPR is exceedingly infectious, and financially critical plaque of sheep and goats. It is caused by an enveloped ribonucleic acid (RNA) virus, called *peste des petits ruminant* virus (PPRV), belongs to the genus *Morbillivirus,* which however has been renamed as *Small ruminant morbillivirus* (SRMV) according to the latest virus taxonomy of the International Committee on Taxonomy of viruses (Amarasinghe *et al.*, 2019). Virions are pleomorphic in nature, varying between 130-390 nanometers in diameter. The genome of the virus has no segment (Gibbs *et al.*, 1979).

The virus has spread far beyond western Africa where it was first described. It is now present in many countries across Africa, Asia, the Middle East and parts of Europe. PPRV strains from lineage I and II are circulating in west Africa, while lineage III is circulating in east Africa and the Middle East (Banyard *et al.*, 2014). Although lineage IV is known to circulating in Asia, a recent realignment in lineage distribution has shown the wide distribution of this lineage in Africa (Albina *et al.*, 2013). PPR is widely distributed in Ethiopia and high sero-prevalence of the disease has been reported from different parts of the country (Delil *et al.*, 2012; Hailegebreal, 2018).

PPR is currently considered to be one of the main trans-boundary animal diseases that constitute a significant threat to sheep and goats production in developing countries. In affected areas, PPR is considered to be a major limiting factor in the development of the small ruminants industry. This is especially evident in many countries in Africa and Asia where sheep and goats play an integral role in sustainable agriculture and employment. The potential and real economic impacts of PPR outbreaks are extremely high and the impact

of the disease on the poorer sections of society is disproportionate, reflecting an intrinsic dependence on sheep and goat farming (Baron *et al.*, 2011).

Due to its high impact on the economy, PPR has been targeted by governments and international organizations with the aim of global eradication by 2030. In 2015, the OIE and FAO have together propelled a global campaign to eradicate PPRV by the year 2030 (OIE and FAO, 2015). To better target eradication control efforts, FAO also launched a global PPRV research network in 2018 with the goal of aligning research efforts to inform strategies for eradication of the virus (Rossiter, 2019).

There are several serological tests targeting antibodies produced against PPRV and widely used in PPR endemic areas. Serological tests like blocking ELISA and competitive ELISA are the leading PPRV antibody detection methods and known for their simplicity and capacity to detect large number of samples in short period of time, hence, they are suitable for sero-surveillances and sero-monitoring (Libeau, 2015). Whereas, serological tests are planned to be touchy and particular, wrong negative and untrue positive comes about to happen; confirmation of new findings by using elective demonstrating strategy is crucial (Diop *et al.*, 2005).

Currently, the N-based competitive ELISA (ID Screen® PPR Competition, ID Vet) is the only commercial kit to detect antibodies produced against PPRV. However, this kit is not affordable by laboratories in developing countries. Therefore, laboratories are seeking for relatively less expensive tests, among which the HPPR b-ELISA® developed by AU-PANVAC was validated for detection of anti-PPRV antibodies (Bodjo *et al.*, 2018). Keeping in view the results of recent studies, the need was felt to assess the suitability, sensitivity and specificity of HPPR b-ELISA® which was recently developed in Ethiopia for detection of PPRV antibodies in different species of animals. Therefore, the objective of this study was to compare and validate the diagnostic performance of HPPR b-ELISA® with that of the ID Screen® PPR c-ELISA (ID Screen® PPR Competition, ID Vet) for detection of anti-PPRV antibodies in ruminants and camels' sera.

2. LITERATURE REVIEW

2.1. The structure of *Small ruminant morbillivirus*

The PPRV has a non-segmented, single-strand negative sense RNA genome with 15,948 nucleotides in length. The genome encodes six structural proteins and two nonstructural proteins namely (Diallo, 1990).

The N protein of the PPRV is the most abundant, antigenically well conserved and foremost constitute of nucleocapsid core virions and plays important role in transcription and replication. The nucleocapsid is much conserved immunogenic core protein and expressed at high level in the infected cells than other viral proteins. This protein is a noble target candidate for diagnosis assay due to its antigenic stability and contain both type specific and cross reactive epitopes (Sereda *et al.*, 2019). N protein inhibits β interferon production by interacting with interferon regulatory transcription factor three to block its activation (Zhu *et al.*, 2019).

The P protein acts as a molecular intermediate that is proposed to bridge components of the ribonucleoprotein during both the replication and transcription activities of the viral life cycle. The P gene of the morbilliviruses is responsible for coding three proteins, including phosphoprotein (P) and two nonstructural proteins (V and C), by overlapping open reading frames and was found to be the most poorly conserved protein of the morbilliviruses. The C protein is a small basic non-phosphorylated protein. High degree of conservation between the different morbillivirus C proteins at the C-terminus was observed with sequence analysis (Mahapatra *et al.*, 2003). V protein of the virus contributes in interfering with host innate immunity by blocking interferon signaling pathways through interacting with signal transducer and activator of transcription 1 and 2 (Chinnakannan *et al.*, 2013) .

The M, F and H proteins are associated with the viral envelope which is derived from the host cell membrane. The matrix protein is found interior of the viral envelope and links the ribonucleoprotein and the external viral proteins, (F and H) and important for virus particle assembly. The H protein is used to bind the host cell receptor during the first step of the

viral infection process. The fusion protein intervenes the combination of the envelope with the cell membrane of the host. The F and H induce protective host immune response against the virus (Bailey *et al.*, 2005). Figure 1 depicts the morphology and genome structure of PPR virion.

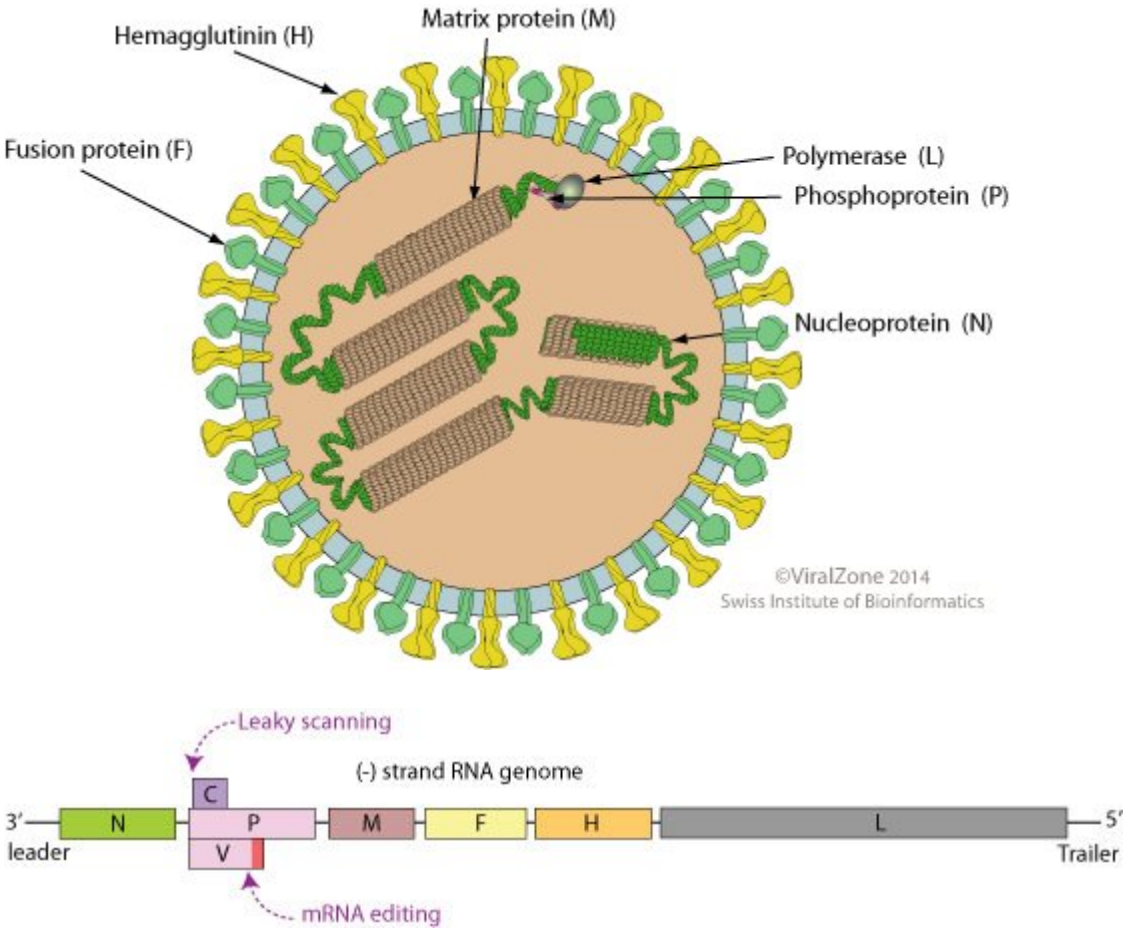

Figure 1: Schematic structure of the peste des petits ruminants' virion (Source: Viral zone, 2014)

2.2. Immune response of hosts to PPRV infection

The relative importance of humoral and cell-mediated responses in the recovery from infection with PPRV is not clear. However, studies suggest that animals that recover from infection have high levels of circulating neutralizing antibody as well as antigen-specific proliferating cluster of differentiation 4 (CD4+) T cells (Baron *et al.*, 2014; Mitra-Kaushik *et al.*, 2001), which indicates either or both responses may be involved in the clearance of the virus. The protective immune response of the host to PPRV infection is obscured to some extent by the generalized immunosuppression which is common to all morbillivirus infections. Immunosuppression induced by morbilliviruses persists after the recovery of peripheral white blood cell count. Peripheral blood leucocytes from animals infected with virulent morbilliviruses show reduced response to mutagens antigens, nevertheless, the vaccine strain of PPRV had only a transient immunosuppressive effect (Rojas *et al.*, 2016).

2.3. Virus transmission and spread

Infected animals are the source of PPRV. Virus transmission is essentially by contact with infected animals, secretions and feces from infected animals. At an early stage of infection, virus excretion is massive in the exhaled air. This probably allows noncontact transmission over at least a few meters. Nasal and ocular discharges, saliva, and feces also contain large amounts of viral antigen. In goats, PPR virus RNA is excreted in the feces during at least 2 months after a natural infection (Ezeibe *et al.*, 2008). Since PPRV is quickly inactivated in the environment, its transmission most often occurs by direct contact between infected and healthy animals. However, indirect transmission through recently contaminated materials should be considered in epidemiological models and control measures. Due to the rapid spread of the virus in immunologically naive flocks, PPRV can only persist in large populations and only if new susceptible hosts are available (Anderson and Wang, 2011).

Livestock trade is the most likely route of PPR virus introduction into new territories. In Africa, more than 600 million small ruminants are at risk of PPR virus infection, and there is a high risk of introduction of the virus to countries that are still free of the disease. PPR is commonly regarded as a seasonal disease, peak infections usually occurring during the cool dry season in endemic areas of Africa, even though, the PPR virus transmission may occur at any season (Abubakar *et al.*, 2009).

2.4. Status of *peste des petits ruminants* in Ethiopia

Among viral diseases of sheep and goats, PPR is the main top constraint to small ruminants' production in Ethiopia. It is widely distributed in the country and high sero-prevalence of the disease has been reported from different parts of the country in small ruminants (Dejene, 2016; Fentie *et al.*, 2018). The occurrence PPR was questionable since its initial suspecting in late 1970s depending on clinical indications in Ethiopia (Pegram and Tereke, 1981). The infectious agent was identified in 1994, and the isolate was reported in 1996 and genetically classified in lineage III (Roeder *et al.*, 1994).

Nevertheless, later reports shown that lineage IV is occurring and proceeded to spread in. This might shows the distribution of PPRV lineage is now changing. Be that as it may be, the relationship between the change of virus strains and epidemiology of the disease is not clear. Thus, to understand the disease epidemiology, sequencing of the occurring strains is vital in Ethiopia (Rume *et al.*, 2019)

2.5. Host range

The PPRV primarily infects sheep and goats, although camels, cattle (Abraham *et al.*, 2005), buffaloes (Khan *et al.*, 2008) and pigs (Nawathe and Taylor, 1979) are susceptible to infection. A clinical case of the virus infection was reported following experimental inoculation of calves and a further report describes an outbreak of clinical disease in buffalo caused by PPRV. However, cattle after experimental infections with PPRV exhibit no sign of viral replication and do not excrete the virus in amounts required to contaminate animals in close contact (Sen *et al.*, 2014).

It is not considered as pathogenic to domestic and wild African buffaloes, although some of them may seroconvert when exposed to the virus in enzootic regions. However, high case fatality rates have been reported and the disease experimentally reproduced in domestic buffaloes in India (Govindarajan *et al.*, 1997).

Additionally, PPR is now recognized as an emerging disease in camels and a respiratory syndrome was the main sign in Ethiopia. It has been shown that camels can seroconvert to the PPRV. High PPR sero-prevalence has been recorded in Nigeria (Bello *et al.*, 2014) and outbreak of PPR had been occurred in camels in Sudan (Khalafalla *et al.*, 2010). Zakian *et al.* (2016) noticed clinical signs such as in appetence, loss of body condition, diarrhea, conjunctivitis, ocular discharges and finally decumbency preceding death of camels infected with PPRV in Iran. Nevertheless, camels after experimental infection with PPRV produce no clinical signs (Fakri *et al.*, 2019), excrete no or very low amount of virus enough to infect the contact animals and didn't transmit the virus to the nearby susceptible animal (Schulz *et al.*, 2019).

PPR was also detected in wild life and study showed that wild small ruminants might play a great role in the epidemiology of the disease, since they might be a source of infections for sheep and goats (Kinne *et al.*, 2010). PPRV infections have been recently reported in dogs (Ratta *et al.*, 2016). Morbilliviruses have a propensity to evolve and change hosts quickly, which reflects their ability to adapt to new cellular environments (Cosby, 2012).

2.6. Diagnostic methods

The launch of the progressive control and eradication program for PPR resulted in increased international demand for validated diagnostic tools and specific reagents for the rapid diagnosis of PPR. PPR is tentatively diagnosed based on clinical findings, and epidemiology. Laboratory confirmation is necessary since PPR can be easily confused with other diseases producing similar clinical signs. Diagnosis of PPR is achieved using various techniques, including virus isolation, antigen detection, and nucleic acid amplification, and indirectly by detection of PPR virus specific antibodies (Couacy-Hymann *et al.*, 2009).
Collection of samples from animals with clinical signs is critical for virus detection, as samples from animals that have recovered seldom allow virus detection. PPR virus is mainly located in lymphoid tissue and the epithelial cells of the respiratory, digestive, and lymphoid systems. It can be isolated from blood during the febrile stage and from biopsies of lungs, lymph nodes, small intestine, and spleen at necropsy. Samples with putative diagnostic value for virology are mostly collected from animals in the erosive mucosal phase of the disease (Zahur *et al.*, 2014).

The use of ocular and nasal discharge samples is becoming increasingly common to detect viral RNA principally because samples can be obtained safely without the risks associated with blood collection and these materials have high viral loads. The use of swabs improves owners' compliance with specimen collection, as the procedure is simple and painless for their animals. Capture and containment of wild animals for PPR diagnosis remains difficult, however, noninvasive tools based on non-contact samples like feces are currently in use and allowed the inclusion of wild animals in the surveillance of the disease (Bataille *et al.*, 2019). The current diagnostic approaches of PPR include clinical signs, viral

isolation and identification, serology and molecular characterizations and are described below.

2.6.1. Clinical signs and pathological lesions

The onset of PPR is marked by sudden dullness and fever (40-41°C). The incubation period ranges from two to six days. The disease is characterized by depression, anorexia, coughing and erosions on the mucus membrane of the buccal cavity coincide with marked salivation (Chauhan *et al.*, 2009).

A clear watery discharge flows from eyes, noses and mouth, which later becomes thick and yellow as a result of bacterial secondary infections. Serous to mucopurulent nasal discharge crust over and occlude the nostrils causing sneezing and difficulty in breathing. Serous mucopurulent ocular discharges ensue causing reddening of conjunctiva and matting together of the eyelids. The mucus membranes of eyes and mouth become much reddened. The epithelial necrosis causes small pin-point grayish areas on the gums, dental pad, palate, lips, inner aspects of the cheeks and upper surface of the tongue (Kihu *et al.*, 2012).

The lining of the mouth is changed to pale and coated with dying cells and the normal membrane may be completely covered by a thick cheesy material. Underneath the dead surface cells, there are shallow erosions. The periorbital edema and cutaneous nodules in PPRV infected animals are usual. Diarrhea is preceded by a sudden drop in body temperature followed by death, in 5 to 12 days after the onset of PPR (Abdalla *et al.*, 2012).

Bronchopneumonia is a common pathological lesion in PPRV infected small ruminants. Lymph nodes including mediastinal and mesenteric are enlarged, congested and become edematous. The A Zebra stripes or markings on rectum and colon are typical lesion produced by the virus (Rahman *et al.*, 2011).

2.6.2. Virus isolation and identification

Virus isolation is still the gold standard test for detection of viral infections. Although several blind passages are needed before cytopathic effect (CPE) is observed, Vero cells are the most commonly used cell line for PPRV isolation. However, recently, virus isolation time has been reduced by using cell lines like Monkey CV1 cell expressing the sheep-goat signaling lymphocyte activation molecule (SLAM) protein (Adombi *et al.*, 2011), the main morbillivirus receptor (Sato *et al.*, 2012) and Marmoset B-lymphoblastoid-B95a cells (Sreenivasa *et al.*, 2006). Vero cells expressing nectin-4 and primary bovine and sheep cells are also useful for virus isolation (Fakri *et al.*, 2016).

PPRV specific CPE manifested after 3-5 days on inoculation. CPE produced by PPRV is distinguished by rounding of the cells that are infected in the clusters, proceeded by fusion of the cell monolayer forming syncytia. Intranuclear and intracytoplasmic inclusion bodies are revealed using hematoxylin staining. Virus isolation take relatively long time to conduct and less sensitive compared to reverse transcription polymerase chain reaction (RT-PCR. (Singh *et al.*, 2010).

2.6.3. Virus neutralization test

Virus neutralization test (VNT) is serological test to detect presence and magnitude of functional systemic antibodies that prevent infectivity of a virus. The VNT is very often used for antibodies titration, sero-monitoring and sero-surveillances, and is considered as the gold standard test, representing the true status of the disease and OIE recommendation for international trade with high sensitivity and specificity. Although VNT is the most reliable test in differentiating the antibodies from different members of morbilliviruses as the level of neutralization is higher in homologous SRMV than other morbilliviruses, it needs cell culture, live viruses and good quality samples, laborious and time consuming (Rossiter *et al.*, 1985)

2.6.4. Agar gel immunodiffusion and counter immuno-electrophoresis

In contrary to virus isolation, virus detection by immunological methods does not require live virus. In agar gel immunodiffusion (AGID), soluble antigens and antibodies diffuse passively in the agar medium, however in counter immuno-electrophoresis (CIE), the antigen and antibody move in opposite direction in the electric field to form the line of precipitation at the point of interaction. AGID is a simple test applied for PPR diagnosis from swabs and tissues of infected animals that is performed using PPRV specific antibodies. CIE is comparatively quick and relatively sensitive. Nevertheless, due to the presence of cross reacting epitopes in PPRV and render pest viruses, both tests could not differentiate the two viruses. These tests are less sensitive at the early stages of infection and with mild forms of the disease (Majiyagbe *et al.*, 1984) .

2.6.5. Immunochromatographic tests

Immunochromatographic tests have been developed following the advances in the field of application of nanotechnology for disease diagnosis. These assays are simple, and they give results within a matter of minutes and require no technical expertise. These tests detect antigen-antibody interactions on a nitrocellulose membrane (NCM) platform by trapping the unknown antigen/ antibody with known antibody/antigen pre-spotted on the NCM. Added samples move along with the colloidal gold labeled PPR antigen, if there is the virus antibody in the sample, it combines with antigen on T-line and show wine red color. These tests detect little as 10^3TCID50/ml of the virus and have good sensitivity and specificity relative to polymerase chain reaction (PCR). These tests can detect all four lineages of the virus from different geographical origins. The result is clear enough to be captured by a smart phone and the picture then sent for control to the veterinary service or the laboratory.

Their direct applications on the flock make them test of choice in the field. Due to its contagious nature, if PPR diagnosis is made at the field level, control measures could be adopted at early stage to prevent the spread of the virus to neighboring flocks (Hussain *et al.*, 2003).

2.6.6. Immunocapture and sandwich enzyme linked immunosorbent assays

These sensitive methods were initially developed for the differential diagnosis of render pest and PPRV. The immunocapture ELISA uses biotinylated anti-N monoclonal antibodies against a cross-reactive epitope of the virus to capture or detection of the virus antigen in clinical samples. The test functions to confirm outbreaks in infected small ruminants and camels (Saeed *et al.*, 2010). Similarly, the sandwich ELISA kit uses monoclonal antibodies directed against an epitope of N-protein of the virus. The sandwich ELISA is efficient having good diagnostic performances in relation to the immunocapture ELISA (Singh *et al.*, 2004a).

2.6.7. Blocking, competitive and indirect enzyme linked immunosorbent assays

Blocking and competitive ELISAs using anti-H monoclonal antibodies were developed for PPR sero-surveillance and sero-monitoring. The blocking ELISA has good diagnostic specificity when compared to the VNT. Both anti-N and anti-H protein monoclonal antibody based competitive ELISAs are available as commercial kits for the detection of antibodies against PPR virus. Similarly, a competitive ELISA based on an anti-H protein monoclonal using partially PPRV is also available. The diagnostic sensitivity and specificity of these tests are equivalent to those of the VNT. The competitive ELISAs based on neutralizing monoclonal antibodies could be used for both sero-surveillance and sero-monitoring of a large number of serum samples (Singh *et al.*, 2004b).

Although a species-specific conjugated secondary antibody is required, this indirect ELISA could be used if the monoclonal antibody used in c-ELISA is lost due to some unavoidable situations or in laboratories where c-ELISA is not available. However, under the current circumstances, c-ELISA might be the best choice for sero-surveillance of various species of domesticated and wild animals (Balamurugan *et al.*, 2007) .

2.6.8. Luciferase immunoprecipitation system (LIPS)

This assay is developed for the specific detection of antibodies in serum samples. LIPS also enable to differentiate antibodies produced against PPRV and render peste virus. PPR-LIPS is highly efficient and no cross-reaction was shown with other morbilliviruses. Therefore, PPR-LIPS is a promising test for particular detection antibodies directed against PPRV (Burbelo *et al.*, 2010). LIPS involve quantifying serum antibodies by measuring luminescence emitted by the reporter enzyme *Renilla* luciferase (Rluc) fused to antigen of interest. The Rluc antigen fusion protein is recognized by antigen specific antibodies and antigen-antibody complexes are captured by protein A/G beads that recognize the Fc region of immunoglobulin G of antibody (Burbelo *et al.*, 2007).

LIPS assay functions by recognizing the action of luciferase from the intercourse of a luciferase fusion protein and virus antigen, antibodies in the test serum sample and protein A/G beads. If there specific antibodies directed against the virus in the test serum, they fixed the fusion protein (Burbelo *et al.*, 2015).

2.6.9. Reverse transcription-polymerase chain reaction

Morbillivirus specific RT-PCRs target the phosphoprotein (P) gene, fusion protein (F) gene and the 3' end of the messenger RNA encoding the nucleoprotein (N) of the virus. RT-PCRs are being applied for research and partly for clinical diagnosis of PPR with good sensitivity, because the N gene is transcribed more than other genes during viral replication. As these assays are laborious and expensive, they are not used for routine clinical diagnosis in resource poor laboratories, especially when the sample size is very large because these assays are laborious and unaffordable (Mao *et al.*, 2010).

This multiplex RT-PCR has higher sensitivity than sandwich ELISA. In single step assays, the reverse transcription and PCR steps are performed in the same micro-tube, thus minimizing cross contamination. Confirmation of the exact expected size of the amplicon

and checking for the absence of cross-reactivity with phylogenetically or symptomatically related viruses by sequence analysis of the PCR product must be carried out for confirmation (Couacy-Hymann *et al.*, 2002). The M gene-based one step and two steps SYBR Green assays were also developed. Both assays were reported to have a detection limit of 0.0001 TCID50/ml for vaccine and field strains and detected the virus nucleic acid as early as 3 days and as late as 20 days post infection in swab materials from experimental samples (Balamurugan *et al.*, 2010).

2.6.10. Real time reverse transcription-polymerase chain reaction

The real time RT-PCR targeting N-gene of *Small ruminants morbillivirus* have added sensitivity and robustness to PPR diagnosis as compared to standard RT-. It generates real-time amplification curve during the cyclic reaction and does not require gel electrophoresis to visualize the PCR amplification. It is simple, elegant and minimize cross contamination and more suitable for standardization. This makes the real time RT-PCR more suitable and efficient to detect minimal amount of a target PPR virus sequence in a sample that might remain undetected by conventional RT-PCR. But it requires a special thermal cycler that can read emitted fluorescence PCR (Bao *et al.*, 2008).

Fluorescent reporters, the double strand Deoxy ribonucleic acid (DNA)-specific dye SYBR Green, in the reaction mixture of real time RT-PCR report the amount of amplified PCR product in terms of fluorescent signals. A disadvantage is that SYBR Green will bind to any double-stranded DNA in the reaction, so primers have to be carefully designed and melt curve analysis must be performed (Balamurugan *et al.*, 2012).

2.6.11. Loop mediated isothermal amplification

The technique can be performed a water bath, or heat block, and amplicons could be recognized by naked eye by color formation of color or precipitate or via conventional agarose gel electrophoresis. Since LAMP is simple, quick, does not require a sophisticated thermo-cycler and sensitive technique, it has become a popular diagnostic platform for many viruses of veterinary importance references needed, even in less resourced

laboratories. LAMP assays used to detect PPRV antigen based on its nucleoprotein and matrix genes (Li *et al.*, 2010).

2.6.12. Reverse transcription-loop-mediated isothermal amplification assay

The reverse transcription LAMP is based upon loop formation of DNA strand on its own self and it excludes the need for thermal cycling. The assays either targeted the M or N gene. The RT-LAMP assay has been evaluated for the detection of viral RNA on a wide spectrum of positive clinical PPRV specimens, and results are obtained in one hour. It is advantageous being simpler and more efficient than conventional and real time RT-PCR. The sensitivity of RT-LAMP assays is ten-fold higher than conventional RT-PCR. As a simple, inexpensive and accurate detection method, reverse transcription LAMP assay is well suited for rapid clinical diagnosis of PPR. Therefore, it can easily replace RT-PCR for disease investigation and surveillance of PPR in field conditions especially in developing countries (Mahapatra *et al.*, 2019).

Currently, reverse transcription LAMP assays have two problems remaining for field use, in that they normally require the prior purification of RNA from clinical samples, requiring significant extra time and equipment and the high sensitivity of the LAMP assays makes them susceptible to false positive results because of cross contamination (Wei *et al.*, 2009).

2.7. Control and prevention

PPR is among sheep and goats diseases that should be controlled for destitution mitigation in developing countries particularly in Africa and Asia. The control of PPR mainly relies on mass immunization of sheep and goats in the areas where the disease is endemic. The launch of rinderpest eradication stimulated the development of vaccine strategies against small ruminant morbillivirus vaccines by the world community and abolished in many countries (Raj *et al.*, 2015).

PPR virus Nigeria 75/1 vaccine is very efficient PPR homologous live attenuated vaccine using the PPR Nigeria 75/1 isolate by continuous passages in Vero cells (Diallo *et al.*, 1989). Anti-PPRV antibodies generated against this vaccine last for at least three years providing long term immunity. It has been demonstrated that the freeze drying of PPRV Nigeria 75/1 vaccine in an excipient that contain trehalose makes it exceptionally thermo-stable and stands high temperatures up to 45°C for two weeks with negligible loss of efficacy (Silva *et al.*, 2011). Since even few mutation might have a genuine effect on the pathogenisity, reducing the passage number is important (Eloiflin *et al.*, 2019).

A live-attenuated PPRV Sungri/96 Vero cell-adapted vaccine against lineage IV is recommended in Asian countries. The PPR vaccine was found to be safe and efficacious in pregnant animals and also has no record of biologically significant immunosuppression. The vaccine provides sterile immunity for more than 6 years. Thus, the Sungri 96 PPR vaccine is safe for mass vaccination campaigns under field conditions To facilitate post vaccination outbreak sero-surveillance, a recombinant marker vaccine that can be either positive or negative and a test that can distinguish infection from vaccinated animals "differentiation of infected and vaccinated animals" (DIVA) are crucial (Sarkar *et al.*, 2003).

3. MATERIALS AND METHODS

3.1. **Study animals**

Four species of animals namely: goats, sheep, cattle and camels from the study areas were included in the study. All sera samples from goats and sheep was collected from Borena zone, Yabelo district, while samples from cattle were collected from Mekele city and camels' sera were collected from Afar regional state, Awash Fentale district.

3.2. Study design and description of samples

3.2.1. Study design

The study compared the diagnostic performance of two anti-PPRV detection tests namely: HPPR b-ELISA® developed by AU-PANVAC, Bishoftu, Ethiopia and ID Screen® PPR c-ELISA developed by a FAO reference laboratory (CIRAD-EMVT, Montpellier, France). A total of 480 sera samples, 120 from each, were collected from goats, sheep, cattle and camels. Briefly, sera samples of goats, sheep and camels were collected during active outbreak reported in 2018, while samples from cattle were collected in 2019 and stored at sera bank of National Animal Health Diagnostic Center (NAHDIC) laboratories. The two tests were first validated using known reference sera. The results of the two tests were then validated with VNT. All tests were performed in duplicates. However, samples that were not possible to categorize as positive or negative were tested furthermore, three times with ID Screen® PPR c-ELISA (N=3) and HPPR b-ELISA® (N=2). The cutoff values for positive results were determined based on the recommended values provided by the manufacturers.

3.2.2. Specimen collection and transportation

Approximately 5 ml blood samples were collected from the jugular vein of each animal using plain vacutainer tubes. Serum was separated from clotted blood by keeping in slanted position overnight of the labeled blood sample. Serum was emptied and aliquoted into 1.8 ml cryovial and kept in an icebox containing icepacks and transported to NAHDIC, Sebeta, Ethiopia for sample processing and laboratory investigations and temporary stored at -20°C until tested.

3.3. **Initial validation of the tests**

The validity of HPPR b-ELISA® and ID Screen® PPR c-ELISA was determined by using a positive sera and negative sera allocated at NAHDIC. The highest dilutions of serum detectable by ID Screen® PPR c-ELISA and HPPR b-ELISA® was determined by testing two fold serial dilutions starting at 1:2 of anti-PPRV antibody positive and negative sera using a cutoff values of 30 and 35% percentage of inhibition for HPPR b-ELISA® and 50 and 60% competition percentage for ID Screen® PPR c-ELISA as described by the manufacturers.

3.4. Testing field sera

3.4.1. The haemagglutinin based-blocking ELISA procedure

The kit uses micro-plates pre-coated with inactivated PPRV antigen. The reagents were equilibrated for 30 minutes at room temperature before use. Then, 75µl of blocking buffer was distributed to all wells. 25µl of blocking buffer was distributed to wells A1 and A2, 25µl of positive control to wells B1 and B2 and 25µl of negative control to wells C1, C2, D1 and D2 and 25µl of each serum per well was distributed to the remaining wells and, the plate was covered and incubated at room temperature (18-25°C) for 1 hour. Following

three washes using 300µl of washing buffer and blot dry in clean paper towels, 100 µl of blocking buffer was distributed in control buffer wells A1 and A2 and 100µl of C4F3-HRP conjugate in the remaining wells. Then, the plates were covered and incubated at room temperature (18-25°C) for 45 minutes. All the wells were washed three times with 300µl of washing buffer and 50µl of tetra-methyl-benzidine (TMB) substrate was distributed to all wells. Then, the plates were covered and incubated in dark room for 15 minutes at 37°C. Then, 50µl of 1 mole H_2SO4 was distributed to all wells and read OD in wells using ELISA reader (Highland Park, LTD, USA) with an inference filter at 450 nanometers connected to a computer loaded with ELISA data information software (Gen 5.3.04) for automated reading and calculation of the percentage inhibition (PI) values. The OD values were converted to PI using the following formula:

$$PI\ (\%) = 100 - \frac{(OD\ Sample - ODCB)}{(ODNC - ODCB)} x100$$

Where, ODCB = optical density of control buffer, ODNC= optical density of negative control and interpreted. The test result is said to be positive if the PI (%) value is ≥35% (Appendix 1).

3.4.2. The ID Screen® PPR competition ELISA procedure

The kit for the detection of PPRV antibodies used micro-plates coated with PPRV recombinant nucleoprotein. The reagents were allowed to come to room temperature (21 ± 5°C) before use and homogenized by vortex. First, 25µl of DB 13 was added to all wells. Next, 25µl of the PC was distributed to wells A1 and B1, and 25µl of the NC was added to wells C1 and D1. At that point 25µl of each serum to be tested were added to the rest wells and incubated for 45 ± 4 minutes at 37± 3°C using micro-plate incubator shaker (AQS manufacturing, LTD, UK). Each well was washed three rounds each using with 300µl of prepared wash solution. Then 100µl of the conjugate 1× diluted in DB 4 was added to each well. After incubating for 30 ± 3 minutes at 21± 5°C, each well was washed three rounds with 300µl of the wash solution. Then, 100µl of TMB solution was added to each well and incubated for 15 ± 2 minutes at 21± 5°C in the dark. Finally, 100µl of H_2SO4 (stop

solution) was added to all wells; The OD was read at 450 nanometers using the ELISA reader (Highland Park, USA).

For each sample, the competition percentage was calculated using the formula:

$$Competition\ percentage = \frac{OD\ sample}{OD\ negative\ control} x\ 100$$

If the percentage of competition (S/N %) was less or equal to 50% (≤50%) the sample was taken as positive; samples presenting the S/N% greater than 60% (>60%) were negative whereas those showing S/N% between the negative and positive values were said to be uncertain (50%<Competition percentage ≤60%) (Appendix 2).

3.4.3. Virus neutralization test procedure

The test sera were thawed and deactivated by heating at 56 °C for 30 minutes in a water bath. Serum samples to be tested was diluted one in five, and following two-fold serially diluted with minimum essential media (100µl/well). Then 100µl of PPRV (vaccine strain PPRV Nigeria 75/1 allocated at NAHDIC laboratories) at 10^3 TCID50/ml was distributed to all wells. The control plate containing both negative and positive control was prepared separately. The NC contain six wells with 200µl cell culture medium without the virus. PC were arranged as six wells each for 100 $TCID_{50}$, 10, 1 and 0.1 $TCID_{50}$/well. Plates were then stayed in the incubator for one hour at 37 °C after which 50µl of the suspension of Vero dog SLAM cells (4×10^5 cells per ml) was distributed to all wells. The plates were put in incubator with of 5 five percent CO_2 at 37 °C. Finally, the plates were followed using an inverted type of microscope, to monitor the CPE effect starting day 3 of incubation. CPE is observed when the serum is negative, in contrary, no CPE was observed if there were an antibodies in the serum against PPRV. Serum was considered positive for PPRV antibodies if the neutralizing dilution was greater than or equal to 1:10 (OIE, 2019).

3.5. Data analysis

The data from serological tests was classified, filtered, coded and entered into Microsoft Excel® 2013. Data analysis was performed using statistical package of STATA version 12.0. The agreement between the tests was determined using Cohen's kappa statistics and two-way contingency table. The kappa value was interpreted as follows: Kappa value (κ)$\leq$ 0 as indicated no agreement, 0.01-0.20 as none to slight, 0.21-0.40 as fair, 0.41-0.60 as moderate, 0.61-0.80 as substantial, and 0.81-1.00 as almost perfect agreement (Viera and Garrett, 2005). The standard formula were used to calculate diagnostic sensitivity of results by HPPR b-ELISA® and ID Screen® PPR c-ELISA kits. The sensitivity was calculated by the formula: Sensitivity =TP/ (TP + FN) $\times$100, where TP= True positive, FN= False Negative. The diagnostic specificity was calculated by the formula: Specificity=TN/ (TN + FP) $\times$100 and expressed as percentage where TN=True negative, FP=False positive (Munro, 2005).

4. RESULTS

4.1. Initial validation of tests using known references sera

Following the standard protocol of HPPR b-ELISA® and ID Screen® PPR c-ELISA, serial dilutions of two known positive sera and two negative sera were tested to validate the test methods. For HPPR b-ELISA®, the highest dilution for specific detection of antibodies against PPRV using a cutoff value of 30 and 35% percentage of inhibition was 1:32 for positive serum 1 and 1:64 for positive serum 2. The result showed no positive inhibition reaction of negative sera (Figure 2a). Similarly, the highest dilution for the detection of anti-PPRV antibodies with ID Screen® PPR c-ELISA was 1:32 for both positive sera using a cutoff value of 50 and 60% competition percentage. No positive competition reaction was observed for dilutions of negative sera (Figure 2b).

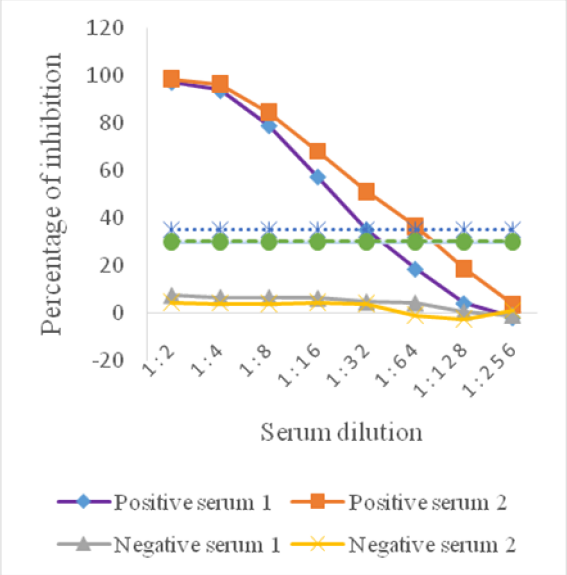

Figure 2a: The analytic sensitivity of b-ELISA

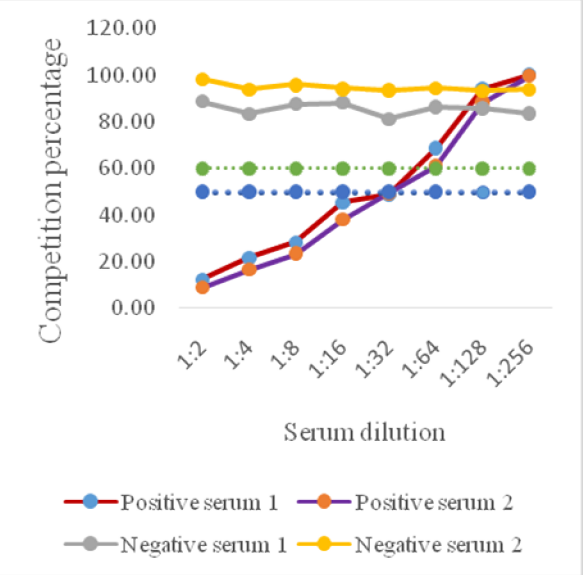

Figure 2b: The analytic sensitivity of c-ELISA

Figure 2: The analytical sensitivity of HPPR b-ELISA® and ID Screen® PPR c-ELISA in the detection of anti-PPRV antibodies

4.2. Comparison of diagnostic performance of HPPR b-ELISA® and ID Screen® PPR c-ELISA tests in ruminants and camels' sera

Out of 480 serum samples tested, 71 (14.79%) were found to be positive with HPPR b-ELISA® and 88 (18.33%) samples were found positive for an anti-PPRV antibodies with ID Screen® PPR c-ELISA. The comparison of HPPR b-ELISA® and ID Screen® PPR c-ELISA kits for PPRV antibody detection is given in table 1.

Table 1: Comparison of diagnostic performance of HPPR b-ELISA® with that of ID Screen® PPR c-ELISA in detecting anti-PPRV antibodies in ruminants' and camels' sera

ID Screen® PPR c-ELISA	HPPR b-ELISA®		
	Negative	Positive	Total
Negative	391 (81.46%)	1 (0.21%)	392 (81.67%)
Positive	18 (3.75%)	70 (14.58%)	88 (18.33%)
Total	409 (85.21%)	71 (14.79%)	480
Agreement = 96.04%	Expected agreement =72.03%		
Kappa (κ) =0.86	Standard error (SE) =0.04	P= 0.00	

The diagnostic sensitivity and specificity of HPPR b-ELISA® compared to ID Screen® PPR c-ELISA were 79.55% (95%CI=69.61-87.40%) and 99.74% (95%CI=98.59-99.99%), respectively. Conversely, the diagnostic sensitivity and specificity of ID Screen® PPR c-ELISA relative to HPPR b-ELISA® were 98.59% (95%CI=92.40-99.96%) and 95.60% (95%CI=93.13-97.37%), respectively.

4.3. Comparison of performance of HPPR b-ELISA® and ID Screen® PPR c-ELISA in detecting anti-PPRV antibodies in goats' sera

The efficacy of the HPPR b-ELISA® was compared with that of the ID Screen® PPR c-ELISA kit by employing of 120 goats' sera. Out of 120 goats sera samples tested, 6 (5.00%) samples were found positive for PPRV antibodies with both tests, and 1 (0.83%) of false-positive and negative was recorded when HPPR b-ELISA® was compared with ID Screen® PPR c-ELISA as shown in table 2. Whereas both the tests were in agreement with each other showing 5 (4.17%) samples as positive and 113 (94.17%) samples as negative. The diagnostic sensitivity and specificity of HPPR b-ELISA® were 83.33 and 99.12%, respectively when compared with ID Screen® PPR c-ELISA in goats. The overall agreements between the two tests were 98.33%, further supported by a kappa value of 0.82 indicating almost perfect agreement between the two tests.

Table 2: Comparison HPPR b-ELISA® and ID Screen® PPR c-ELISA in the detection of antibodies directed against PPRV in goats' sera

ID Screen® PPR c-ELISA	HPPR b-ELISA®		
	Negative	Positive	Total
Negative	113 (94.17%)	1 (0.83%)	114 (95.00%)
Positive	1 (0.83%)	5 (4.17%)	6 (5.00%)
Total	114 (95.00)	6 (5.00%)	120
Agreement =98.33%	Expected agreement = 90.50%		
κ= 0.82	SE= 0.09	P =0.00	

4.4. Comparison of diagnostic performance of HPPR b-ELISA® and ID Screen® PPR c-ELISA tests in detecting anti-PPRV antibodies in sheep sera

The efficacy of the HPPR b-ELISA® was compared with that of the ID Screen® PPR c-ELISA kit by employing of 120 sheep sera. The HPPR b-ELISA® was found to detect 31 (25.83%) anti-PPRV antibodies, while ID Screen® PPR c-ELISA detected anti-PPRV antibodies in 32 (26.67%) in sheep sera. Further analysis of samples revealed that 1 (0.83%) samples showed negative by HPPR b-ELISA® was found positive with ID Screen® PPR c-ELISA. Both the tests were in agreement with each other showing 31 (25.83%) samples as positive and 88 (73.33%) samples as negative (Table 3).

The diagnostic sensitivity and specificity of HPPR b-ELISA® relative to ID Screen® PPR c-ELISA were 96.88 and 100.00%, respectively. Conversely, the sensitivity and specificity of Screen® PPR c-ELISA in relation to HPPR b-ELISA® were 100.00 and 98.88%, respectively in detecting antibodies against PPRV in sheep sera. The overall agreements between the two tests were 99.17%, further supported by the kappa value of 0.98 suggesting almost perfect agreement between the two tests.

Table 3: Comparison of diagnostic performance HPPR b-ELISA® with that of ID Screen® PPR c-ELISA in detecting anti-PPRV antibodies in sheep

ID Screen® PPR c-ELISA	HPPR® b-ELISA		
	Negative	Positive	Total
Negative	88 (73.33%)	0 (0.00%)	88 (73.33%)
Positive	1 (0.83%)	31 (25.83%)	32 (26.67%)
Total	89 (74.17%)	31 (25.83%)	120
Agreement= 99.17%	Expected agreement= 61.28%		
κ=0.98	SE=0.09	P=0.00	

4.5. Comparison of diagnostic performance of HPPR b-ELISA® and ID Screen® PPR c-ELISA test in detecting anti-PPRV antibodies in cattle sera

The HPPR b-ELISA® was found to detect 34 (28.33%) anti-PPRV antibodies, while ID Screen® PPR c-ELISA detected 46 (38.33%) antibodies directed against PPRV in cattle sera. Twelve 12 (10.00%) samples that were negative with HPPR b-ELISA® were shown to be positive with ID Screen® PPR c-ELISA. Both the tests were in agreement with each other showing 34 (28.33%) samples as positive and 74 (61.67%) samples as negative (Table 4).

The relative sensitivity and specificity of HPPR b-ELISA® in comparison with that of ID Screen® PPR c-ELISA in cattle were 73.91 and 100.00%, respectively. On the other hand, the sensitivity and specificity of ID Screen® PPR c-ELISA relative to HPPR b-ELISA® was 100.00 and 86.05%, respectively. The overall agreements between the two tests were 90.00%, further supported by the kappa value of 0.78 suggesting substantial agreement between the two tests.

Table 4: Comparison of HPPR b-ELISA® and ID Screen® PPR c-ELISA in detecting anti-PPRV antibodies in cattle sera

ID Screen® PPR c-ELISA	HPPR® b-ELISA		
	Negative	Positive	Total
Negative	74 (61.67%)	0 (0.00%)	74 (61.67%)
Positive	12 (10.00%)	34 (28.33%)	46 (38.33%)
Total	86 (71.67%)	34 (28.33%)	120 (100.00%)

Agreement= 90.00% Expected agreement = 55.06%

κ= 0.78 SE= 0.09 P= 0.00

4.6. Comparison of performance of HPPR b-ELISA® and ID Screen® PPR c-ELISA tests in detecting PPRV antibodies in camels sera

None of the camels' sera was found positive to anti-PPRV antibodies with HPPR® b-ELISA. Four (3.33%) samples that showed negative result with HPPR b-ELISA® were shown positive with ID Screen® PPR c-ELISA, whereas samples that were negative by ID Screen® PPR c-ELISA were not positive with HPPR® b-ELISA. Both the tests were in agreement with each other showing no samples as positive and 116 (96.67%) samples as negative (Table 5).

The diagnostic specificity of HPPR b-ELISA® in relation to ID Screen® PPR c-ELISA was 100.00%, while that of ID Screen® PPR c-ELISA compared to HPPR b-ELISA® was 96.67%, in camels.

Table 5: Comparison of HPPR b-ELISA® and ID Screen® PPR c-ELISA in the detection of antibodies directed against PPRV in camels' sera

ID Screen® PPR c-ELISA	HPPR b-ELISA®		
	Negative	Positive	Total
Negative	116 (96.67%)	0 (0.00%)	116 (96.67%)
Positive	4 (3.33%)	0 (0.00%)	4 (3.33%)
Total	120 (100.00%)	0 (0.00%)	120
Agreement= 96.67%	Expected agreement= 96.67%		
$\kappa = 0.00$	SE= 0.00	P= 0.50	

4.7. Validation of HPPR b-ELISA® and ID Screen® PPR c-ELISA results with VNT

A total of 80 sera samples of all species were selected for testing. The samples that were positive with either HPPR b-ELISA® (N=1) or ID Screen® PPR c-ELISA (N=15), negative (N=43), and positive (N=21) with both tests were used to validate the results of the two ELISA tests with VNT. Compared with VNT, the HPPR b-ELISA® had a sensitivity and specificity of 80.00 and 96.36%, respectively (κ=0.78). The agreement between HPPR b-ELISA® and VNT was almost perfect in goats (κ=0.83) and sheep (κ=0.89), moderate in cattle (κ=0.50), and no agreement in camels (κ=0.00). The relative sensitivity and specificity of the HPPR b-ELISA® test compared to VNT in each species of animals is illustrated in table 6.

Table 6: The diagnostic sensitivity and specificity of HPPR b-ELISA® test relative to VNT in ruminants and camels

Parameters	Species of animals				
	Goats	Sheep	Cattle	Camels	Total
Sensitivity	100.00%	100.00%	50.00%	--------	80.00%
Specificity	94.12%	87.50%	100.00%	100.00%	96.36%

The ID Screen® PPR c-ELISA had 92.00% diagnostic sensitivity and 76.36% specificity when compared with the gold standard test with a substantial agreement (κ=0.61). The agreement between ID Screen® PPR c-ELISA and VNT was substantial in goats (κ=0.69) and sheep (κ=0.78), fair in cattle (κ=0.30), and no agreement in camels (κ=0.00). The relative sensitivity of the ID Screen® PPR c-ELISA test compared to VNT in each species of animals is presented in table 7.

Table 7: The diagnostic sensitivity and specificity of ID Screen® PPR c-ELISA test with that of VNT in ruminants and camels

Parameters	Species of animals				
	Goats	Sheep	Cattle	Camels	Total
Sensitivity	100%	100%	80.00%	--------	92.00%
Specificity	88.24%	75.00%	50.00%	80.00%	76.36%

5. DISCUSSION

A diagnostic test for mass application should be simple, convenient, rapid, cost-effective, and efficient for intensive surveillance and routine diagnose of diseases. Conventional serological techniques and virus isolation are generally used to detect antibodies produced against morbilliviruses infections. Among the serological tests that have been used in the past for the diagnosis of PPR, VNT and ELISA are the popular tests for the detection of anti-PPRV antibodies (Barrett, 1999).

The present investigation aims to compare the diagnostic performance of HPPR b-ELISA® test with that of a commercial ID Screen® PPR c-ELISA and validate the results of the tests against VNT for specific detection of antibodies directed against PPRV in small ruminants, cattle and camels. Both tests were initially validated using known reference sera. At 1:2 dilutions, the PI was found to be nearly 100% for HPPR b-ELISA® and the competition percentage was below 10% for ID Screen® PPR c-ELISA which indicates these tests are valid to detect antibodies directed against PPRV.

5.1. Comparing HPPR b-ELISA® with ID Screen® PPR c-ELISA in detecting antibodies directed against PPRV in ruminants and camels

The c-ELISA test is being used extensively under field conditions for sero-surveillance and sero-monitoring of PPRV antibodies in sheep and goats (Durrani *et al.*, 2010) cattle (Agga *et al.*, 2019) and camels. The HPPR b-ELISA® was also developed and tested to detect anti-PPRV antibodies in sheep and goats. Therefore, comparing the performance of the HPPR b-ELISA® with the ID Screen® PPR c-ELISA and validation of the results with VNT (the gold standard test) is crucial to further validate and use the HPPR b-ELISA® in detecting antibodies produced against PPRV in ruminants and camels.

The investigation was based on screening of 480 sera samples of goats, sheep, cattle and camels' collected from different parts of Ethiopia. In the present study, HPPR b-ELISA® test detected PPRV antibodies in 14.79%, while the ID Screen® PPR c-ELISA could detected in 18.33% of sera samples of ruminants and camels. The HPPR b-ELISA® had a diagnostic sensitivity and specificity of 79.55 and 99.74%, respectively compared to the commercial ID Screen® PPR c-ELISA. On the other hand, the sensitivity and specificity of ID Screen® PPR c-ELISA compared to HPPR b-ELISA® were 98.59 and 95.60%, respectively. The HPPR b-ELISA® and ID Screen® PPR c-ELISA showed good correlation, with a kappa agreement at 0.86. McHugh (2012) indicated that kappa value greater than 0.6 shows adequate agreement between raters.

The diagnostic sensitivity and specificity of HPPR b-ELISA® test relative to ID Screen® PPR c-ELISA were 83.33 and 99.12%, in goats, 96.88 and 100% in sheep, 73.91 and100% in cattle, 0.00% and 100% in camels, respectively. Conversely, the ID Screen® PPR c-ELISA had a diagnostic sensitivity and specificity of 100 and 88.24% in goats, 100 and 98.88% in sheep, 100 and 86.05% in cattle. The agreement between the two tests in detecting antibodies against PPRV was almost perfect in goats (κ=0.82) and sheep (κ=0.98) and substantial in cattle (κ=0.78). These results indicate the two tests agree well in detecting anti-PPRV antibodies in ruminants' sera. This shows that the HPPR b-ELISA® test can be used as an alternative or as a confirmatory test to ID Screen® PPR c-ELISA in detecting anti-PPRV antibodies in small ruminants and cattle. Mapaco *et al.* (2019) used ID Screen® PPR c-ELISA as a screening test, followed by HPPR b-ELISA® as a confirmatory test to detect anti-PPRV antibody in Mozambique.

In contrast to other species, HPPR b-ELISA® and ID Screen® PPR c-ELISA had no agreement in detecting anti-PPRV antibodies in camels' sera (κ=0.00). Further investigation is needed why the two tests do not agreed in detecting antibodies against PPRV in camels sera and weather new protocol is needed in either or both tests to use them in detecting anti-PPRV antibodies in camels' sera.

The result indicated that the HPPR b-ELISA® directed against the H-protein is slightly more specific than the ID Screen® PPR c-ELISA directed against the nucleoprotein of PPRV. This could be clarified by the reality the Haemeagglutinin protein is not so much constant between morbilliviruses and produce more particular antibodies (Barrett *et al.*, 2006). The epitope of monoclonal antibodies C4F3 could be localized in a specific region of the H-protein. In contrast, the N- protein is antigenically well conserved among morbilliviruses and expressed at a high level in the infected cells compared to other viral proteins. The region of nucleoprotein examined determine the degree of related sequence between different morbilliviruses i.e., high degree sequence similarity regions produce the cross-reacting antibodies among the morbilliviruses. In addition, the need for the region specific test is essential due to cross-reaction between members of morbilliviruses such as *Measles morbillivirus* or *Canine morbillivirus* (Diallo *et al.*, 1994).

Assays comparisons showed the HPPR b-ELISA® was slightly less sensitive than ID Screen® PPR c-ELISA. This might be related to the fact that the N-protein is very important protein produced during virus replication that induce a high level of antibodies in the beginning of morbilliviruses infections or vaccination (Barrett *et al.*, 1993). Antibodies against N-protein are easily perceptible and might contribute to the slight contrast in sensitivity compared to the HPPR® b-ELISA. The H-protein produce most of the neutralizing and defensive antibodies when susceptible animals are infected or vaccinated with morbilliviruses (Giraudon and Wild, 1985). Nevertheless, anti-H-antibodies are also produced at a level similar to that of anti-N antibodies, and the anti-H-antibodies could be detected beginning at 7 days post-infection or vaccination. Antibodies against the H-protein are at the high level at 21 days post-infection or vaccination (Graves *et al.*, 1984).

5.2. Validation of HPPR b-ELISA® and ID Screen® PPR c-ELISA results with VNT

The sensitivity of HPPR b-ELISA® and ID Screen® PPR c-ELISA were 80.00 and 92.00%, respectively relative to the VNT. The HPPR b-ELISA® test had higher specificity (96.36%) than the ID Screen® PPR c-ELISA (76.36%) compared to the VNT. Both ELISA tests had excellent sensitivity (100.00%) in goats and sheep compared to the VNT. The HPPR b-ELISA® test had higher specificity (94.12%) than the ID Screen® PPR ELISA (88.24%) in compared with the gold standard test in goats. Jacobson (1998) described that a diagnostic test is considered to be efficacious when diagnostic sensitivity and specificity are around 90% and above compared to the gold standard test.

Similarly, both ELISA tests had acceptable specificity, which was 75.00 and 87.50% for the ID Screen® PPR c-ELISA and HPPR® b-ELISA, respectively in sheep. The ID Screen® PPR c-ELISA and HPPR b-ELISA® had a sensitivity of 80.00 and 50.00%, respectively, while the diagnostic specificity of ID Screen® PPR c-ELISA and HPPR b-ELISA® was 50 and 100%, respectively with a fair agreement for ID Screen® PPR c-ELISA and moderate agreement for HPPR b-ELISA® in cattle. The relative sensitivity and specificity of ID Screen® PPR c-ELISA in reference laboratories were 94.5 and 99.4%, respectively (Libeau *et al.*, 1995). Saliki *et al.* (1993) found that the blocking ELISA using two neutralizing monoclonal antibodies had 98.9% specificity and 90.4% sensitivity in comparison to the VNT.

These results indicated both ID Screen® PPR c-ELISA and HPPR b-ELISA® are suitable tests to detect anti-PPRV antibodies goats, sheep and cattle sera. In camels, four sera were found to be positive using ID Screen® PPR c-ELISA, while all sera were negative using both VNT and HPPR b-ELISA®. This indicates the ID Screen® PPR c-ELISA shows false positive in detecting antibodies against PPRV in camels. VNT negative sera that were found positive with ID Screen® PPR c-ELISA and HPPR b-ELISA® might be due to the reactivity with related antibodies produced by other morbilliviruses (Burns *et al.*, 2019; Vinayagamurthy *et al.*, 2020). In fact, ruminants and camels on farms are often in contact with dogs and could share canine distemper virus infection and undergo sero-conversion.

However, this assumption needs to be confirmed by VNT for specific detection of antibodies against other morbilliviruses including canine distemper virus (Munir *et al.*, 2013).

6. CONCLUSION AND RECOMMENDATIONS

In conclusion, both the HPPR b-ELISA® and commercial ID Screen® PPR c-ELISA tests were efficient for the specific detection of antibodies against PPRV in all species of ruminants. However, there was no agreement between the two tests in detection of antibodies directed against PPRV in camels. The HPPR b-ELISA® test could be used as alternative to ID Screen® PPR c-ELISA or in combined with the ID Screen® PPR c-ELISA in detecting anti-PPRV antibodies in goats, sheep and cattle sera. This study suggests that ID Screen® PPR c-ELISA and HPPR b-ELISA® for detecting the presence of PPRV antibodies in ruminants' sera are sensitive methods relative to the gold standard test and such kits can be used for research and clinical purposes. The good susceptibility and satisfactory specificity of these tests bolster their capacity to replace the VNT. However, the ID Screen® PPR c-ELISA showed high rate of false positives in detection of anti-PPRV antibodies in camels' sera and the use of this test in detecting specific antibodies against PPRV needs further investigation.

Based on the above conclusion the following recommendations were forwarded:

- The anti-PPRV HPPR b-ELISA® kit is convenient kit for detecting specific antibodies directed against PPRV in goats, sheep and cattle.
- The HPPR b-ELISA® has a potential as an alternative test or used in combination for detection of specific directed antibodies against PPRV since it compared well with the commercial ID Screen® PPR c-ELISA test.
- Although, both ID Screen PPR c-ELISA and HPPR b-ELISA® tests had good sensitivity and specificity compared to the VNT, it is not yet possible to differentiate vaccinated and naturally infected animals using these tests, which warrants DIVA vaccine for PPRV.
- It is highly recommended to use confirmatory test before reporting the presence of anti-PPRV antibody using ID Screen® PPR c-ELISA in camels' sera since the rate false positives is high when compared to VNT.

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8. APPENDICES

Appendix 1: HPPR Blocking ELISA (HPPR b-ELISA®): Protocol version 2019-2

Assay components

- ✓ Pre-coated plates (inactivated PPRV antigen)
- ✓ C4F3-HRP conjugate: 1.5 ml
- ✓ Positive control (PC):1.5 ml
- ✓ Negative control (NC):1.5 ml
- ✓ Dilution buffer (DB): 250 ml
- ✓ Washing buffer (WB) 25×: 250 ml
- ✓ TMB: 60 ml
- ✓ Stop solution (H_2SO4 1Mole): 60 ml
- ✓ Skim milk powder: 50 gram

1. Buffers

1.1. Blocking buffer, freshly prepared: dilution buffer + skim milk (3%).

1.2. Washing buffer: for 1000 ml of washing buffer, 960 ml of fresh distiller or deionized water and 40 ml of washing buffer 25×.

2. Assay procedure

Pre-coated plate and reagents must be equilibrated for 30 minutes at room temperature before use.

2.1. Distribution of controls and test samples

- ❖ Distribute 75 µl of blocking buffer to all wells.
- ❖ Distribute the controls:
  - ➢ Control buffer: 25µl of blocking buffer in wells A1 and A2
  - ➢ Positive control (PC): 25µl in wells B1 and B2
  - ➢ Negative serum (NC): 25µl in wells C1, C2, D1 and D2
- ❖ Distribute test samples: 25µl of each serum per well.

- ❖ Cover the plate and incubate at room temperature (18-25°C) for 1 hour

2.2. Washing

- ❖ Wash all the wells 3 times with 300 µl of washing buffer.
- ❖ At the last washing, blot the plate against clean paper towels to remove the remaining buffer.

2.3. Prepare dilution of C4F3-HRP conjugate at 1:100. For one plate take 9.9 ml of blocking buffer and add 100 µl of C4F3-HRP conjugate stock

2.4. Control buffer and distribution of conjugate

2.5.1. Control Buffer: distribute 100 µl of blocking buffer in control buffer wells A1 and A2

2.5.2 Distribution of conjugate: 100 µl of conjugate in the wells (except A1 and A2).

2.53 Cover the plate and incubate at room temperature (18-25°C) for 45 minutes

2.5. Wash all the wells three times with 300 µl of washing buffer

2.6. Distribution of TMB substrate

- ❖ 50µl of TMB to all wells
- ❖ Cover and incubate the plate in dark room 15 minutes at 37°C.

2.7 Stop the reaction (1mole H_2SO4) and read the plate

- ❖ Distribute 50µl of 1mole H_2SO4 to all wells.
- ❖ Read the OD in wells using ELISA reader with a filter at 450 nanometers

3. Criteria for test plate validation

3.1. Optical density of wells CB and NC

- ❖ Average OD of wells CB (ODCB) ≤ 0.1
- ❖ Median OD of wells NC (ODNC) ≥ 0.6

3.2. The PI calculation

$$PI\ (\%) = 100 - \frac{(OD\ Sample - ODCB)}{(ODNC - ODCB)} x100$$

4. Validation of PI control wells PC and NC

- ❖ PI value of the 2 wells of PC > 60%
- ❖ At least PI value of 3 wells NC < 15%

5. Interpretation of each samples result

- ❖ Negative serum: PI ≤ 30%
- ❖ Doubtful serum: 30% < PI < 35
- ❖ Positive serum: PI ≥ 35%

Appendix 2: Protocol for the detection of antibodies against the PPRV with ID Screen® PPR c-ELISA

1. Kit components/ reagents

- ➢ Microplates coated with PPR recombinant nucleoprotein
- ➢ Anti-nucleoprotein-HRP concentrated conjugate (10×)
- ➢ Positive and negative controls
- ➢ Dilution buffers 4 and 13
- ➢ Wash concentrate (20×)
- ➢ Stop solution (H_2SO4 0.5 Mole)
- ➢ Substrate solution (TMB)

2. Sample preparation

In order to avoid differences in incubation times between samples, it is possible to prepare a 96 well plate containing the test and control samples, before transferring them into an ELISA microplate using a multi-channel pipette.

3. Wash solution preparation

- ➢ Wash solution (1×) is prepared by diluting the concentrated wash solution (20×) in deionized or distilled H_2O after mixing gently.

4. Testing procedure

All reagents are homogenized by vortex after kept at room temperature for 30 minutes.

i. 25µl of DB 13 is added to all wells, 25µl of the PC is added to wells A1 and B1, 25µl of the NC is added to wells C1 and D1, 25µl of each test samples are distributed to the rest wells.

ii. Each well is washed three rounds with 300µl of the prepared washing solution after putting in the incubator at 37± 3°C for 45 ± 4 minutes.

iii. 100 µl of diluted the HRP conjugate (1×) is added to each well

iv. Each well is washed 3 times with 300 µl of the wash solution after incubating for 30 minutes at 21°C.

v. Add 100 µl of the substrate solution to each well and incubate for 15 ± 2 minutes at 21± 5°C in the dark.

vi. Add 100 µl of H_2S04 to all wells to terminate the reaction.

5. Validation

The test is valid if the mean value of the ODNC is > 0.70 and the mean value of the ODPC is < 30% of the ODNC.

6. Interpretation

For each sample, the following formula is used calculate the competition percentage:

$$\text{Competition percentage} = \frac{OD\ sample}{OD\ NC}\ x\ 100$$

Samples showing a S/N%≤ 50% are taken as positive; sample presenting a S/N%> 60% are said to be negative; whereas sample presenting samples presenting between positive and negative are uncertain.