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Development and Validation of a Cost-Effective Haemagglutination Inhibition Assay Using Vero Cell Line for Peste des Petits Ruminants Virus Diagnosis in Resource-Limited Settings

Tahira Hanif[1]*, Aamer Bin Zahur[1], Jawaria Ali Khan[2], Asma Latif[1], Najma Hanif[3], Aman Ullah[1], Nabeela Haneef[4], Tanveer Ibrahim[4], Muhammad Avais[2], Aftab Ahmad Anjum[2], Aneela Zameer Durrani[2] and Munib Hussain[1]

[1]*Animal Health Laboratories, Animal Science Institute, National Agriculture Research Center, Islamabad, Pakistan*
[2]*Department of Clinical Medicine and Surgery, University of Veterinary and Animal Sciences, Lahore, Pakistan*
[3]*MCS, National University of Sciences and Technology, Rawalpindi, Pakistan*
[4]*Division of Nutrition, National Institute of Health, Islamabad, Pakistan*

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ABSTRACT

Peste des petits ruminants (PPR) is a highly contagious viral disease causing significant economic losses due to high morbidity and mortality in sheep and goats, particularly in Pakistan. The commonly used diagnostic tests including serum neutralization test (SNT) and competitive enzyme-linked immunosorbent assays (c-ELISA), are prohibitively expensive for field application. Consequently, there is a pressing need for a cost-effective diagnostic method suitable for small regional laboratories. This study presents the development and validation of a haemagglutination inhibition (HI) assay for PPRV diagnosis, utilizing antigen prepared from Vero cell lines. A total of 610 sera samples from non-vaccinated sheep and goats were analyzed using HI assay. The HI assay results were compared with SNT and c-ELISA, and the analysis showed a perfect agreement between HI assay and SNT (kappa=0.9115). The sensitivity and specificity of the HI assay were 95% and 96%, respectively. These findings demonstrate that the HI assay is a highly convenient, sensitive, and cost-effective method for PPR diagnosis, making it particularly suitable for resource-limited settings.

INTRODUCTION

Peste des petits ruminants virus (PPRV; small ruminant *Morbillivirus*) significantly contributes to morbidity and mortality among sheep and goats in various regions, including Asia, the Middle East, West Africa, Turkey's Marmara area, and European countries such as Georgia and Bulgaria (Abdollahpour *et al*., 2006; Babaoglu *et al*., 2023; Ishag *et al*., 2023; Özkul *et al*., 2002). The virus primarily affects small ruminants but has expanded its host range to include other livestock, such as large ruminants, impacting various domestic animals (Kumari *et al*., 2021; Tully *et al*., 2023; Zakian *et al*., 2016). PPRV has also been detected in wild small ruminants such as the *Dorcas gazelle* (Gazellinae), *Nubian ibex* and Laristan sheep (Caprinae), gemsbok (Hippotraginae), and Sindh ibex (*Capra aegagrus* blythi) in various countries, including Pakistan and the UAE (Abubakar *et al*., 2011; Kinne *et al*., 2010; Kumari *et al*., 2021). PPRV, the causative agent of peste des petits ruminants (PPR), belongs to the genus *Morbillivirus* within the order Mononegavirales and the family Paramyxoviridae (Amarasinghe *et al*., 2019). PPRV was initially classified into four lineages (I-IV) based on gene sequencing (Courcelle *et al*., 2024). Lineages I-III were predominantly found in various African countries, while lineage IV, known as the Asian lineage, was primarily observed in the Middle East and Asia (Banyard *et al*., 2010; Dhar *et al*., 2002; Milovanović *et al*., 2022; Shaila *et al*., 1996). Recent research has determined that the

* Corresponding author: dr.tahirahanif@gmail.com
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sequenced PPRV in regions of China and West Africa belongs to lineages IV and II (Ba *et al*., 2024; Biguezoton *et al*., 2024; Wang *et al*., 2015). PPRV transmission can occur among animals, such as goats and sheep, through aerosols, inhalation, and direct contact with contaminated feed troughs, water, nasal and ocular discharges, and feces (Alemu *et al*., 2019; Loul *et al*., 2020; Zahur *et al*., 2009). The incubation period of PPR disease is 2-7 days and most commonly 4-5 days (Dhar *et al*., 2002; Parida *et al*., 2019).

The clinical signs of PPR disease are highly noticeable. Clinical examination of animals infected with PPR reveals pyrexia (106-107˚F), mild ocular and nasal discharges, mild conjunctivitis, congestion of the third eyelids, and diarrhea. Erosive lesions can also be observed on the inner side of the upper lip of infected animals (Baron *et al*., 2011; Rahman *et al*., 2024; Yousaf *et al*., 2023; Zahur *et al*., 2009). The morbidity and mortality rates range from 0 to 90%, depending on husbandry practices, age, breed, and the virulence of the PPRV (Diallo, 2006). PPRV is responsible for a highly contagious disease, leading to significant economic losses in domestic animals and endangering the conservation of wild herbivores. Accurate diagnosis is essential for the global control and eradication plan of PPR. The use of on-site tests will enhance diagnostic capabilities in extremely remote areas and vulnerable wildlife ecosystems, where maintaining the optimal cold chain for clinical sample transportation is challenging (Aboah *et al*., 2024; Kinimi *et al*., 2020).

In developing countries such as Pakistan, sheep and goats, predominantly raised in rural areas, play a crucial role in meeting the country's meat and wool demands. Unfortunately, PPR is a lethal, rapidly spreading disease that results in the death of millions of these animals. Early diagnosis is vital for effective treatment, requiring sensitive and specific assays. However, research laboratories in small villages and hilly areas lack the facilities for c-ELISA and serum neutralization tests, which are the most reliable for PPR diagnosis. This study aims to develop a cost-effective serodiagnostic assay as an alternative for use in less-equipped laboratories.

MATERIALS AND METHODS

This study was conducted at the Animal Health Laboratories, National Agriculture Research Center (NARC) in Islamabad, Pakistan, from September 2022 to July 2023. A total of 610 serum samples were collected from 352 goats and 258 sheep across various regions, including Gilgit, Kotli, Mansehra, and Dera Ghazi Khan. The samples were obtained from non-vaccinated sheep and goats of all ages. The blood samples from non-vaccinated sheep and goats were collected by jugular vein, clotted and centrifuged to separatesera were saved at -20 ºC, serum neutralization test (SNT) and competitine ELISA (c-ELISA) tests. SNT was performed using microtiter method designated by OIE Terrestrial manual (OIE, 2019) Briefly, SNT was carried out in 96-well microtiter plates and vero cells were used. Sera samples were heat inactivated and then different dilutions were prepared against PPRVs using cell line in microtiter plates. These plates were read after 1 and 2 weeks of incubation and the wells showing neutralization were calculated. For detection of PPRV antibodies the c-ELISA kits, manufactured by Innovative Diagnostics (ID. vet), CIRAD, France. Kits were used according to the manufacturer's instructions. The competitive ELISA micro-plates were read with ELISA reader at 450nm filter (Libeau *et al*., 1995).

Preparation of PPR antigen

PPR isolate (PAK-Fjg-07/NARC 4) was originally isolated from an outbreak in village of Taxila, district Rawalpindi. The isolate was attenuated serially onto the vero cell lines up to 17 passages. The harvest was collected when 70-80% flask was infected showed CPEs. Then the harvest was centrifuged at 700rpm for 5 min and supernatant was used as positive control. Afterwards, the antigen was titrated by haemagglutination (HA) assay with washed chicken RBCs. The highest HA titer (512) was observed with PPR antigen at 17 passaged using 0.6% chicken RBCs.

Haemagglutination procedure (HA)

The HA test was performed as described by Wosu (1985) and Ezeibe *et al*. (2004). Briefly, twofold serial dilutions of PPR antigen was made in U bottom microtitration plate with 25 µL of phosphate buffer saline (PBS) of pH 6.8. Then 25µL of RBCs were added to all wells. HA test was optimized with different concentration of chicken RBCs (0.5%,0.7%,1%) to all wells and plates were incubated at 4°C, 25°C, 37°C for 25, 45, 60 min, respectively. After incubation results were noted and four HA units of PPR antigen was calculated. The positive and negative controls were used in all HA plates.

Haemagglutination inhibition (HI)

Haemagglutination inhibition (HI) test was carried out, as designated by Alexander and Chettle (1977). All sera samples were heat inactivated before HI test. Briefly, twofold serial dilutions of each serum sample were made with 25 µL of PBS in U bottom HA plate. Then 25µL of diluted PPR antigen were added to all wells and plate was kept at 4°C for one h. A 0.6% chicken RBCs were added

to all wells and plate was again set aside at 4◦C for one h. Results were validated on the base of RBC's control.

Statistical analysis

The overall agreement among the results of HI, SNT and c-ELISA respectively was determined by using Kappa statistics (Dohoo *et al*., 2009).

RESULTS

Table I summarizes the distribution of serum samples collected from non-vaccinated sheep and goats across four different areas: Gilgit, Kotli, Mansehra, and Dera Ghazi Khan (DG Khan). Overall, 610 serum samples were collected, with 352 from goats and 258 from sheep, indicating the distribution and total count of samples used in the study.

Table I. Distribution of sera samples from non-vaccinated sheep and goats by area.

Areas	Species		Total samples
	Goat	Sheep	
Gilgit	63	50	113
Kotli	49	90	139
Mansehra	100	36	136
DG Khan	140	82	222
Total	352	258	610

Figure 1A illustrates the relationship between the HA titer and the concentration of RBCs used in the assay. As the RBC concentration increases from 0.5% to 0.6%, the HA titer also increases, reaching its peak at 512. Beyond 0.6%, the HA titer decreases significantly, whereas the highest HA titer (512) was obtained using 0.6% chicken RBCs at the 17th passage. It drops to 128 at a 0.7% RBC concentration and continues to decline to 16 at a 1% RBC concentration. This indicates that 0.6% RBC concentration yields the highest HA titer, suggesting it is the optimal concentration for this assay.

Figure 1B illustrates the relationship between HA and various temperatures. The HA titer, which measures the virus's ability to cause haemagglutination, is highest at the lowest temperature tested. At 4°C, the HA titer is at its peak, with a value of 512. As the temperature increases to 24°C, the HA titer progressively decreases to 128. Further increases in temperature to 39°C result in a continued decline of the HA titer to a minimal value of 4. This trend indicates that the haemagglutination activity of the PPRV antigen is optimal at lower temperatures and significantly decreases as the temperature rises.

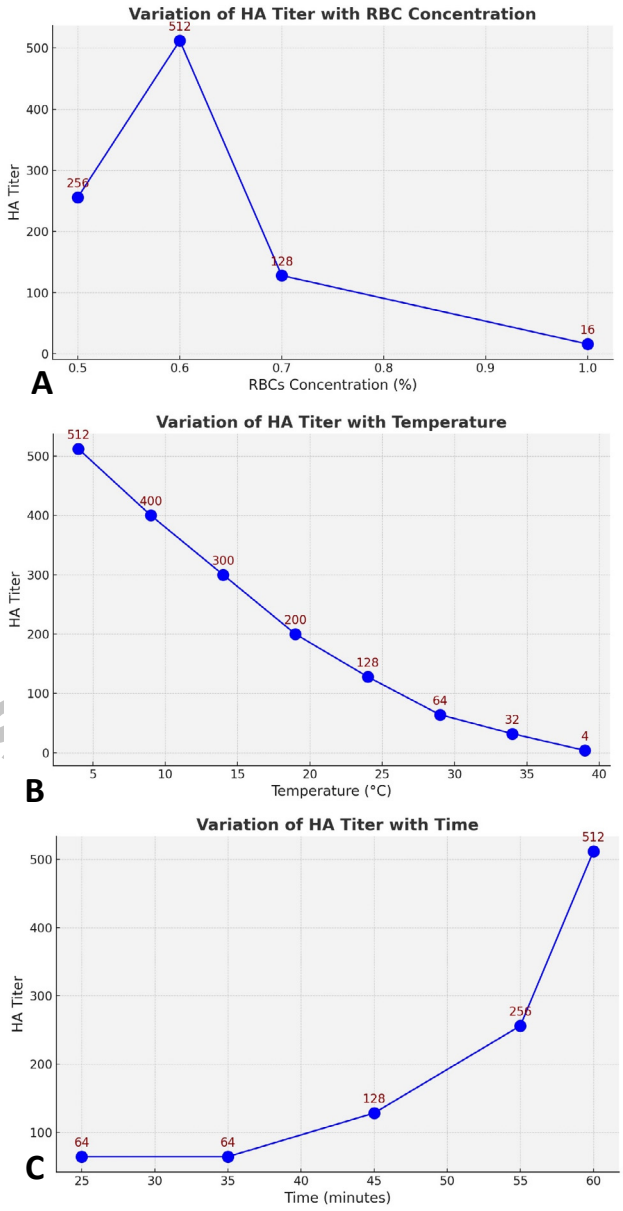

Fig. 1. Variation of HA titer with different concentration of RBCs (A), different temperatures (B), and at different time intervals (C).

Figure 1C illustrates the relationship between the HA titer and various time intervals during the assay. The HA titer, which measures the virus's ability to cause haemagglutination, increases with the duration of the incubation time. At 25 min, the HA titer starts at 64. The titer shows a slight increase to 128 by 45 min. After 45 min, the HA titer rises sharply, reaching 512 at 60 min. This indicates that the haemagglutination activity of the PPRV

antigen significantly improves with longer incubation times, with optimal activity observed at 60 min.

Table II compares the results of 610 sera samples tested using both HI and c-ELISA assays to determine the presence of PPRV antibodies. Both HI and c-ELISA identified 299 samples as positive. Additionally, HI identified 9 samples as positive that were negative by c-ELISA.

Table II. Comparative sensitivity and specificity of HI and c-ELISA and SNT.

Tests		Positive	Negative	Total
c-ELISA HI	Positive	299	9	308
	Negative	19	283	302
	Total	318	292	610
SNT HI				
	Positive	296	12	308
	Negative	15	287	302
	Total	311	299	610

Both HI and c-ELISA identified 283 samples as negative. HI identified 19 samples as negative that were positive by c-ELISA. HI identified a total of 308 positive samples and 302 negative samples, while c-ELISA identified 318 positive samples and 292 negative samples.

The overall agreement between the two tests is calculated as the sum of the concordant results (both positive and negative) divided by the total number of samples. Concordant results are 299 + 283 = 582, leading to an overall agreement of approximately 95%.

The sensitivity of HI, which is the ability to correctly identify positive samples (true positives) as determined by c-ELISA, is calculated as the proportion of true positives identified by HI out of the total positives identified by c-ELISA: 299/318 ≈ 94%. The specificity of HI, which is the ability to correctly identify negative samples (true negatives) as determined by c-ELISA, is calculated as the proportion of true negatives identified by HI out of the total negatives identified by c-ELISA: 283/292 ≈ 97%. The Kappa value (κ) measures the agreement between the two tests beyond what would be expected by chance alone, taking into account both the observed agreement and the expected agreement. A Kappa value of $\kappa = 0.9082$ indicates a perfect or near-perfect agreement between the HI and c-ELISA tests. In the c-ELISA, a cutoff was established where samples exhibiting optical density (OD) values below 50% of the OD of the positive control were classified as positive

These findings suggest that the HI test has excellent sensitivity (94%) and specificity (97%) for detecting PPR disease, with a Kappa value indicating a very strong correlation between HI and c-ELISA results. This demonstrates that the HI test is highly reliable for diagnosing PPR in resource-limited settings.

Table II compares the results of 610 sera samples tested using both HI and SNT assays to determine the presence of PPRV antibodies. Both HI and SNT identified 296 samples as positive. Additionally, HI identified 12 samples as positive that were negative by SNT. Both HI and SNT identified 287 samples as negative. HI identified 15 samples as negative that were positive by SNT. HI identified a total of 308 positive samples and 302 negative samples, while SNT identified 311 positive samples and 299 negative samples.

The overall agreement between the two tests is calculated as the sum of the concordant results (both positive and negative) divided by the total number of samples. Concordant results are 296 + 287 = 583, leading to an overall agreement of approximately 96% (583/610).

The sensitivity of HI, which is the ability to correctly identify positive samples (true positives) as determined by SNT, is calculated as the proportion of true positives identified by HI out of the total positives identified by SNT: 296/311≈95%. The specificity of HI, which is the ability to correctly identify negative samples (true negatives) as determined by SNT, is calculated as the proportion of true negatives identified by HI out of the total negatives identified by SNT: 287/299 ≈ 96%. The Kappa value (κ) measures the agreement between the two tests beyond what would be expected by chance alone, taking into account both the observed agreement and the expected agreement. A Kappa value of $\kappa = 0.9115$ indicates a perfect or near-perfect agreement between the HI and SNT tests. For SNT, serum samples were subjected to heat inactivation and serial dilution before exposure to the PPRV. The absence of cytopathic effects in the wells post-incubation was used to assess the presence of neutralizing antibodies, with effective neutralization confirming their presence. Quantitative analysis revealed that 92% of the wells demonstrated neutralization, reflecting a significant efficacy in inhibiting the PPRV. The study also highlighted the importance of even minimal concentrations of neutralizing antibodies, as evidenced by the positive results achieved at a dilution threshold of 1:10.

These findings suggest that the HI test has excellent sensitivity (95%) and specificity (96%) for detecting PPR disease, with a Kappa value indicating a very strong correlation between HI and SNT results. This demonstrates that the HI test is highly reliable for diagnosing PPR in resource-limited settings.

DISCUSSION

PPR disease is widespread in the Arabian Peninsula, the Indian subcontinent, and the Middle East. The presence of PPR in Pakistan has been recorded since 1991 (Abubakar *et al*., 2015; Amjad *et al*., 1996; Benfield *et al*., 2023). Various molecular and serological tests are currently employed for diagnosing PPR, including immunocapture enzyme-linked immunosorbent assay (Ic-ELISA), c-ELISA, cell culture isolation, and polymerase chain reaction (PCR). However, these diagnostic methods are costly, necessitate technical expertise, and require well-equipped laboratories. Additionally, virus isolation techniques are not suitable for routine diagnostics due to their time-consuming and labor-intensive nature (OIE, 2008). Agar gel immunodiffusion (AGID) is a cost-effective and straightforward test that can be conducted in any laboratory, including field settings. However, AGID lacks sensitivity and cannot detect mild forms of PPR due to the low levels of viral antigen excreted. Similarly, HI is an inexpensive and simple assay used for detecting PPR disease (Ezeibe *et al*., 2010).

In the present study, the HI assay was performed using a serially attenuated PPRV isolate. This isolate underwent serial attenuation on Vero cell lines for up to 17 passages. The hemagglutination activity of the PPR cultured antigen was evaluated after each passage using chicken RBC concentrations of 0.5%, 0.6%, 0.7%, and 1%. The highest HA titer of the PPRV antigen (512) was observed after the 17th passage at a 0.6% concentration of chicken RBCs (Wosu, 1984). The hemagglutination activity of the PPRV antigen was assessed at various temperatures (4°C, 8°C, and 37°C) and different time intervals (25, 45, and 60 min). The optimal activity of the PPRV antigen was observed at 4°C for 60 min. Additionally, the PPR cultured antigen was capable of causing agglutination of chicken RBCs in PBS with a pH of 6.8, which aligns with the value used by Ezeibe *et al*. (2004) and Wosu (1991).

Four hemagglutination (4HA) units of PPRV antigen were calculated, and the HI test was conducted. In this test, samples were classified as seronegative for PPR if the sera had a titer of less than 1:16, while sera with titers greater than 1:8 were considered positive.

To validate the results of the newly developed HI assay, the tested sera samples were compared with SNT and c-ELISA. The overall agreement between the results of c-ELISA and HI was 95%. The sensitivity and specificity of the HI assay were observed to be 94% and 97%, respectively. The kappa value ($\kappa = 0.9082$) indicated a perfect agreement between HI and c-ELISA.

However, when comparing the HI assay with the SNT, it was concluded that the overall agreement between HI and SNT was 96%. The sensitivity and specificity of the HI assay were found to be 95% and 96%, respectively. Kappa analysis indicated a perfect agreement between HI and SNT ($\kappa = 0.9115$). These findings suggest that the HI assay has excellent sensitivity and specificity for the diagnosis of PPR disease.

In this study, the HI assay was compared with the c-ELISA and the SNT. All these tests successfully detected PPRV antibodies in sera samples. The results indicated that c-ELISA and SNT are highly sensitive and specific for detecting PPRV antibodies. However, c-ELISA is very expensive, and SNT is labor-intensive and impractical for field use. Therefore, the HI assay was found to be more advantageous than other techniques for titrating PPR antibodies. The HI test is easy, fast, reliable, and can be performed in less-equipped laboratories, making it a suitable technique for field conditions.

CONCLUSION

This study developed and validated a cost-effective HI assay for the diagnosis of PPRV in resource-limited settings. Utilizing antigen prepared from Vero cell lines, the HI assay demonstrated high sensitivity (95%) and specificity (96%) when compared to the SNT and c-ELISA. The overall agreement with SNT was nearly perfect ($\kappa = 0.9115$). These results highlight the HI assay's reliability, convenience, and cost-effectiveness, making it a suitable diagnostic tool for small regional laboratories and remote areas lacking advanced facilities. The HI assay offers a practical alternative for early and accurate diagnosis of PPR, which is critical for controlling and eradicating the disease, ultimately reducing economic losses in the livestock sector.

DECLARATION

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IRB approval

The research program was approved by the Program Executive Committee of research for Regional Agricultural Development Program (RADP) under the approval No. As 12/RADP/11-Pak.

Ethical statement

All procedures performed during this research were conducted in accordance with institutional guidelines and

approved by the relevant authority RADP.

Statement of conflict of interest

The authors have declared no conflict of interest.

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