Comparison of In-house Developed Indirect ELISA with Commercially Available Kit for Diagnosis of Peste des Petits Ruminants (PPR) Virus

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December 2021

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A Thesis

Submitted to Quaid-i-Azam University, Islamabad in the Partial fulfillment of the requirements for the degree of

MASTER OF PHILOSOPHY
IN
ANIMAL GENOMICS AND BIOTECHNOLOGY

BY

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AUTHOR'S DECLARATION

I would like to declare that the data presented in this thesis **"Comparison of In-house Developed Indirect ELISA with Commercially Available Kit for Diagnosis of Peste des Petits Ruminants (PPR) Virus"** is generated myself from original research work under the supervision of Dr. Muhammad Naeem Riaz at Animal Biotechnology Program, National Agriculture Research Centre (NARC), Islamabad, Pakistan. The results and material used in this thesis never presented anywhere else earlier.

Abid Hussain

Dated: December 2021

ACKNOWLEDGMENTS

I am grateful to **ALLAH** Almighty who is most merciful and beneficent, and **HOLY PROPHET MUHAMMAD (P.B.U.H**) who is the torch bearer of knowledge. The whole facilities, opportunities, abilities, powers, and strength are blessed by **ALLAH** almighty which helped me to reach this destination.

I would also like to thank **Dr. Syed Murtaza Hassan Andrabi**, Head, Department of Animal Genomics and Biotechnology.

Furthermost, I would like to pay special thanks to my supervisor **Dr. Muhammad Naeem Riaz,** Assistant Professor, Animal Genomics and Biotechnology, PARC Institute of Advance Studies in Agriculture, Quaid-i-Azam University, Islamabad, Pakistan, for his continuous support and efforts for my thesis research work and moral support and mentorship during whole academic session.

I am highly obliged and thankful to my mentor, **Dr. Tahira Kamal** for being morally and technically supportive during whole academic period.

I would love to thanks to all my friends/colleagues (**Rafia Sameen, Dr. Abubakar Siddique, Shan-e-Zahra**, **Zunaira Fatima**, **Dr. Ambreen Gul** and **Sarfraz Mehmood**) for their continuous support during whole academic session. I am also thankful to my brothers and sisters for being very supportive and encouraging in life whenever I stuck in different matters. I would like to especially thank to my elder brother **Asif Hussain Mastoi** for always being there for me as a friend. I am forever indebted to him giving me the opportunities and support.

Last but not least, I am truly grateful to my parents **Mr. & Mrs. Ghulam Hussain Mastoi** who always supported me at every step to reach this level.

Abid Hussain

Dedication to

My

FATHER

For earning an honest living for us and for supporting and encouraging me to believe in myself.

My

MOTHER

A strong and gentle soul who taught us to believe in ALLAH, believe in hard work and that so much could be done with little

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LIST OF ABBREVIATION

AGID	Agar Gel Immunodiffusion
c-ELISA	Competitive- Enzyme Linked Immunosorbent Assay
CIE	Counter Immunoelectrophoresis
CI	Confidence Interval
CPE	Cytopathic Effect
ELISA	Enzyme Linked Immunosorbent Assay
FAO	Food and Agricultural Organization
I-ELISA	indirect-Enzyme Linked Immunosorbent Assay
LAMP	Loop Mediated Isothermal Amplification
OD	Optical Density
OIE	World Organization for Animal Health
PCR	Polymerase Chain Reaction
PI	Percentage of Inhibition
PP	Percent Positivity
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

ABSTRACT

Peste des petits ruminants (PPR) is a major disease of sheep, goats, camel, and wild ruminants. It can affect entire populations of immunologically immature hosts. PPR may lead to epidemics that affect the economy of any country to a certain extent. In order to control the disease, an accurate and reliable diagnosis is the first step. So, there is a need for a series of cost-effective alternative tools for rapid diagnosis of PPR infection. The aim of this study was to compare inhouse developed indirect ELISA (i-ELISA) with commercially available c-ELISA for the detection of antibodies against PPRV. A total of 694 serum samples were obtained from sheep and goats based on clinical signs from different regions of Pakistan. The relative sensitivity and specificity of i-ELISA was evaluated, and results were also compared with c-ELISA and VNT in terms of sensitivity and specificity. The agreements between the tests were determined using Cohen's Kappa statistics and two-way contingency table. Specificity and sensitivity of i-ELISA was found to be 95.37 & 89.84% respectively as compared to cELISA, similarly these results were also compared with VNT as 100 & 81% respectively. Moreover, cost of i-ELISA for 96 well plate was estimated $36 as compared to commercially available kits which ranges from $400-$500 making it more affordable as compared to commercial c-ELISA for developing countries like Pakistan.

Key words: Peste des petits ruminants, Ruminants, Antibodies, Indirect ELISA, c-ELISA, VNT.

CHAPTER 1

INTRODUCTIO

1. INTRODUCTION

Peste-des-petits-ruminants (PPR) is a notifiable transboundary disease of small ruminants. It is an acute and highly contagious viral disease caused by the Peste des petits ruminants virus (PPRV). It belongs to morbillivirus of the Paramyxoviridae family. It causes severe respiratory and digestive system complications. PPRV has high morbidity and mortality rate. PPR is regarded as an Office International des Epizooties (OIE) list A disease.

1.1 History of outbreaks of PPR and its global distribution

PPR was formerly associated with a region of Western Africa. Peste Des Petits Ruminants is a French name, as French was the native language of Western Africans. Its name alludes to the disease's resemblance to Rinderpest (Diallo *et al.*, 2007). During World War II, in 1942, (Gargadennec and Lalanne, 1942) reported an RP that seemed like a catastrophic sickness in Cote d'Ivoire, Western Africa. Only sheep and goats were affected, while animals in proximity of these diseased animals were unaffected. Other African countries, such as Nigeria, Senegal, and Ghana, later reported the sickness. Goat Plague, Kata, Pseudo Rinderpest, and later Stomatitis pneumo-enteritis complex were its common names (Otte, 1960; Rowland and Bourdin, 1970; Braide, 1981). PPR was regarded to be a Western African problem for three decades until the disease in goats in Sudan, which was first thought to be RP in 1972, was confirmed as PPR (Diallo *et al.*, 1987).

PPRV is endemic in several East African nations. In 1996, genetic typing confirmed that the virus found in East Africa belongs to PPRV lineage III. Previous isolations were carried out on animals from Oman that were connected to the same lineage as those from East African countries. Molecular tests in Sudan, Uganda, and Tanzania also confirmed the link between viruses and lineage. Viruses of lineage IV have also been identified in several parts of the continent (Khalafalla *et al.*, 2010).

In the year 2000, the situation of PPRV in Saudi Arabia was investigated (Al-Naeem *et al.*, 2000). in April 2002, the country saw a disease outbreak in goats and sheep that resulted in a 100% mortality rate (Housawi *et al.*, 2004; El-Rahim *et al.*,

2005). Camels was thought to be the source of the virus's spread in goats (ABD EL-HAKIM, 2006). Jordan and Lebanon both have also reported goat and sheep PPR in seroprevalence studies. In 2006, PPRV was discovered in a Kenyan district. This disease subsequently spread to other parts of the country, wreaking havoc on food security and the livelihoods of the local population. PPRV also struck Somalia in 2006, wreaking havoc on the country's central regions (Kihu *et al.*, 2012). However, the spread of disease was halted in this case due to prompt implementation of appropriate precautions. Furthermore, ring vaccination was implemented across the country to prevent the virus from spreading. PPRV infection has also been found in North African countries. In 1987 and 1990, there were a lot of cases of this disease, especially in Egypt. In the year 2006, a spike in PPRV cases occurred in Egypt's Aswan governorate. This epidemic demonstrated the ability of diseased goats to remain asymptomatic for an extended period. Following this time, these animals acquired a severe clinical illness (El-Hakim, 2006). Except for Egypt, all North Africa's countries were supposed to be PPR-free until a large-scale outbreak of PPRV infection in Morocco in 2008. In 2010, however, viruses from both lineages III and IV were discovered from goats in Qatar (Al-Dubaib 2009; Elzein et al. 2004).

This suggested that PPR has a longer history than previously assumed as shown in Fig. 1.1. (FAO, 2015). Because of its transboundary nature, economic significance, and ability to manipulate global efforts to reduce poverty, the disease is gaining in popularity (Lefèvre and Diallo, 1990a; Zahur *et al.*, 2008). Approximately 70 countries have either reported infection with PPRV or are suspected of being infected with the virus at this time. Africa (including North Africa), Asia, and the Middle East account for 60 percent of the world's countries. Another 50 countries are at risk of being affected by PPR.

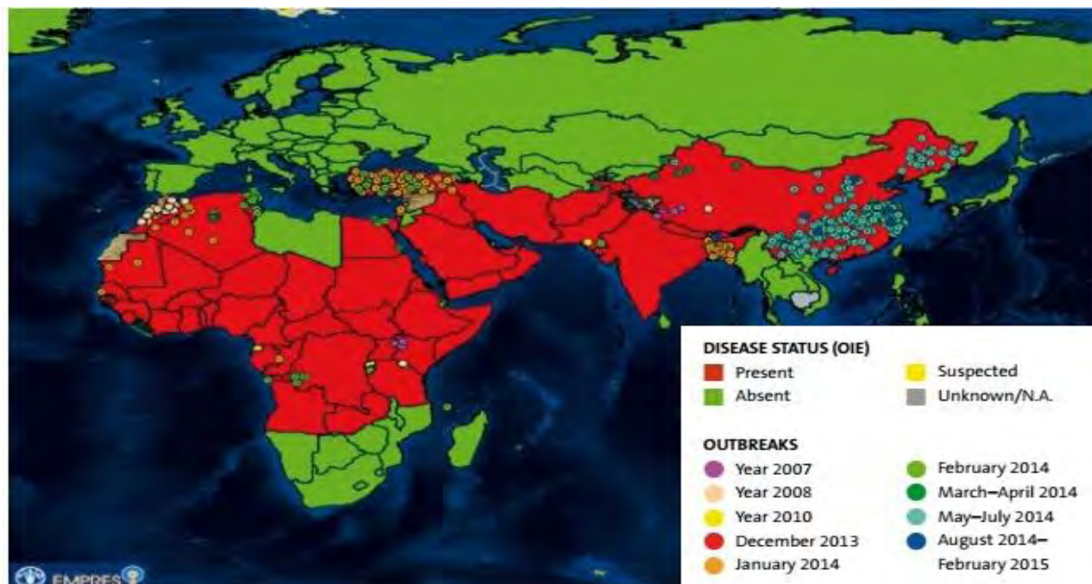


Figure 1.1. Global PPR situation (FAO, 2015)

1.2 PPR in Pakistan and Economic losses

During the last decade, the number of PPR outbreaks in Pakistan has increased to an alarming level, involving more recent areas. (Ali, 2004). PPR virus can destroy entire populations of hosts who are immunologically immature/innate. Pakistan's PPR virus is found in 48.30 percent of the population in different districts throughout the country. Several studies (Abubakar *et al.*, 2008) have reported dramatic consequences, including morbidity rates of 80–90 percent and mortality rates ranging from 50 to 80 percent. And it may lead to epidemics that affect the economy of any country to a certain extent. As per government of Pakistan’s estimates that it causes economic losses of Rs. 20.5 billion (US$ 0.24 billion) per year in the country alone (Abubakar et al., 2015).(Abubakar *et al.*, 2015).

1.3 Etiology

PPR is caused by the Peste des Petits Ruminants virus (PPRV). It belongs to the Morbillivirus genus, which belongs to the Mononegavirales order in the Paramyxoviridae family (Gibbs *et al.*, 1979b). Because it includes Measles virus (Disease in Humans), CDV (in Dogs), RPV (in Bovines), Phocine distemper virus (in Dolphins), and the morbilliviruses of domestic animals living in seas and oceans, this class of virus is extremely important in the medical and veterinary fields. New

morbilliviruses have recently been discovered, such as feline morbilliviruses (in cats) (Woo *et al.*, 2012) and rodent/bat morbilliviruses (Drexler *et al.*, 2012).

1.4 Physiochemical properties of the virus

Because of the virus's weak nature, it is unable to withstand environmental conditions outside of the host. At 56°C, it has a half-life of 22 minutes, and at 37°C, it has a half-life of approximately three hours (Hamdy and Dardiri, 1976). Alcohol, ether, and detergents are all detrimental to PPRV. At -20oC, it can live in tissues for an extended period (Abubakar and Munir, 2014)

1.5 Viral Genome

Unlike other Morbillivirus members, the PPRV genome is non-segmented and has a negative-sense strand. PPRV is made up of 15948 nucleotides that code for eight proteins: nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin protein (H), polymerase protein (L), and two nonstructural proteins (C and V). In the entire genome, the PPRV genome is arranged into six contiguous, nonoverlapping transcription units that correspond to the genes of the six structural viral proteins in the sequence 3'-N-P-M-F-H-L-5' (Rima *et al.*, 1986; Sidhu *et al.*, 1993). Between each gene, there are conserved areas. There are 30 and 50 untranscribed sequences in the PPR genome which serve a critical function in the replication of regulatory elements (Bailey *et al.*, 2005). The viral leader region includes the N gene's 30 untranslated region (UTR), which forms the genome's promoter region (GP), and the L gene's 50 UTR, which forms the promoter against the genome's promoter (AGP). When compared to other UTRs found throughout the viral genome, the UTR between the F and M gene ORF is unusually lengthy (1080 nucleotides) and very rich in G and C nucleotides (68–72 percent GC across the region). In general, the viral genome is very restricted, with the maximum nucleotide and amino acid sequence diversity of 12 percent and 8%, respectively (Muniraju *et al.*, 2014).

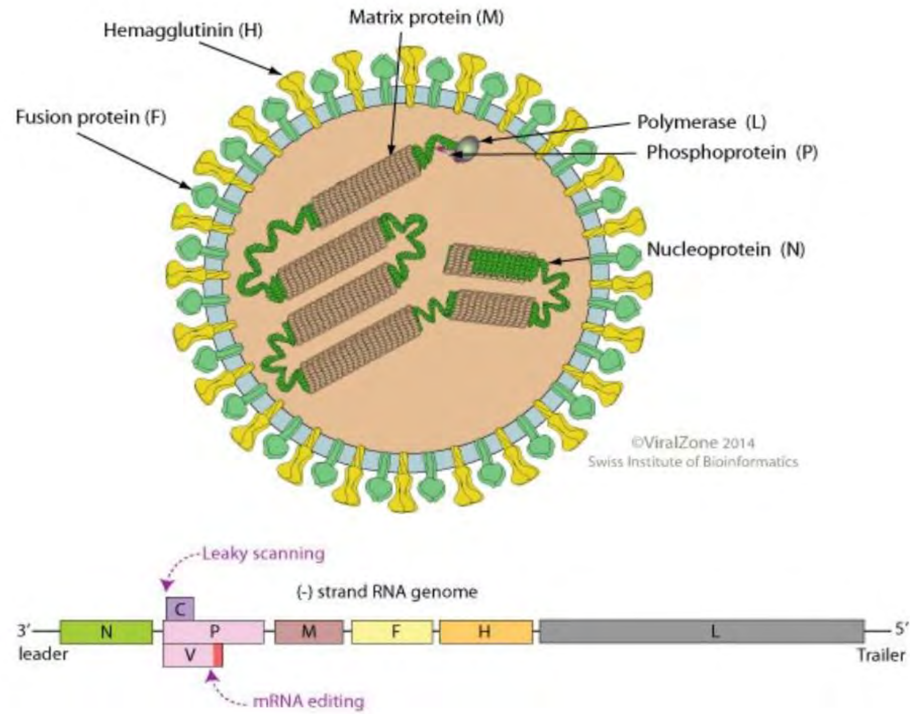


Fig. 1.2: Schematic diagram of PPRV Viral Genome (Source: Viral Zone@, 2014)

1.6. Viral Protein

1.6.1. N Protein

Nucleocapsid (N) protein is one of the most important structural proteins in morbilliviruses. It reacts with other N proteins in the same family. The development of the nucleoprotein core is influenced by the N-N composition. The freshly synthesized genome was completely encased by this core. The N protein forms a connection with the P and L proteins during replication and transcription. They move on polyacrylamide gels with apparent molecular weights ranging from sixty to sixty-eight kDa for PPR and RPV, respectively (Diallo *et al.*, 1987). According to published data, the N-protein sequence of morbilliviruses has 525 amino acids for PPRV, RPV, and MV, but only 523 amino acids for DMV, CDV, and PMV (Muthuchelvan *et al.*, 2006). This indicates that the sequence contrast between N proteins from various morbilliviruses varies from 67 to 74 percent between different amino acid (Diallo *et al.*, 2007).

1.6.2. P Protein

The P protein is phosphorylated protein. Between the proteins P, N, and L, a viral polymerase complex is formed. It aids in the production of RNA envelopes. The protein's true molecular weight is 54-55 kDa, however it runs on SDS-PAGE with a larger molecular weight of 72-86 kDa (Diallo et al. 1987). This unique shift is caused by the protein's copious threonine and serine being phosphorylated post-translationally. PPRV has a 509-amino-acid P protein, whereas DMV has 506-amino-acid P protein, and all other morbilliviruses have 507-amino-acid P proteins. The P protein is one of the virus's least long-lasting proteins (Mahapatra *et al.*, 2003).

1.6.3. Matrix (M) protein

Inside the capsid is where the Matrix protein is found. It aids in the virus's morphogenesis and division. The M protein has ability to form cytoplasmic ribonucleoprotein complexes. It also interacts with the proteins that make up the viral envelope. The sequence of amino acids for M Protein is the most similar among all morbilliviruses. RPV and PDV/CDV have a 76 percent similarity, CDV and PDV have a 91 percent similarity, while PPRV and RPV have an 84 percent similarity (Diallo *et al.*, 2007)

1.6.4. Fusion (F) protein

The fusion protein is a glycoprotein found on the surface of the cell. This protein is embedded in the envelope and protrudes as spikes. It aids the virus's entry into the host cell by interfering with the cellular and viral membranes on the cell's surface. F protein cannot carry out this activity without the assistance of H protein 1, which catalyses F protein's action (Moll *et al.*, 2001). Its highly conserved areas are similar to those found in the M protein. 2410 nucleotides make up the PPRV F gene. Paramyxoviruses produce an inactive precursor (F0). The host cell proteases break F0 into two proteins (F1 and F2) that are linked by an active disulphide bond. This proteolytic cleavage is critical for the protein's biological function (Smith *et al.*, 2009).

1.6.5. H Protein

The H protein is also a glycoprotein found on the surface of cells. It assists the virus in binding to a cell receptor, which is the first stage in the infection process. It also catalyzes the F protein's fusion activity (Das *et al.*, 2000). Inside the rough endoplasmic reticulum, it forms a complex with glycogen (RER). Varying morbilliviruses have different lengths. DMV has 604 amino acids, PPRV has 609 amino acids, and MV has 617 amino acids. Its less preserved characteristic is reflected in its sequence variation. This high level of sequence diversity aids in the virus's adhesion to the host cell. H protein is the primary focus of the host humoral reaction because of its exceptional capacity. Antibodies used to neutralize viruses are mostly directed against the H protein. PPRV's H protein, like the haemagglutinin-neuraminidase (HN) protein seen in paramyxoviruses, possesses neuraminidase activity (Seth and Shaila, 2001).

1.6.6. Large Protein

Large proteins encode for a region near the genome's 5' end. It is the biggest protein in comparison to all other proteins, yet RNA-dependent RNA polymerase is less abundant (RdRp). On polyacrylamide gels, they reveal a molecular weight of 200 kDa. In MV, RPV, PPRV, and DMV, it has 2183 amino acids (Baron and Barrett, 1995; Bailey *et al.*, 2005). It is also conserved among the morbilliviruses, despite its enormous size. RdRp is thought to perform all the processes required for genomic RNA replication and transcription. The RdRp functions in the presence of a cofactor, the P protein (Moyer and Horikami, 1991).

1.7. Epidemiology

1.7.1. Transmission

The virus is prevalent in an animal's oral, nasal, and ocular discharges, as well as the faeces. Infected animals' secretions, which are released into the air while coughing and sneezing, spread the virus throughout the surroundings (Johnson and Ritchie, 1968). PPR spread to susceptible animals present in the close vicinity of infected animals (Özkul *et al.*, 2002). Inhaling aerosols created by infected animals' sneezing and coughing, as well as direct contact with excrement, can spread the virus

(Zakian *et al.*, 2016). Water, bedding material and feed troughs contaminated through an infectious agent are also the source of dissemination of the disease (Biruk, 2014). Virus excretion was noticed two days after the infection began. PPR outbreaks are mostly caused by the introduction of new animals, animal migrations, livestock markets, community grazing, and stress (Miraglia *et al.*, 2009).

1.7.2. Pattern of disease incidence

In general, morbidity is more visible in the full prone goat population than in the sheep population. In sheep and fractionally resistant goat herds, a moderate form of illness can arise (Abraham *et al.*, 2005). It has been observed that the virus has a stronger affinity for caprine species than for ovine species, and that the virus causes more mortality in goat populations, particularly in young goats, than in susceptible sheep (Singh *et al.*, 2004b).

In diverse ecological systems and geographical areas, there is a sufficient variance in the disease's epidemiological pattern. In the humid Guinean zone, where PPR occurs as an epizootic, morbidity and death rates can reach 90% and 80%, respectively. PPR is rarely fatal in dry and semi-arid locations, although it usually appears as a silent or undetectable symptom that leads to the diagnosis of pasteurellosis (Lefèvre and Diallo, 1990b). Saudi Arabia has recorded morbidity rates as high as 90% and death rates as high as 70% (Abu-Elzein *et al.*, 1990). When compared to the rest of the months, the illness is most common between the months of March and June (Singh *et al.*, 2004b).

PPR disease is prevalent in most places, although incidence spiked towards the end of fall and the beginning of the winter season (Dec and Jan). This might be due to the season of kidding and lambing, a lack of nutrition, or stressed animals being more susceptible to disease (Zahur *et al.*, 2008). Season, on the other hand, is important since it imposes stress in severe situations. As a result, PPR is widespread in Sindh Pakistan throughout the summer. Animal movements, on the other hand, have a higher impact on disease incidence. PPR is also frequent in several Indian locations during exceptionally hot temperatures (Singh *et al.*, 2004b). PPR cases in Nigeria rose throughout the winter and wet seasons (Opasina and Putt, 1985). To summarize, the season not only increases the danger of disease development, but also causes stress

due to a variety of factors such as dietary shortages, housing capacity, animal migrations, and a lack of understanding of the quarantine system (Zahur *et al.*, 2011).

1.7.3. Clinical signs

In sheep and goats, PPRV caused three different kinds of disease: peracute, acute, and subacute(Abdollahpour *et al.*, 2006; Banyard *et al.*, 2006). In the peracute variant of PPR, the illness manifests clinically after three days of incubation. High pyrexia is seen up to 107.6°F. The animal becomes stressed, dull, and anorexic. This stage may include conjunctivitis and nasal/buccal cavity congestion. In general, diarrhea and oral erosion are not seen in this type of the illness. In acute cases, the disease manifests clinically after three to four days of incubation, resulting in fever and other PPR-specific clinical symptoms such as occulo-nasal discharge, congestion of the upper GIT mucosal lining, conjunctivitis, and vulvovaginitis. This is followed by watery diarrhea and then bloody diarrhea (Abubakar *et al.*, 2008). In neglected animals, dehydration leads to death at the end. In the acute stage of the disease, the animal exhibits the following general symptoms in order:

- The incubation period is two to seven days, with a fever ranging from 103°F to 106°F appearing in three to ten days.
- Conjunctivitis and oral mucosa congestion develop after two to three days of pyrexia.
- Occulo-nasal secretions begin four to seven days after infection and last two to four days; with time, this severe discharge becomes mucopurulent, and these congestions eventually manifest as oral lesions.
- These lesions can sometimes appear as an ulcer five to nine days following infection in severe cases. Caseous-material also appears around the tongue and soft palate.
- Diarrhoea starts after four to ten days of infection, emaciated, weight loss, dehydration and death.
- In mild cases, however, the animal itself will recover within ten to fifteen days of infection.

The mild form of the illness is the sub-acute form. The severity of clinical signs and symptoms is usually modest, and the condition lasts longer with a low death rate.

1.8. Pathology:

1.8.1. Pathogenesis

Epithelial and lymphoid tissues are the locations where PPRV targets and multiplies rapidly. PPRV enters the body through the respiratory route, and the virus is found in the tonsils, pharyngeal, and mandibular lymph nodes. Viremia occurs two to three days after contact with PPRV infection, causing PPRV to spread to the gastro-intestinal mucosae, respiratory tract, bone marrow, and spleen (Gibbs *et al.*, 1979b). The PPR virus induces immunological suppression by causing leucopenia and lymphopenia (Rajak *et al.*, 2005).

1.8.2. Gross Lesions

PPRV affects two systems: the gastrointestinal and respiratory systems, resulting in severe abnormalities in both organs (Zahur *et al.*, 2011). External examination reveals a PPRV-infected carcass that is dehydrated, emaciated, and has watery diarrhea around the perineal region. Mucopurulent secretions can also be seen around the eyes, nose, and mouth. Cheesy material is seen inside the buccal cavity, as well as severe hemorrhages on the mucosal lining. Zebra striping is a symptom of widespread congestion in the large intestine (caecum, colon, and rectum). Hemorrhages can be observed on the ileocecal valve, and in severe cases, edematous and ulcerative mucosa can be seen throughout the GIT (Munir *et al.*, 2013).

In the afflicted animal's upper respiratory route, hyperemia with erosions can be seen. Lungs become reddish, purple, and pneumonic in appearance. The epithelia of the anterior and cardiac lobes of the lungs becomes stiff and hard. These pathognomonic lesions have never been seen or reported in a Rinderpest patient (Brown *et al.*, 1991). The inner surface of the rumen may have some congestion. On the inner surface of the abomasum, advanced kinds of hemorrhages can be seen. The spleen may become somewhat enlarged because of the congestion (Toplu, 2004).

1.9. Immunity

Immunosuppression is seen in PPR virus-infected host species. As a result, afflicted animals are more likely to develop additional illnesses, resulting in a high mortality. After the occurrence of the disease, the host achieves a lifetime safe immunological level, regardless of immunosuppression (Schlender *et al.*, 1996). Morbilliviruses' surface glycoproteins hemagglutinin (H) and fusion protein (F) are the most effective in inducing and conferring protective immunity (Sinnathamby *et al.*, 2001). If the dam has been vaccinated or has been exposed to PPR infection, the PPR vaccine has the ability to be passed on to newly born kids and lambs. Up to 6 months of age, antibodies acquired from the dam can be detected (Balamurugan *et al.*, 2012).

1.10. Diagnosis

1.10.1. In field diagnosis

PPR is often diagnosed based on its distinct clinical signs and symptoms. However, similar clinical symptoms can be confused with RP, sheep and goat pox, CCPP, CPD, and a variety of other diseases. Although these clinical signs help to narrow down the diagnosis, laboratory diagnosis is required for viral confirmation and characterization.

1.10.2. PPR Laboratory Diagnosis

PPR diagnosis is not certain based on clinical signs and symptoms, necropsy, or epidemiology. Different serological methods such as indirect ELISA i-ELISA, CIE, and AGID have been used to diagnose this disease, however it is unable to distinguish PPR from other related diseases (Liermann *et al.*, 1998; Balamurugan *et al.*, 2012). For sensitive and specific detection of PPRV, molecular-based and cell culture methods (virus isolation) can be utilized. Although cell culture methods are time-consuming and labor-intensive. molecular techniques like as RT-PCR, real-time PCR, and LAMP are not only sensitive and specific for PPR diagnosis, but they are also highly quick (Cartee *et al.*, 2003). PPRV is detected via nucleic acid sequencing in addition to viral isolation and antibody detection (Diallo *et al.*, 1989). Molecular-

based methods, on the other hand, are more trustworthy and aid in the definite diagnosis of PPRV.

1.10.3. Virus Isolation Methods (Cell culture Technique)

It is regarded as the gold standard in the diagnosis of PPR. Primary sheep and bovine cells can be used to isolate viruses (Scheid and Choppin, 1974). In addition, cell lines such as Vero and B95a are increasingly commonly utilized to isolate PPRV (Diallo *et al.*, 2007). During PPRV infection, the cytopathic effect (CPE) occurs, which includes structural changes in infected cells (round shape), vacuolation, cell cytoplasm granulation, monolayer cell fusion, and syncytia formation. Many blind passages are necessary due to the failure of viral separation during the first passage. To address this issue, cell line derivatives with morbillivirus receptors have been devised that allow virus isolation in less than a week without the need of blind passages, such as CV1 with goat SLAM and Vero cell line with dog SLAM. Although the viral isolation technique cannot be used frequently for diagnosis due to its time commitment, it is highly successful for isolating viruses from various field samples, which are subsequently investigated using molecular techniques (Adombi *et al.*, 2011).

1.10.4. Antigen detection methods

1.10.4.1. Virus neutralization (VNT)

VNT is a serological test that detects antibodies that are specific for PPR. This test may be performed using both primary cells (Lamb kidney cells) and cell lines (Vero). This test may be used to distinguish PPR virus from Rinderpest (RP) (Horvath *et al.*, 1992) using either simple tubes or a microtiter plate (96 well) referred to as micro-VNT. PPRV may be distinguished from RP, CDV, and MV using this test (Rapaport and Shai 1994). Although this test is useful because of its sensitivity and specificity, it is also time consuming because it is laborious and expensive (Gibbs *et al.*, 1979a).

1.10.4.2. Hemagglutination Assay (HA)

For PPRV diagnosis, HA is an effective, low-cost, simple, and quick test that does not require specialized laboratory equipment. (Wosu, 1985) was the first to demonstrate PPRV's hemagglutination activity in 1985. PPRV can cause agglutination of RBCs in chicken, pig, goat, and even human blood (Group O). The HA test is particularly useful for diagnosing PPR in live animals. Agglutination time is calculated in this test, which is the time it takes RBCs to settle down in wells, whereas there is no agglutination in wells with positive samples. However, because elution can begin after agglutination in some situations, HA test results should be obtained within a specific time frame, such as one hour in the case of PPRV. However, this test is not particularly sensitive, and some studies have found that using the HA test might result in false positive findings (Ezeibe *et al.*, 2004).

1.10.4.3. Agar gel immunodiffusion (AGID)

AGID is a low-cost, simplistic test that may be performed in both the lab and in the field. This test is not just for detecting antibodies, but also for detecting antigens (Obi and Patrick, 1984). This test has a number of flaws, including the inability to distinguish PPRV from RP, despite its 92 percent specificity, and the inability to identify mild PPR due to its low sensitivity (Obi and Patrick, 1984; Abraham *et al.*, 2005).

1.10.4.4. Counter immunoelectrophoresis (CIEP)

By identifying both antibodies and antigen, CIEP is a rapid and precise test for PPR diagnosis. When AGID findings were compared to CIE results, it was evident that CIE (80.3%) was more effective and sensitive for PPRV detection than AGID (42.6%). It is simply performed by utilizing an electrophoresis bath connected to a high-voltage source (Obi and Patrick, 1984).

1.10.4.5. Immuno-capture enzyme-linked immunosorbent assay (icELISA)

This test was initially developed in the CIRAD-EMVT (World Reference Laboratory in France) and has since gained widespread recognition for detecting

PPRV (antigen). PPRV antigen is captured using a biotinylated monoclonal antibody (N) for PPRV epitope in this assay.

Antibodies against PPRV have also been identified using MAPs (multiple antigenic peptides). This test uses MAPs that are highly specific for PPRV polypeptides, resulting in a high titer in ELISA. The use of two MAbs against N protein in an icELISA allows for faster detection of PPRV (Diallo *et al.*, 1995).

1.10.5. Antibodies detection methods

1.10.5.1. Hemagglutination Inhibition (HI)

This test may be used to quantify antibodies against PPRV that are present in serum samples. In a microtitre plate, serum samples are diluted twofold. The antibody dilution required for agglutination inhibition can be used to determine the serum sample titer. Titration of PPRV antigen can also be accomplished using HAI and HA assays (Wosu, 1985).

1.10.5.2. Competitive ELISA (c-ELISA)

Competitive ELISA was devised, in which MAbs for the PPRV H protein were utilized to differentiate PPRV from RPV (Anderson *et al.*, 1991). The competition between monoclonal antibodies and antibodies in the test serum is the basis for this test. PPRV was identified using a c-ELISA based on specific MAbs against H protein (Anderson *et al.*, 1991; Saliki *et al.*, 1993; Singh *et al.*, 2004a) and N protein (Diallo et al. 1995). When compared to VNT, this test demonstrated to be more sensitive and specific (Singh *et al.*, 2000).

1.10.5.3. Indirect ELISA (i-ELISA)

In comparison to competitive ELISA, an i-ELISA for the detection of PPR antibodies was developed (Balamurugan *et al.*, 2007). i-ELISA had a relative diagnostic specificity of 95.09 percent and a diagnostic sensitivity of 90.81 percent when compared to competitive ELISA(Singh *et al.*, 2004c). Despite the need for a species-specific conjugated secondary antibody, this i-ELISA could be utilized if the

MAb clone used in the competitive ELISA is lost owing to unavoidable circumstances or in laboratories where the competitive ELISA is not readily available.

1.10.6. Molecular diagnostic techniques

1.10.6.1. RT-PCR

Because of its ability to detect minute amounts of DNA or RNA even in samples with low viral titer, it is regarded an effective and sensitive technique. Compared to the ELISA method, this test has a higher sensitivity (Forsyth and Barrett, 1995). It is very suitable for PPRV detection due to its rapidity and specificity (Nanda et al. 1996). RT-PCR based on two PPRV proteins, Phospho (P) and Fusion (F), was primarily used to identify and differentiate PPRV from RPV (F). For PPR diagnosis, N (Nucleoprotein) based RT-PCR has now been designed. The sensitivity of RT-PCR is 1000 times greater than that of virus isolation (Couacy-Hymann *et al.*, 2002). PPRV can be detected through RT-PCR using ocular samples even before symptoms appears. PCR products can also be used in nucleic acid sequencing to establish a link between isolates and geographic locations (Shaila *et al.*, 1996). Different types of PCR, such as multiplex and qRT-PCR, (Bao *et al.*, 2011; Batten *et al.*, 2011; Kwiatek *et al.*, 2011), have also been shown to be more efficient for PPRV identification than traditional PCR. When compared to real-time PCR, LAMP appeared to be a viable technique for PPRV diagnosis with similar sensitivity (Khalafalla *et al.*, 2010).

1.10.6.2 Real-Time Quantitative RT-PCR (qRT-PCR)

The gold standard test for PPRV detection is RT-PCR, however it is a time-consuming, labor-intensive technique. That is why several researchers have devised quantitative RT-PCR, which, despite its high cost, provides rapid and quantitative results. The quantitative RT-PCR/Real-time RT-PCR test is more sensitive and specific than the conventional RT-PCR technique. It is a better test than others because it provides a quick results, lowers the risk of contamination, takes less time, and is more specific (Agüero *et al.*, 2007; Shaw *et al.*, 2007). The number of steps in real time RT-PCR lowers to achieve a final conclusion; results are shown on screen as

amplification occurs; there is no need to load on an agarose gel or perform further steps as in conventional RT-PCR (Bao *et al.*, 2008)

(Bao *et al.*, 2008) developed a one-step real-time quantitative reverse transcription PCR (qRT-PCR) assay based on TaqMan for the detection of PPRV. this assay produced rapid and more specific results. For detection of PPRV, he applied N-gene sequence-based primers and probe. This assay could detect virtually all PPRV lineages. This assay exhibited greater specificity and sensitivity than traditional RT-PCR (Bao et al., 2008). Similarly, Batten et colleagues. conducted a research in 2011 for fast detection of PPRV using qRT-PCR (Batten et al. 2011 (Batten *et al.*, 2011).

(Balamurugan *et al.*, 2012) improves SYBR green qRT-PCR technique for PPR detection in 2011 utilizing M-Gene sequence. In comparison to (Bao *et al.*, 2008; Batten *et al.*, 2011) probe-based assay, this assay is faster, more sensitive, and less costly. He also compared it to TaqMan RT-PCR and conventional RT-PCR. This assay, he claims, is more sensitive than TaqMan RT-PCR and traditional RT-PCR. For early detection of PPRV, this is a suitable alternative to TaqMan qRT-PCR and conventional RT-PCR (Balamurugan *et al.*, 2012). Later, (Abera *et al.*, 2014) developed a SYBR green based RT-PCR for the detection of PPRV utilizing the N-gene sequence. N-gene was utilized in this assay since it is the highest expressed gene in PPRV. This was a two-step assay, but it was more sensitive, specific, and quick in detecting PPRV (Abera and Thangavelu, 2014).

1.10.6.3 Nucleic acid hybridization

This technique was also shown to be effective for diagnosing PPRV from field samples. The N gene of PPRV is being used to deploy cDNA probes (radiolabeled)(Banyard *et al.*, 2006; Muthuchelvan *et al.*, 2006). This test has the advantage of being able to distinguish between PPRV and RP without the need of a viral isolation method. cDNA probes that target F, M and P gene are not very specific. Furthermore, because to their short half-life, probes cannot be utilized widely, and the sensitivity of this assay is lower than that of PCR (Muthuchelvan *et al.*, 2006).

1.10.6.4 Pennside tests

As a Penside test for PPRV identification, a dot-ELISA test using MAbs for N (Saravanan *et al.*, 2006) and M (Obi and Patrick, 1984) might be utilized. It allows for the screening of a larger number of samples suspected of containing PPRV and may be used both in the field and in the lab. It has a higher specificity and sensitivity than s-ELISA (Singh *et al.*, 2009). The lateral flow test can also be used to diagnose PPR, although it has not been done routinely until recently. However, because it is quick, simple, and straightforward, it may be utilized as a penside test for diagnosis. Dipsticks by utilizing MAb for M protein have also been developed. In addition, a c-ELISA test for rapid PPRV diagnosis was created (Choi *et al.*, 2005). Immunofiltration ELISA has a high specificity and sensitivity, making it suitable for use as a PPRV screening test (Raj *et al.*, 2008).

Polyclonal antibodies against M protein (recombinant) have now been developed, with higher specificity in immunofluorescence and western blot. As a result, they are considerably better and safer, and they can be used for PPRV surveillance and diagnosis in both non-enzootic and enzootic regions around the world (Robinson and Knight-Jones, 2014).

1.11. Phylogenetic Analysis

In most cases, amplified DNA may be easily studied using sequencing technologies, and the newly generated sequence data can then be compared to previously collected sequence data in order to construct a "phylogenetic tree" dendrogram (Haas and Barrett, 1996). A phylogenetic tree for various morbillivirus species was created using this method. Morbillivirus phylogenetic relationships were determined using a universal primer pair based on conserved sequences of the respective P genes. For this sort of study, (Haas and Barrett, 1996) found that identical findings were achieved utilizing sequencing data from either N or P genes.

1.12. Prevention and Control

1.12.1. Vaccination

Because PPR is a viral infection, it cannot be cured. However, while a small number of antibiotics are suggested to make animals immune to subsequent pulmonary infections, this type of management is prohibitively expensive in the event of an outbreak. As a result, the disease can be managed by implementing cleanliness and prophylactic measures; nevertheless, rigorous sanitary measures are clearly impractical in less developed nations. Vaccination is the only method to prevent the disease. Rinderpest Virus cell culture attenuated vaccine gave effective PPR protection. This vaccination has no negative effects on the health of goats of all breeds, and it has a protective titer that lasts for over a year (Nawathe and Taylor, 1979).

To avert large epidemics, an efficient vaccine and appropriate vaccination administration are required (Talley and Salama 2003). The following qualities must be present in an effective vaccine:

1. It must stimulate a sufficient immune response,
2. It must be efficient throughout storage, and
3. It must have enough immunogenicity.

Multiple passages of the PPRV strain resulted in favorable attenuation (Diallo *et al.*, 1989). In field conditions, the homologous strain of vaccine used against PPR was proven to be safe for pregnant animals and to generate a sufficient immunological response in 98% of vaccinated animals. A single dosage of vaccination provides protection for at least three years, which is an animal's usual productive life. In general, after 63 passages in the Vero cell line, PPR vaccination elicited a robust immune response that lasted at least 3 years (Diallo *et al.*, 1995).

The vaccinations have no effect on the physiological processes that occur in sheep and goats during pregnancy. Furthermore, this vaccination stimulates the production of colostral anti-PPR antibodies, which are detectable even in three-month-old children (Awa *et al.*, 2002). It is suggested that kid and lambs born to

vaccinated or exposed mothers be vaccinated at the ages of 4 and 5 months, respectively (Awa *et al.*, 2002). Similarly, antibodies generated because of PPR infection may impair vaccination efficacy. As a result, before mass vaccination against PPR, the status of titer against PPR infection should be evaluated, particularly in enzootic areas (Banik *et al.*, 2008).

Aims & Objectives of study:

- Evaluation of indirect ELISA (i-ELISA) for specificity & sensitivity check.
- Determination of cost effectiveness of In-house developed indirect ELISA (i-ELISA)
- Evaluation of field samples /surveillance studies using both ELISA kits

CHAPTER 2

MATERIAL & METHODS

2. MATERIAL AND METHODS

2.1 Study design

The study was designed to compare the diagnostic performance of in-house build i-ELISA at Animal Biotechnology Program NARC, Islamabad Pakistan with commercially available c-ELISA kit (ID Screen® PPR Competition, Montpellier, France) for the detection of PPRV antibodies. Briefly, sera samples of sheep and Goat collected during active outbreaks reported in 2019-2020 from Islamabad, Gilgit, Fateh jhang, Attcok, Jaffarbad, and Quetta, Pakistan (Fig. 2.1). Initial validation of the two assays was carried out using known reference sera. Virus neutralization test (VNT) was then used to validate the findings of the two tests. All the experiments were carried out in replicates.

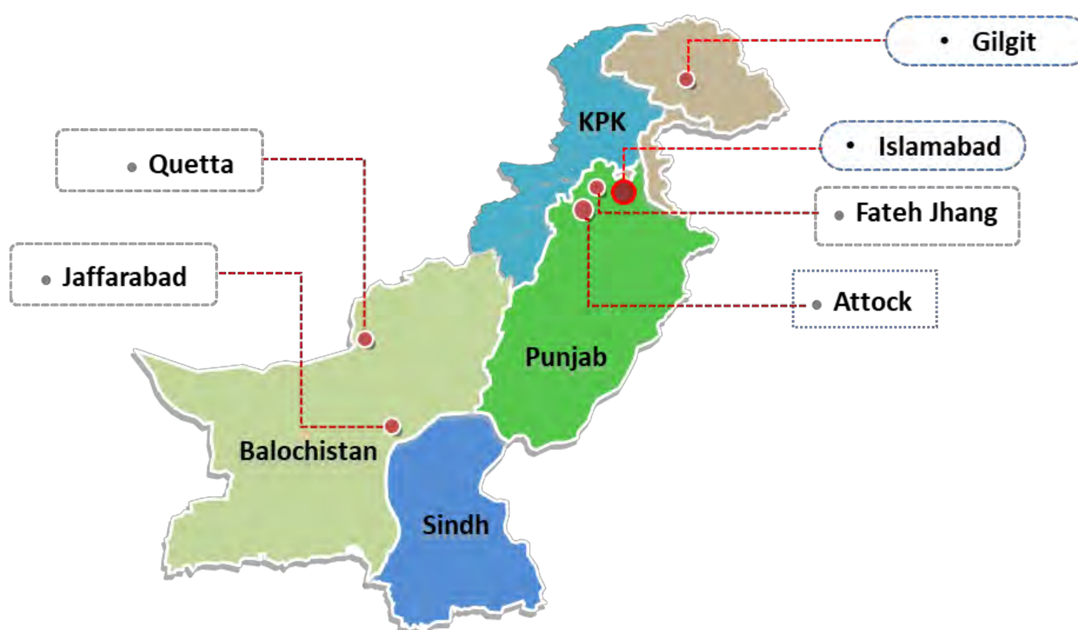
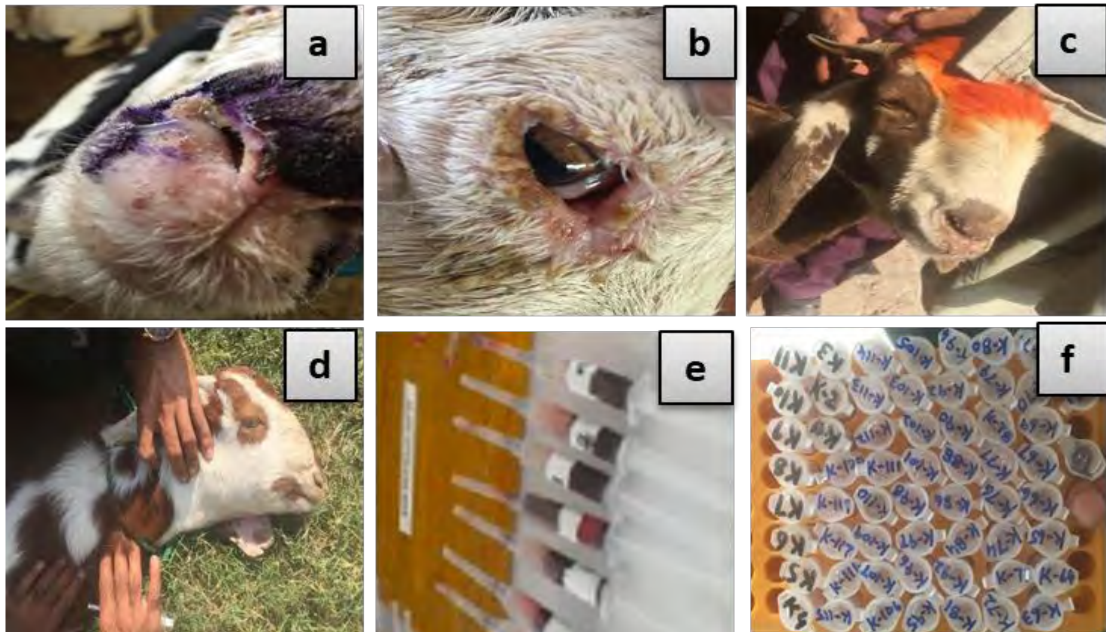


Fig. 2.1: Map of Pakistan showing sites of samples collection

2.2 Collection, transportation, and processing of samples

Infected sheep and goats were used to collect clinical samples including nasal swabs and whole blood for testing. Simple vacutainer tubes were used to collect roughly 5 mL of blood from each animal's jugular vein, which was then analyzed. Serum of blood samples was separated by keeping it overnight in tilted position. The

serum was then taken-off, aliquoted in 1.8 mL cryo-vials and transported to ABP, NARC, Islamabad by keeping it in an icebox with icepacks for further processing and laboratory examinations (Fig. 2.2). Samples were initially kept at -20°C until laboratory testing. Spleen, lymph nodes, gut, kidney, and lungs tissue samples were also taken from dead animals. All tissue samples kept at 20°C until needed.



F.2.2: Field sample collection animals showing clinical signs: (a) mucoid nasal discharge (b) lacrimation with peri-oral lesions (c) mucoid nasal and oral discharge. (d) blood collection (e) and (f) sample processing.

2.3 Initial validation of tests

The c-ELISA kit and i-ELISA were validated by using a positive and negative sera. Two-fold successive dilutions of anti-PPRV antibody positive and negative serum starting from 1:2 was tested to determine the highest detectable dilution of serum from both type of ELISA. According to manufacturer's guide, for c-ELISA kit, a cutoff value of 50 and 60 percent competition percentage was used whereas for i-ELISA cutoff value of 50% percent positivity was used.

2.4 Laboratory testing of field sera

2.4.1 PPR Viral Antibody Detection Using a Competitive ELISA

A c-ELISA kit used to collect serum samples from clinically sick and seemingly healthy goats demonstrated antibodies to the pestis des petits ruminant's virus nucleoprotein. (ID Screen PPR Competition, Montpellier, France. Appendix-I) Detection of PPRV antibodies with reference to competition percentage was carried out using this kit. The microplates were coated with purified recombinant PPR nucleoprotein (NP). The samples to be examined and the controls were added to the pre-coated plates after they had been diluted in the Dilution buffer included with the kit. At 37 °C, the plates were incubated for 45 minutes. After three washes with washing buffer, the anti-NP conjugate dissolved in Dilution buffer was added to the micro wells and incubated for 30 minutes at 21 °C. After washing the plates three times with washing buffer, the substrate (TMB) was poured. The reaction was halted after 15 minutes of incubation in the dark at 21 °C, and the data were read in an ELISA reader, with the OD measured at 450 nm. Samples with a competition percentage of less than or equal to 50 percent were considered positive for the presence of PPRV antibodies, while samples with a competition percentage of more than 50 percent and less than or equal to 60 percent were considered doubtful, and samples with a competition percentage of more than 60 percent were considered negative. (Appendix II.) Finally, the formula was used to compute the sensitivity and specificity of the c-ELISA. (Appendix III.)

2.4.2 Indirect ELISA (i-ELISA)

The i-ELISA assay was performed as reported by (Balamurugan *et al.*, 2007) with some modification at ABP, NARC, Pakistan. 96-well, flat bottomed, polystyrene microtiter plates (Nunc Maxisorp) were used using partly pure attenuated PPR virus (locally isolated) as coating antigen (in 100µl volume) diluted in in carbonate-bicarbonate buffer (pH 9.6. Appendix IV). For each phase, the plate was incubated at 4°C for 1 hour in humid chamber. After incubation, the wells were washed three times with washing buffer PBS-T (0.002 mol/L PBS with 0.05 percent Tween 20 Appendix V), to remove unbound antigen, and then blocked with 100µL of blocking solution [PBS-T containing 5 percent of Bovine serum albumen (BSA) (Appendix VI)]. After incubation for 2 hours at 37°C and washing of the plate, 100µL of serum

to be tested (including positive antiserum with VNT titer ≥ 1:164 and negative serum obtained from uninfected goat) diluted in dilution buffer (PBS-T containing 5 percent of Bovine serum albumen (BSA) was added and incubated for 2 hours at 37°C followed by washing. The anti-goat–HRPO conjugate [(horseradish peroxidase) (Abcam, Cambridge, UK)], pre-diluted in blocking buffer (1:1000) was then added (100 l/well) and plate incubated at 37°C for 1 hour. The colour reaction was developed for 15 minutes by adding substrate to each well. Then reaction was stopped with 1 mol/L H_2SO_4 (Appendix VII) and absorbance was measured using an automated plate reader at a wavelength of 492 nm. The results were expressed as PP (percent positivity) ratio which were calculated as given below.

Negative control (NCx) = Mean of Negative Control

Positive control (PCx) = Mean of Positive Control

$$\frac{S}{P} ratio = \frac{Sample\ OD - NCx}{2}$$

$$PP\ value = \frac{Sample\ OD\ x\ 100}{Mean\ OD\ of + ive\ Control (PCx)}$$

Where OD is the optical density. PP values greater than 50% were considered positive

2.4.3 Virus Neutralization Test

For the detection of antibodies to PPRV using serum samples, a gold standard virus neutralization assays was carried out as reported by (Bandyopadhyay *et al.*, 1999) in vero cells. Briefly The test sera were thawed and kept in a water bath at 56 degrees Celsius for 30 minutes to deactivate. The serum samples to be examined were diluted one-to-five and serially diluted two-fold up 1:128 concentration with minimum essential media (100µL/well). After that, 100 micro-liters of PPRV vaccine strain (PPRV Nigeria 75/1) was added into all wells at a known concentration (100TCID50/ml) upto 8th well from left to right. Separately, a control plate with both negative and positive controls was also prepared. After one hour of incubation at 37°C, 50 L of Vero dog SLAM cell solution (4x105 cells per ml) was evenly distributed across all wells. The plates were placed in a 37°C incubator with 5% CO2. Finally, CPE effect, starting on day 3 of incubation, was monitored by observing the

plates using an inverted type of microscope. When the serum was negative, CPE was seen; however, when the serum was containing antibodies against PPRV, no CPE was seen. For neutralizing dilution ≥ 1:10, the serum was considered positive for antibodies against PPRV.

2.5 Data Analysis

Serological test data was categorized, filtered, coded, and then entered into Microsoft® Excel® for Microsoft 356 MSO (version 2110). The statistical package GraphPad Prism 5.01 was used to analyze the data. Two-way contingency table and Cohen's kappa statistics were used to determine the degree of agreement between the tests.

The statistical formula of (Samad *et al.*, 1994)was used to perform statistical analysis for the comparison of specificity and sensitivity of both diagnostic assays. The following (Table 2.1) is a description of the statistical formula that was employed.

Table 2.1: Comparison of experimental assay with gold standard assay

	Gold standard assay		**Total**
Diagnostic Assay to be tested	**Positive**	**Negative**	
Positive	a (TP)	b (FN)	a + b
Negative	c (FP)	d (TN)	c + d
Total	a + c	b + d	a + b + c + d

a =Number of samples that passed both diagnostic assays (True Positive).

b = The number of samples that were positive in the conventional test but not in the gold standard test (False Negative).

c = Number of samples that failed conventional testing but passed the gold standard test (False Positive).

d = The number of samples that were found to be negative in both diagnostic assays (True Positive).

a+**b**+**c**+**d** = Total samples (N)

The diagnostic sensitivity of the results obtained with c-ELISA kit and i-ELISA was calculated using a standard formula.

The sensitivity of assays was estimated as under;

$$\text{Sensitivity} = \frac{\text{TP}}{\text{TP+FN}} \times 100$$

Where TP stands for True Positive, and FN stands for False Positive.

The specificity of assays was estimated as under

$$\text{Specificity} = \frac{\text{TN}}{\text{TN+FP}} \times 100$$

Where, TN stands for True Negative, and FP stands for False Positive (Munro, 2005).

Kappa value (κ)≤ 0 indicates no agreement, 0.01-0.20 indicates none to minor agreement, 0.21-0.40 indicates fair, 0.41-0.60 indicates moderate, 0.61-0.80 indicates significant, and 0.81-1.00 indicates practically perfect agreement (Viera and Garrett, 2005). (Viera and Garrett, 2005).

CHAPTER 3

RESULTS

3. RESULTS

3.1 Collection of Sample

Blood samples (n=694) from sheep and Goat collected during active outbreaks reported in 2019-2020 from Islamabad, Gilgit, Fateh jhang, Attcok, Jaffarbad, and Quetta, Pakistan (Fig. 2.1). Number and percentage of samples received from each district is shown in Table 3.1 and Fig 3.2.

Table: 3.1 Collection of sample from different areas

Name of City	No. of Samples		
	Goat	Sheep	Total
Islamabad	98	74	172
Gilgit	39	26	65
Fateh Jhang	51	42	93
Attock	73	52	125
Jaffarabad	83	47	130
Quetta	44	65	109
Total	388	306	694

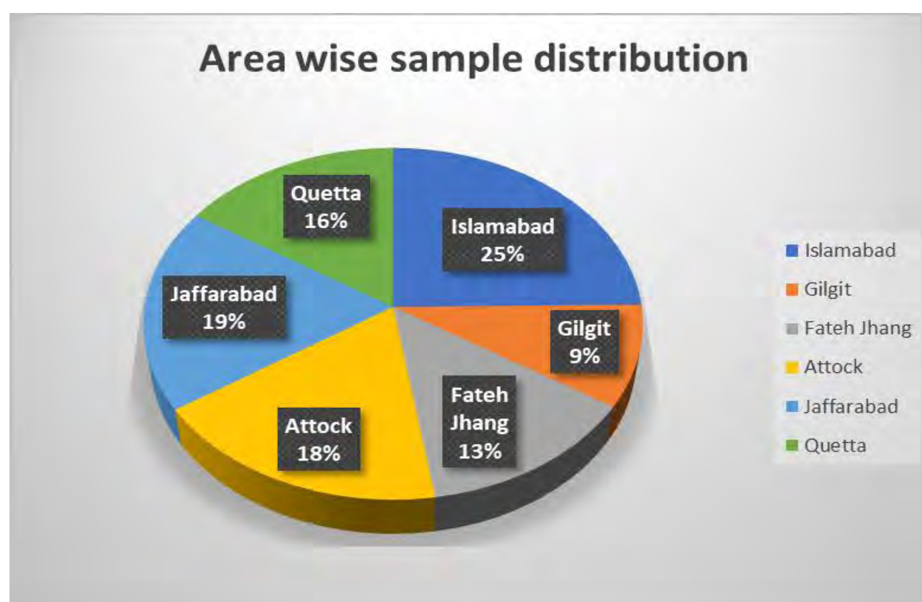


Fig. 3.1 Diagrammatic representation of area wise distribution of collected samples

3.2 Initial validation of tests using known references sera

To validate the testing methods, serial dilutions of two known positive sera and two known negative sera were examined using the standard protocol of the i-ELISA and c-ELISA kits. For i-ELISA, the maximum dilution for specific detection of antibodies against PPRV was calculated using a cutoff value of 50% Percent Positivity (PP) Value (Fig 3.2). Similarly, the maximum dilution for detecting anti-PPRV antibodies using a c-ELISA kit was 1:32 for both positive sera using a 50% and 60% competition percentage threshold value. For dilutions of negative sera, no positive competitive reaction was detected (Figure 3.3).

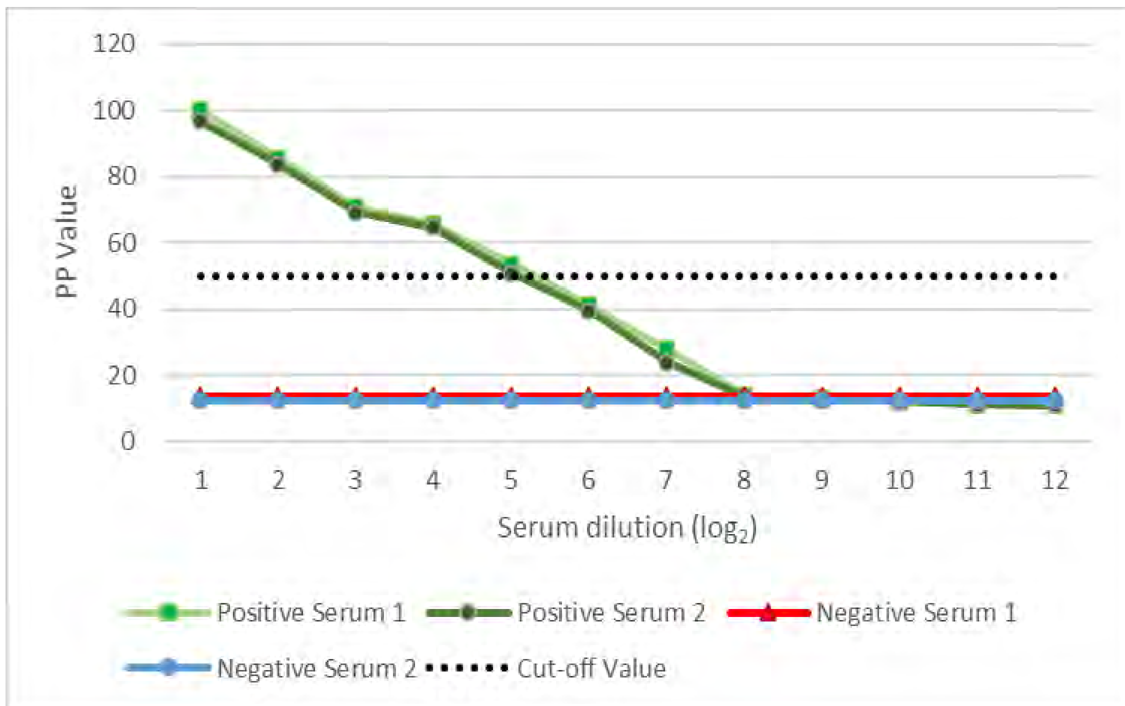


Figure: 3.2 The analytical sensitivity of indirect ELISA (i-ELISA) in detection of PPRV antibodies

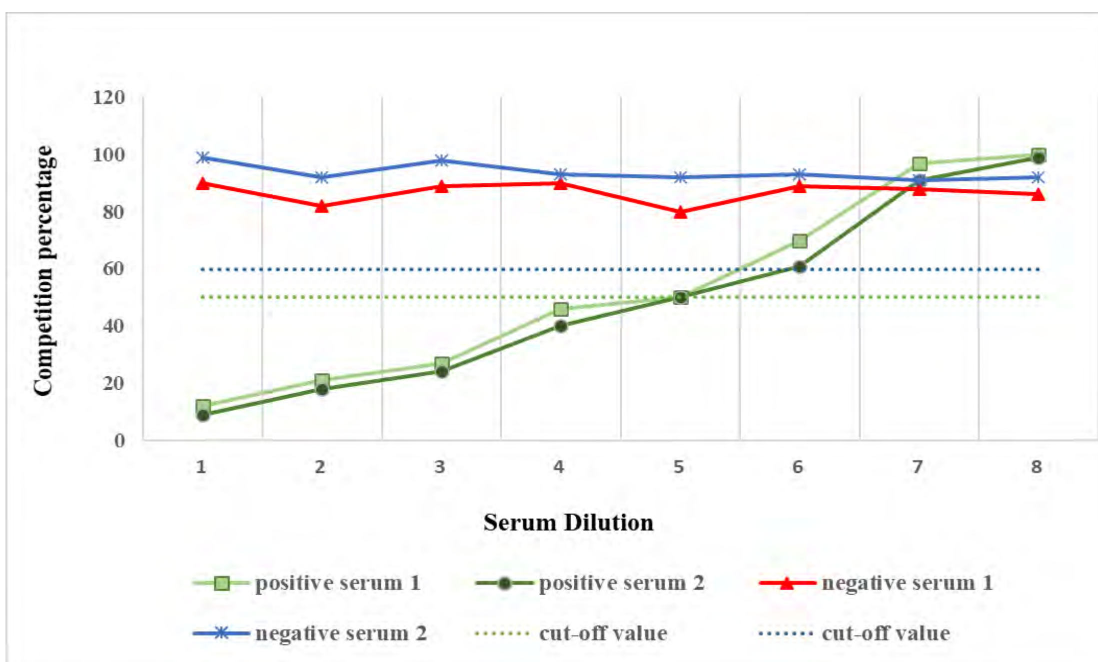


Figure. 3.3. The analytical sensitivity of ID Screen® PPR c-ELISA in detection of PPRV antibodies

3.3 Evaluation of field samples with Inhouse build i-ELISA and c-ELISA tests in ruminants

A total 694 field samples from sheep and Goat collected, processed, and analyzed Inhouse build i-ELISA (Fig 3.4) and c-ELISA kit (Fig 3.5). Equal Sample number and sequence was run parallelly in each plate to ensure results of both kits were compared smoothly and accurately.

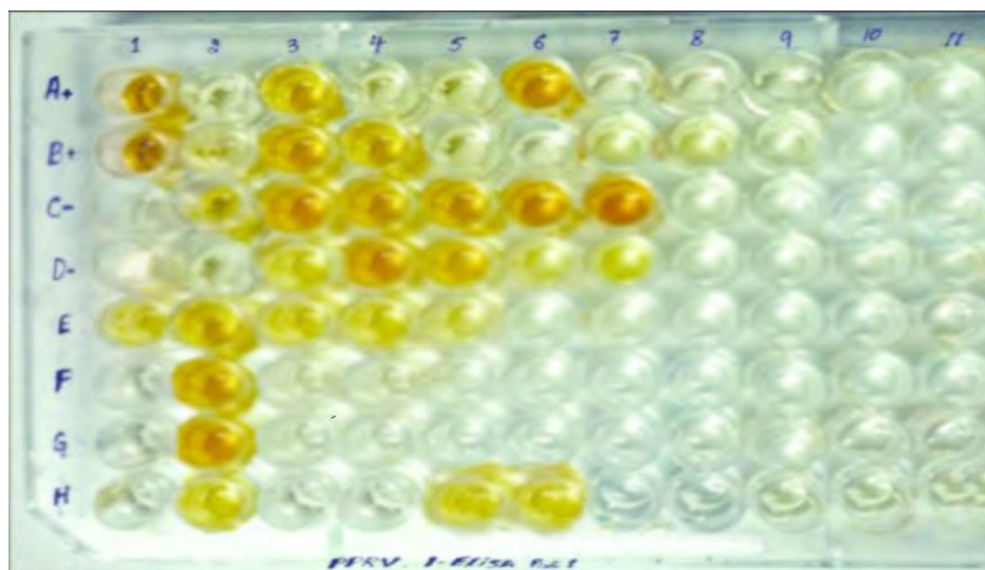


Fig. 3.4 Analysis of field samples with Indirect ELISA(i-ELISA)

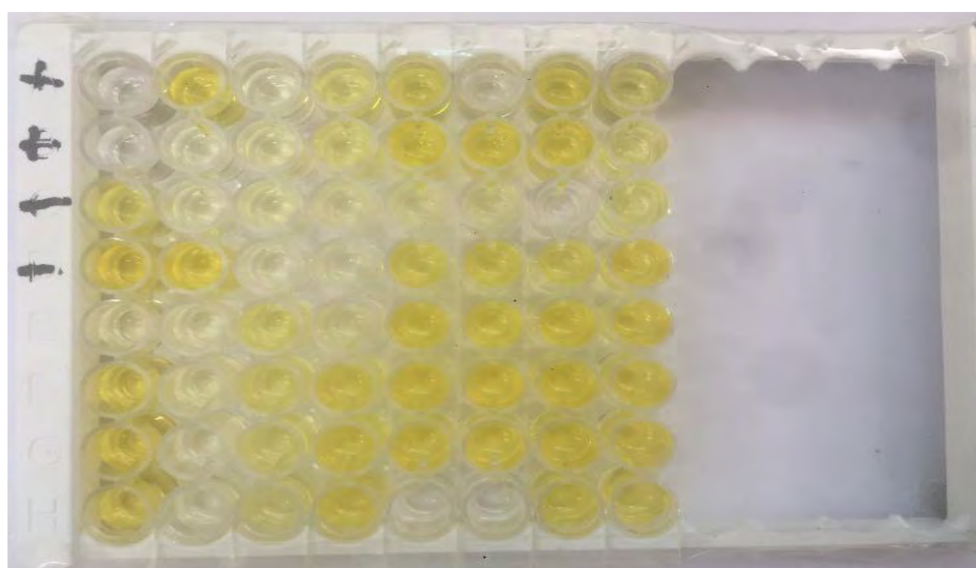


Fig. 3.5 Analysis of field samples with c-ELISA kit

3.4. Comparison of diagnostic performance of Inhouse build i-ELISA and c-ELISA tests in ruminants

Anti-PPRV antibodies were detected in 42.07 percent of the 694 serum samples tested, whereas anti-PPRV antibodies were detected in 43.95 percent of the samples tested. Sensitivity and specificity of i-ELISA and c-ELISA for PPRV antibody detection are shown in Table 3.2.

Table 3.2: Comparative diagnostic performance of i-ELISA and c-ELISA in small ruminants

Inhouse build indirect ELISA (i-ELISA)	ID Screen® PPR c-ELISA		
	Positive	**Negative**	**Total**
Positive	274	18	292
Negative	31	371	402
Total	305	389	694

Kappa= 0.856, SE of kappa = 0.020 (95% CI = 0.817 -0.895) P value: < 0.0001
Specificity of i-ELISA: (371/371+18) x 100 = 95.37 (95% CI= 0.9279-0.9723)
Sensitivity of i-ELISA: (274/274+31) x 100 = 89.84

3.5. Comparison of diagnostic performance of Inhouse build i-ELISA and c-ELISA kit tests in Goats

Anti-PPRV antibodies were detected in 173 (44.59%) of 388 goat serum samples tested with i-ELISA and 166 (42.78%) of 388 samples tested with c-ELISA. Table 3.3 compares sensitivity and specificity of i-ELISA and c-ELISA kits for the detection of PPRV antibodies.

Table 3.3: Comparative diagnostic performance of i-ELISA and c-ELISA in Goats

Inhouse build indirect ELISA (i-ELISA)	ID Screen® PPR c-ELISA		
	Positive	**Negative**	**Total**
Positive	154	12	166
Negative	19	203	222
Total	173	215	388

Kappa= 0.842 SE of kappa = 0.028 (95% CI = 0.742 to 0.942) P value: < 0.0001
Specificity of i-ELISA: (203/203+12) x 100 = 94.42 (95% CI= 0.9045-0.9708)
Sensitivity of i-ELISA: (154/154+19) x 100 = 89.02 (95% CI= 0.8338-0.9326)

3.6. Comparison of diagnostic performance of Inhouse build i-ELISA and c-ELISA kit in Sheep

Out of 306 serum samples from sheep, 126 (41.18%) were found to be positive for PPRV using i-ELISA whereas 132 (43.13%) samples were found positive while using c-ELISA. The data of comparison of i-ELISA and c-ELISA for sensitivity and specificity has also shown in table 3.4.

Table 3.4: Comparative diagnostic performance of i-ELISA **and c-ELISA in sheep.**

Inhouse build indirect ELISA (i-ELISA)	ID Screen® PPR c-ELISA		
	Positive	**Negative**	**Total**
Positive	120	6	126
Negative	12	168	180
Total	132	174	306

Kappa= 0.886 SE of kappa = 0.020 (95% CI = 0.7729-0.9985) P value: < 0.0001
Specificity of i-ELISA: (168/168+6) x 100 = 96.55 (95% CI= 0.9265-0.9872)
Sensitivity of i-ELISA: (120/120+12) x 100 = 90.91(95% CI= 0.8466-0.9521)

3.7. Validation of Inhouse build i-ELISA with VNT in small ruminants

i-ELISA used in this study also validated with VNT he most reliable test for detection of morbillivirus antibodies. Relative specificity and sensitivity of the two assays was compared for the detection of PPRV antibodies as under in Table 3.5.

Table 3.5: Validation of diagnostic sensitivity and specificity of inhouse build i-ELISA with VNT in small ruminants.

Inhouse build indirect ELISA (i-ELISA)	Virus Neutralization Test (VNT)		
	Positive	**Negative**	**Total**
Positive	292	0	292
Negative	65	337	402
Total	357	337	694

Kappa= 0.814 SE of kappa = 0.022 (95% = 0.771-0.856) P value: < 0.0001
Specificity of i-ELISA: (337/337+0) x 100 = 100 (95% CI= 0.9891-1.000)
Sensitivity of i-ELISA: (292/292+65) x 100 = 81.79 (95% CI= 0.7739-0.8566)

3.8 Evaluation of field sample of PPRV with i-ELISA

Out of 694 samples tested with i-ELISA, 292 (42%) samples were tested positive for *PPRV,* and 402 (58%) samples found negative. Seroprevalence in sheep and goat were found 40% and 43% respectively. A total of 41% samples from Islamabad Capital Territory, 45% sample from Gilgit, 40% samples from Fateh Jhang, 49% samples from Attock, 35% samples from Jaffarabad, and 46% samples from Quetta were tested positive for *PPRV*. Percentage of positive samples from sheep and goat from different areas of Pakistan is as shown in fig 3.6.

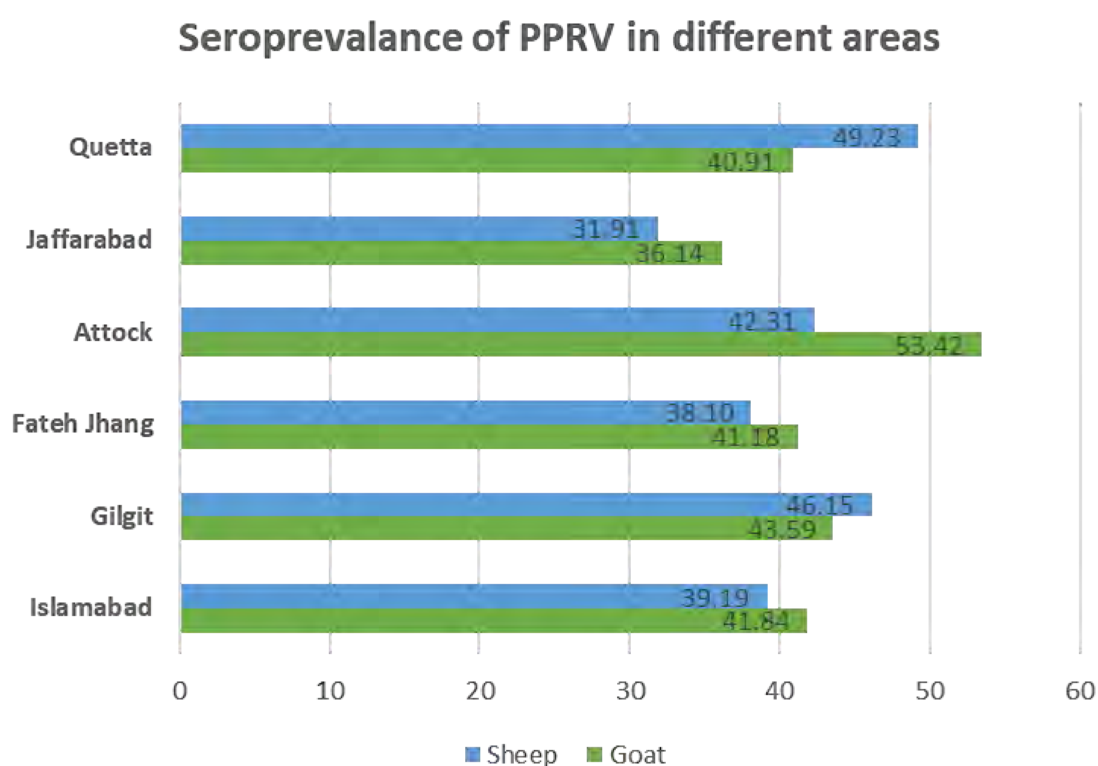


Fig 3.6. Graphical representation of Seroprevalence of PPRV in samples obtained from different areas of Pakistan

3.9 Cost evaluation of inhouse build Indirect ELISA (i-ELISA)

Table 3.6 shows the costs of the components needed for i-ELISA. The cost of 92 samples evaluated using a 96-well ELISA plate as a reference for comparison. The prices listed here are based on current exchange rates in Pakistani rupees and are subject to change. Analysis of 92 samples was estimated to cost 36 US dollars. When compared to commercially available kits, which typically cost between $400 and $500.

Table 3.6: Cost evaluation of Indirect ELISA (i-ELISA)

Material	Unit	Unit Price (US. $)	Quantity required per plate (Unit)	Cost per plate (US. $)
ELISA plate	No.	2.2	1	2.2
Coating antigen	uL	4.1	5	20.3
Coating buffer	ml	1.3	1	1.3
Washing buffer	ml	0.2	39	8.5
Blocking buffer	ml	0.3	9.6	2.4
Dilution buffer	ml	0.1	9.6	1.2
Conjugate	ml	313	0.001	0.3
Substrate	g	5.0	0.01	0.1
Stop solution	ml	0.03	9.6	0.2
Total				**36**

CHAPTER 4
DISCUSSION

4. DISCUSSION

Peste des petits ruminants (PPR) is a highly contagious viral disease of sheep and goats. PPRV has morbidity rate ranges from 80-90% and a mortality rate of 50% which may reach up to 80% in small ruminants (Abubakar *et al.*, 2008). PPRV can affect entire populations of immunologically immature hosts. It may lead to the epidemics that affect the economy of any country to a certain extent. In Pakistan during the last decade, PPR outbreaks have increased to an alarming level (Ali, 2004). It is estimated that only in Pakistan it causes economic losses of worth Rs. 20.5 billion (US$ 0.24 billion) annually (Abubakar *et al.*, 2015).

PPR is regarded as an Office International des Epizooties (OIE) list A disease because of its impact on the livestock farming community and related economics. The OIE and FAO are trying their best to eradicate the PPR as Rinderpest (RP) was eradicated from the globe. For the effective eradication operation, accurate diagnostics is the first step to determine the scope and variability of a disease among susceptible populations (Banyard *et al.*, 2010). Rapid diagnosis of PPR infection necessitates the development of inexpensive and alternative diagnostic methods. To ensure that diagnostic tests employed by field laboratories achieve a minimum diagnostic performance requirement, strict standards are required (Wright, 1998).

Various serological and molecular assays have been developed and being applied for the investigation of PPR disease. These include AGD, VNT, ELISA (s-ELISA, c-ELISA, Indirect ELISA etc.) and PCR (RT-PCR, qPCR & RT-LAMP) etc. These assays vary in their specificity, sensitivity, reliability, and reproducibility. Although these assays precisely detect PPRV but in developing countries like Pakistan, there is a need for the development of cost-effective diagnostic assays which are also applicable in field conditions. The aim of this study was to compare inhouse developed i-ELISA with commercially available c-ELISA kit, which could be a good alternative to c-ELISA for the detection of antibodies against PPRV and can successfully be applied for PPR sero-epidemiological surveys.

In the current study, the validation of i-ELISA and c-ELISA was confirmed using known reference sera prior to analysis of field samples, which showed that with the decrease of serum antibodies, the OD values decreases as well. So, a positive

serum gave higher titers till 1:32 dilutions (Fig. 3.2) while in a negative serum sample even in its purest form, titers were below the cutoff value (50%). In this case, antibodies against the PPR virus in the test sample are specific for binding. Similarly, the maximum serum dilution for detecting anti-PPRV antibodies using a c-ELISA was 1:32 for both positive sera using a 50% and 60% competition percentage threshold value. For dilutions of negative sera, no positive competitive reaction was detected.

The relative sensitivity and specificity of i-ELISA was evaluated using two-sided contingency table method. Similarly, results were also compared with c-ELISA and VNT in terms of sensitivity and specificity. Out of 388 goat serum samples tested, 166 samples were found to be positive using the i-ELISA. This method compared very well with c-ELISA, with a high degree of specificity 94.42% (95% CI= 0.9045-0.9708) and sensitivity 89.02% (95% CI= 0.8338-0.9326) shown in Table 3.3. In a similar fashion, out of 306 sheep serum samples tested, 122 samples were found positive, demonstrating high specificity 96.55% (95% CI= 0.9265-0.9872) and sensitivity 90.91% (95% CI= 0.8466-0.9521) when compared to the c-ELISA shown in Table 3.4. When compared to c-ELISA and VNT, the overall specificity and sensitivity of i-ELISA were 95.37% and 89.84%, and 100% and 81%, respectively (Tables 3.2 and 3.4). The results were in line with previously reported (Balamurugan *et al.*, 2007).

Before launching a control program for any infectious disease, it is necessary to thoroughly investigate detailed epidemiological characteristics of a disease through extensive clinical and serological surveillance. The test distinguishes clearly between the infected and uninfected populations and performs extremely well when compared to the c-ELISA. Seroprevalence in sheep and goat were found 40% and 43% respectively. A total of 41% samples from Islamabad Capital Territory, 45% sample from Gilgit, 40% samples from Fateh Jhang, 49% samples from Attock, 35% samples from Jaffarabad, and 46% samples from Quetta were tested positive for PPRV. Previous studies carried out in Pakistan estimated a seroprevalence of PPR more than 45% (Khan *et al.*, 2008; Abubakar *et al.*, 2009; Zahur *et al.*, 2011; Abubakar *et al.*, 2016; Abubakar *et al.*, 2017). Recent study of (Rasheed *et al.*, 2020) reported

seroprevalence of the PPR Virus in sheep and goats of Gilgit Baltistan 44% and 46% respectively.

The comparative cost estimation conducted in this study showed an inhouse build i-ELISA would cost approximately US$ 36 for 92 samples, as compared to commercially available kits that cost US$ 400-500 for same number of samples. The results of the current study has proven that the i-ELISA used in this work seems to be both cost-effective and as successful as the commercial cELISA in identifying PPRV antibodies as previously reported.

The results of the present study concluded that if i-ELISA commercially produced in a country, it may be a valuable tool for the detection of PPR virus at a low cost and with high reliability, like other ways of PPRV isolation and detection such as VNT, c-ELISA, and others.

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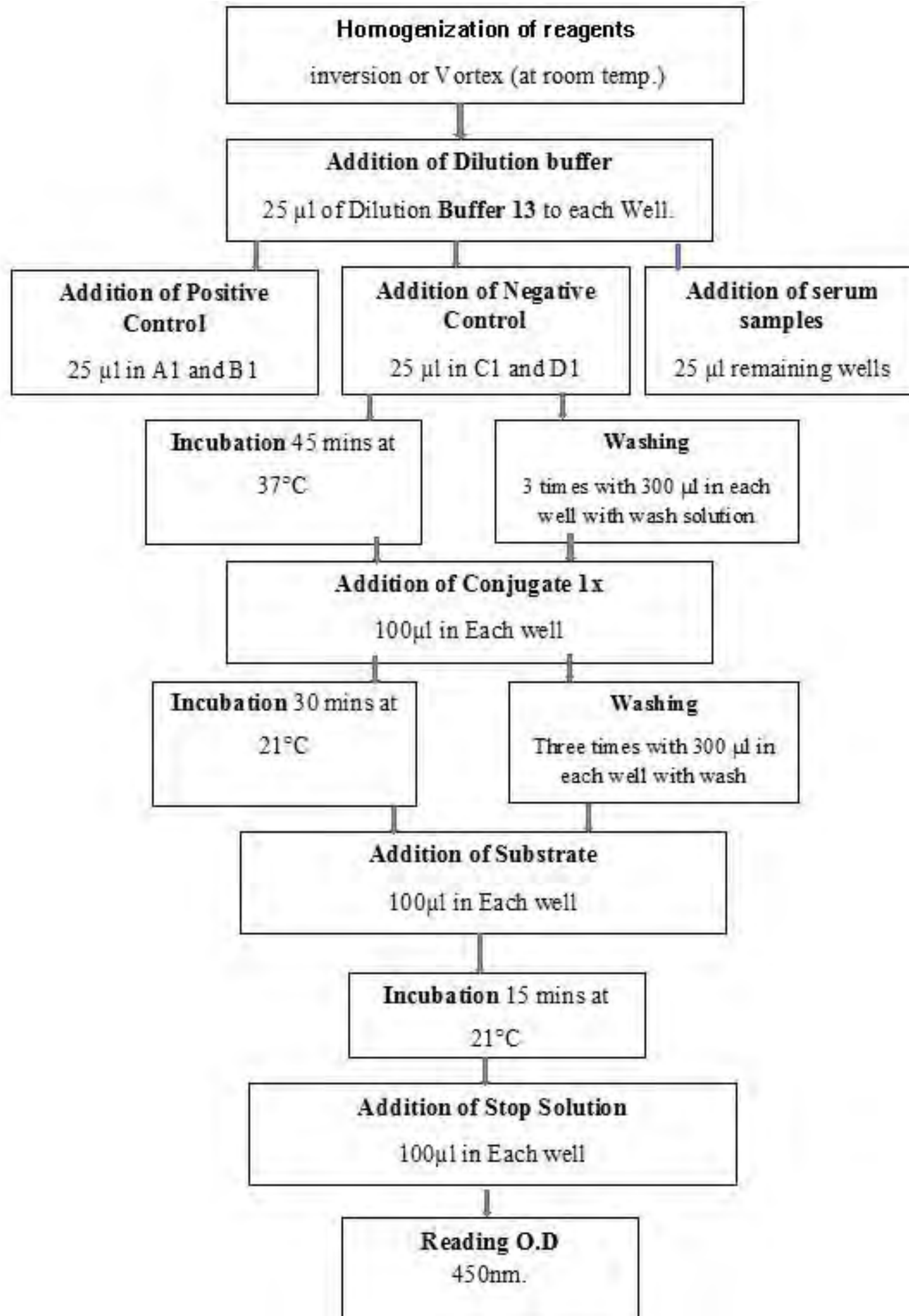
APPENDICES

APPENDICES

Appendix i: Protocol for ID screen PPR c-ELISA



Appendix ii. Validation of ID screen PPR c-ELISA kit

The test is validated if:

- The mean value of the negative Control O.D (OD_{NC}) is greater than 0.7

$$OD_{NC} > 0.700$$

- The mean value of the Positive Control O.D (OD_{PC}) is less than 30% of OD_{NC}.

$$OD_{PC}/OD_{NC} < 0.300$$

Appendix iii. Interpretation for ID screen PPR c-ELISA kit

- For each sample, calculate the competition percentage (S/N %).

$$S/N\ \% = \frac{OD_{sample}}{OD_{NC}} \times 100$$

Samples presenting a S/N %:

- Less than or equal to 50% are considered **POSITIVE**.
- Greater than 50% and less than or equal to 60% are considered **DOUBTFUL**.
- Greater than 60% are considered **NEGATIVE**.

Result	**Status**
$S/N\ \% \leq 50\%$	**Positive**
$50\% < S/N\ \% \leq 60\%$	**Doubtful**
$S/N\ \% > 60\%$	**Negative**

Appendix iv. Preparation of 50mM carbonate/bicarbonate buffer, pH 9.6

- Na2CO3 1.59g
- NaHCO3 2.93g
- Dissolve in 1liter deionized water
- Thimerosal 0.10g/liter (can be added as preservative if necessary).

Appendix v. Preparation of Phosphate buffered saline, pH 7.4, containing 0.05% Tween 20 (PBS-T)

- NaCl 8.00g
- KH2PO4 0.20g
- Na2HPO4 1.15g
- KCl 0.20g
- Thimerosal 0.10g (optional)
- Tween 20 0.5ml

Dissolve in deionized water and bring up to a final volume of 1 liter.

Appendix vi. Preparation of 5% BSA PBS-T

- To make 5% BSA PBS-T, add 5g bovine serum albumin (heat shocked fraction BSA, Sigma A-7030) to 100ml PBS-T. discard if 5% BSA PBS-T becomes cloudy.

Appendix vii. Preparation of 1M H_2SO_4

- To make 5% BSA PBS-T, add 5g bovine serum albumin (heat shocked fraction BSA, Sigma A-7030) to 100ml PBS-T. discard if 5% BSA PBS-T becomes cloudy.