# Development of a Thermostable Vaccine for Control of Peste des Petits Ruminants (PPR)


*By*

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**2020**

# Development of a Thermostable Vaccine for Control of Peste des Petits Ruminants (PPR)


A Thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

In

Microbiology

*By*

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# DEDICATION

*I dedicate this effort to my lovely parents specially my late father for her dream of my higher education, my siblings and to my loving husband and daughter*

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| | |
|---|---|
| AGID | Agar Gel Immunodiffusion |
| ATCC | American Type Cell Culture |
| cDNA | Complementary Deoxyribonucleic Acid |
| CDV | Canine Distemper Virus |
| CPE | Cytopathic Effect |
| cELISA | Competitive ELISA |
| DMV | Dolphin Morbillivirus |
| EDI | ELISA Data Interchange |
| EDTA | Ethylene Diamine Tetra Acetic Acid |
| ELISA | Enzyme Linked Immunosorbent Assay |
| FAO | Food and Agriculture Organization |
| FBS | Fetal Bovine Serum |
| F gene | Fusion Protein Gene |
| FMD | Foot and Mouth Disease |
| GKC | Goat Kidney Cells |
| GMEM | Glasgow Modified Minimum Essential Medium |
| HA | Haemagglutination Assay |
| H protein | Haemagglutinin Protein |
| IAEA | International Atomic Energy Agency |
| IcELISA | Immunocapture ELISA |
| L protein | Large Protein |
| Mabs | Monoclonal Antibodies |
| M protein | Matrix Protein |
| MV | Measles Virus |
| NARC | National Agricultural Research Centre |
| NGS | Next Generation Sequencing |

| | |
|---|---|
| N protein | Nucleo Protein |
| OIE | Office International des Epizooties |
| OD | Optical Density |
| OPD | Ortho Phenylene Diamine |
| PARC | Pakistan Agriculture Research Council |
| PCR | Polymerase Chain Reaction |
| PCV | Packed Cell Volume |
| PDV | Phocine Distemper Virus |
| PI | Percent Inhibition |
| PMV | Porpoise Morbillivirus |
| PP | Percent Positivity |
| PPR | Peste des Petitis Ruminants |
| PPRV | Peste des Petitis Ruminants Virus |
| P protein | Phospho Protein |
| RNA | Ribonucleic Acid |
| RP | Rinderpest |
| RPV | Rinderpest Virus |
| RT-PCR | Reverse Transcriptase Polymerase Chain Reaction |
| SDS-PAGE | Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis |
| SISPA | Sequence-independent, single-primer amplification |
| TADs | Transboundary Animal Diseases |
| $TCID_{50}$ | Tissue Culture Infectivity Dose Fifty |
| TCRV | Tissue Culture Rinderpest Vaccine |
| TPB | Tryptose Phosphate Broth |
| Vero cells | African Green Monkey Kidney Cells |
| VI | Virus Isolation |
| VNT | Virus Neutralization Test |

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# ACKNOWLEDGEMENTS

All praises for **ALMIGHTY ALLAH**, the Merciful, the most Compassionate and the beneficent with whose countless blessings I was able to complete this assignment. I offer my courteous thanks from the deepest of my heart to **HOLY PROPHET, HAZRAT MUHAMMAD (PBUH)** who is forever model of guidance and knowledge for all humanity. I have numerous people to thank who were instrumental to make this day possible.

First and foremost, I would like to thank Dr. Rabaab Zahra, Associate Professor, Department of Microbiology, Quaid-I-Azam University, who as my supervisor always encouraged and helped me to complete this task. I am thankful to ma'am for her scholarly criticism and encouragement during my thesis write-up. I am also thankful to Dr. Rani Faryal, Chairperson, Department of Microbiology for her kind and soft behavior and great help during the course of my study at University.

I would like to express my great appreciation and thanks to my co-supervisor, Dr. Aamer Bin Zahur, Director, PARC/Director General, NVL, Islamabad for his prestigious interest and support throughout my academic and research career. He served as my mentor who motivated me to carry out studies on this project of global concern. He always encouraged and facilitated me during the entire research work. His intellectual guidance, valuable suggestions and accommodative attitude make it very easy for me to complete my task.

I am extremely thankful to Dr. Muhammad Afzal, Project Director, FAO, Pakistan for his expert opinion, guidance, technical support and advice during my research work. I am also thankful to my Supervisor at CIRAD, France, Dr. Genevieve Libeau for her kind attitude, technical support and precious time during my studies abroad. Special thanks to Dr. Asim Janjua, BPO, VRI, Lahore and Dr. Anees, Research Officer for facilitating me in completion of my task (vaccine formulation) under their kind supervision.

I would like to extend my special thanks and gratitude to all of my colleagues, support staff and students at Animal Health Program, ASI, NARC, particularly Dr. Aman Ullah, Dr. Hamid Irshad, Dr. Muneeb Hussain, Dr. Umer Farooq, Dr. Naila Siddique, Dr. Riasat Wasee Ullah and Dr. Aitezaz Ahsan for their co-operation and support during my research work.

I am highly thankful to Dr. Khalid Naeem, former Director ASI and Dr. Manzoor Hussain, former Project Director, FAO, Pakistan for their guidance and moral support during my research career.

I am also thankful to my university fellows Shazira, Ameer, Saba, Amna, Anum and Afshan for their kind words and moral support during my stay at university.

Allah has blessed me with sincere friends. I am thankful to all of my friends for their continuous encouragement and moral support during the course of my studies. I extend my special thanks to Rubina Kausar, Sofia Anjum, Hira Imran, Huma Naseer, Tahira Hanif and all others.

At this precious moment of my life, I am greatly missing my loving father (late). May Allah rest his soul in peace and grant him a place in Jannatul Firdaus. My father was the person who motivated me to carry out higher studies and excel in life. However, elders of my family, especially my maternal grandfather (Nana Abu), my brothers (Kamran and Usman), and my sisters (Najma, Uzma and Lubna) never let me feel alone during hard times of my life. I find myself short of words to pay thanks to all of them. Special thanks also to my nieces, Dua, Anabia and Hassan for their love, affection and alluring gestures. I appreciate my father-in-law (Ghulam Murtaza) for his encouragement and moral support during my doctoral studies.

I am grateful to my lovely husband Irfan Ahmed for his keen interest, patience and tremendous love. He overwhelmingly supported me during my research and thesis write-up. Thanks are also to my lovely daughter, Eshaal Irfan who is very close to my heart. You both are and will be a source of inspiration during the rest of my life.

Last but not the least, I feel indebted to my mother for her love, help, selfless commitment and patience during my studies and in my life. Without her, I was unable to accomplish this goal. My doctoral endeavors had numerous hard times. My mother stood by me through all those difficult moments.

In the end, I am thankful to all my family, friends, lab colleagues, fellows and students again. May Allah showers his countless blessings on all of us!

**Asma Latif**

## Abstract

The current study describes the development of a homologous lineage, live attenuated, thermostable vaccine for control of Peste des Petits ruminants (PPR). The objectives of the study were to isolate and characterize PPR virus (PPRV) from PPR outbreaks in Pakistan, to evaluate the thermostability potential of PPRV local isolates, to attenuate the most thermo-tolerant strain of PPRV and assessing its pathogenicity in goats, to develop a live attenuated thermo-tolerant PPR vaccine and to evaluate the comparative efficacy of different chemical stabilizers on the thermostability of newly developed PPR vaccine. In order to achieve the first objective, a total of 239 clinical samples were collected from 43 suspected outbreaks of PPR reported in different provinces of the country during 2012-2014. The samples were confirmed by Ic-ELISA and RT-PCR. The study revealed an appraisal of 70.17% of positive samples by RT-PCR than Ic-ELISA which confirmed 61.58% of samples positive for PPR antigen. It was concluded that RT-PCR is more sensitive assay for confirmation of PPRV from suspected field outbreaks. The results achieved an overall 65.58% of PPR positive samples. The phylogeny and epidemiology of PPRV circulating among small ruminant population were studied by conducting molecular characterization of N gene. The data was analyzed by constructing phylogenetic studies. The analysis of data consorted all the present study isolates into lineage IV. Moreover, Pakistani isolates showed more diversity and were found more closely associated with other isolates from neighboring countries like China, India and Iran.

Full genomes of three local PPRV isolates were sequenced using two sequencing methods; random sequencing method or SISPA (Sequence-independent single-primer amplification) and amplification with specific primers. The results showed that amplification with specific primers resulted in high PPRV specific reads (96.7%) as compared to SISPA which did not cover the entire PPRV genome. Considering the cost of amplification and number of reads obtained with the method, sequencing with specific primers is highly suggested. Phylogenetic analysis of full genomes clustered the three isolates within lineage IV clade sharing high sequence identity at nucleotide level with other PPRV strains from same lineage. Moreover, partial sequence of N gene was found informative for a preliminary investigation of origin of a strain. The genome organization of these three strains was also same and was in agreement with the “rule

of six" for Paramyxoviridae. Comparative analysis of full genomes of these local strains and one Nigerian strain (75/1) revealed differences at nucleotide and protein level.

A comparative analysis of Vero-76 and BTS-34 cells was undertaken by inoculating PPRV isolates on both cell lines. BTS cells showed more pronounced CPEs associated with PPRV along with high virus titer in short time than on Vero cells. The present study also describes the appraisal of thermostability potential using 7 local isolates of PPRV in order to select the most suitable candidate for development PPR vaccine. Results of the thermostability experiments revealed that PPRV was relatively stable at 4°C with no radical loss of infectivity for 3 weeks. PPRV in cell culture medium was impressible to heat and could be inactivated in 7 and 5 minutes at 60°C and 65°C. However, at 56°C, 20 to 25 minutes were required to eliminate PPRV infectivity. Ultraviolet irradiation effectively inactivated PPRV within 2-3 minutes. One isolate (PAK-LRS-13/NARC) appeared more resistant at high temperatures maintaining reasonable required titer after heat treatments. Based on thermostability characteristics, the attenuation of a local thermo-tolerant strain of PPRV was conducted to develop a homologous lineage PPR vaccine by serially passaging the strain upto $60^{th}$ passage onto Vero cells. The pathogenicity trials were also conducted in goats to monitor the degree of attenuation and progressive loss of pathogenicity after $20^{th}$, $40^{th}$ and $60^{th}$ passage. It showed that after $20^{th}$ passage, slight hyperthermia and oculo-nasal discharge were observed in goats. However, after $40^{th}$ and $60^{th}$ passage, no clinical manifestations were noticed. It was concluded that $60^{th}$ passage virus could safely be used for development of PPR vaccine in order to minimize the risks associated with the reversion of attenuated strain to wild type. PPR vaccine was prepared by thermo-stabilization method used for RP vaccine. A comparative study was also conducted to evaluate the effects of three different stabilizers on the thermostability of PPR vaccine. The results were compared by accelerated stability tests. Among the three chemical stabilizers, the loss in viral titer during lyophilization in case of Lactalbumin Hydrolysate-Sucrose (LAH) was only 0.23 $TCID_{50}$/ml as compared to the Trehalose dehydrate (TD) (0.37 $TCID_{50}$/ml) and Weybridge medium (WBM) (0.45 $TCID_{50}$/ml) which suggests LAH as the stabilizer of choice for enhancing thermostability of PPR vaccine.

The efficacy of this newly developed PPR vaccine was evaluated by carrying out experimental immunization in goats. Dynamics of humoral immune response was monitored. Challenge protection studies were carried out in animals previously

inoculated with PPR vaccine made by Nigerian strain (75/1) and newly developed thermostable vaccine formulation. A virulent field isolate catalogued as PAK-Fjg-14/NARC was used in challenge protection studies. It was found that antibodies developed after 3 days post vaccination. The results were comparable to the Nigerian strain (75/1) vaccine. The animals combated the challenge and did not produce any clinical signs of disease throughout the experimental period. PPRV was not detected in any of nasal, ocular and oral secretions by RT-PCR. Results of humoral immune response revealed that immunization with vaccine prepared from local thermostable strain of PPRV with the new formulation provided equally good protection in goats. The study concluded that the newly developed thermostable vaccine would be beneficial in eliminating cold chain associated problems present in existing vaccine and also help in progressive control of PPR in the country.

# Chapter 1

## Introduction and Review of Literature

# Chapter 1: Introduction and Review of Literature

## 1.1 Introduction

Livestock plays a noteworthy job in livelihood of poor people by providing food, income and employment to the rural communities in developing countries. But the countries with limited resources remain vulnerable to various livestock diseases which become endemic making their control costly (Rossiter and Al Hammadi, 2009). Among the infectious diseases, TADs (Transboundary animal diseases) are of great concern due to their serious consequences (Negesso et al., 2016). TADs may be described as 'highly contagious diseases having potential for rapid spread across borders affecting the livelihood of poor farmers due to their high morbidity and mortality' (FAO, 1999). Serious economic losses have been encountered by livestock farmers due to TADs such as rinderpest (RP), pest des petites ruminants (PPR), foot and mouth disease (FMD), classical swine fever (CSF), contagious bovine pleuro pneumonia (CBPP) and Rift Valley fever (RVF) (Seyoum and Teshome, 2017). Among other TADs, Peste des petits ruminant (PPR) is an enormusly contagious animal disease affecting predominantly goats and sheep and some wild species of small ruminants (Bwihangane et al., 2017). The causative agent is classified in the genus morbillivirus which also includes viruses known to cause rinderpest, measles and distemper in cattle, humans and dogs respectively (Mariner et al., 2017). PPR has been declared as the next eradicating target by Food and Agriculture Organization (FAO) of United Nations and World Organization for Animal Health formerly Office International des Epizooties (OIE) after successful eradication of rinderpest in 2011 (Latif et al., 2018). The disease has gained a tremendous economic significance because of its massive morbidity (100%) (Hailat et al., 2018) and mortality (0-90%) in suseptible animals (Rojas et al., 2017).

Rapid spread of PPR in Asia, Middle East, Africa and other neighbouring countries is further intensified by restricted vaccination and illegal cross border animal movements (Bouslikhane, 2015). Without effective control measures, the disease can severely affect small ruminants' productivity and ultimately the livelihood of poor livestock holders (Jones et al., 2016).

## 1.2 Historical Background

The first documented evidence of PPR was provided by Gargadennec and Lalanne in 1994 when they reported a disease in West Africa (Cote d' Ivoire) which was identical to RP (rinderpest) but it infected only sheep and goats whereas cattle in contact stayed apparently uninfected (Balamurugan et al., 2014). Soon after, the disease was reported in other neighboring countries of Africa like Nigeria, Senegal and Ghana (Kozat and Sepehrizadeh, 2017). For a long time, it was considered that PPR belonged to Western Africa until a disease similar to RP appeared in 1972 in goats of Sudan which was later identified as PPR (Fentahun and Woldie, 2012). Before 1987 in India, it was believed that infection in sheep and goats was caused by rinderpest. But the continuous occurrence of PPR after eradication of rinderpest drew attention towards the fact that many outbreaks affecting small ruminants in India previously associated with rinderpest were actually due to PPR (Kumar et al., 2014). Due to its rapid spread to a number of other African and Asian countries and sharing of more than one lineage has made PPR a disease of global concern (Libeau et al., 2014). The disease has become more important due to its transboundary nature, socio-economic implications on livestock and the complexities it has made in rinderpest eradication (Couacy-Hymann et al., 2002).

Besides the commonly used French name PPR, the ailment has many other acronyms like goat plague, Kata, plague of small ruminants or ovine rinderpest (Kumar et al., 2014).

## 1.3 The Causative Agent

The plague is caused by a virus, believed to be a modified rinderpest virus acclimated to sheep and goats with the loss of virulence for cattle (Mornet et al., 1956). However, based on morphological changes, growth characteristics, physicochemical attributes and composition antigens, it was demonstrated that RP and PPR viruses were distinguishable (Hamdy et al., 1976). PPR virus has been allocated the family *paramyxoviridae* and genus *morbillivirus* (Gibbs et al., 1979). *Morbilliviruses* also include measles virus (MV), rinderpest virus (RPV), canine distemper virus (CDV), phocid morbilliviruses (PMV), dolphin morbilliviruses (DMV), and porpoise distemper virus (PDV) (Gibbs et al., 1979; Kumar et al., 2014). Differential diagnosis by ELISA based on monoclonal antibodies (Libeau et al., 1995), hybridization studies employing specific nucleic acid probes (Diallo

et al., 1989) and sequencing techniques (Forsyth and Barrett, 1995) had confirmed PPRV as a separate entity.

### 1.4 Virion Structure

PPRV when examined under electron microscope is pleomorphic in shape and the diameter is in the range of 130-390nm. The virus particle consists of an envelope with a thickness of 8.5-14.5nm having spikes measuring 8-15nm in length. The ribonucleoprotein strands have hearing bone-like appearance that are 14-23nm thick (Durojaiye et al., 1985). PPR virus like other members of its genus has a single segmented RNA genome of 15,948 nt in length and of negative polarity (Diallo et al., 2007; Rahman et al., 2016). It encodes for eight proteins (Six structural and two non-structural). Among the structural proteins are hemagglutinin (H), Fusion (F), nucleocapsid (N), matrix (M), phosphoprotein (P) and large protein (L) while V and C are designated as nonstructural proteins (Figure 1.1) (Diallo et al., 1994). F and H, being external to the capsid play important role in initiation of infection process. These external proteins are also involved in inducing specific immunity against the virus (Diallo, 1990). The N and F protein genes have commonly been targeted for molecular detection of viral genome. Comparative studies of N and F genes showed that N protein gene emerged to be more pertinent for segregating between closely related but geographically distinct PPR viruses (Kwiatek et al., 2007; Rahman et al., 2016).

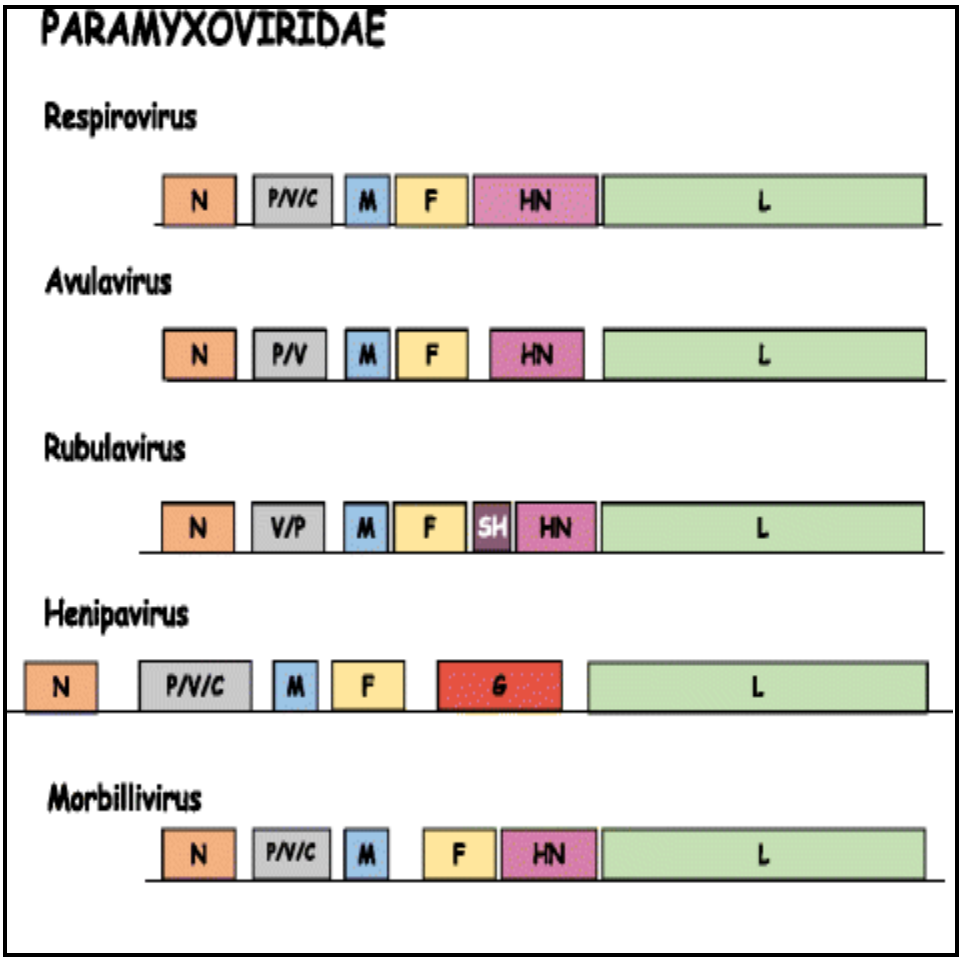



**Figure 1.1:** Representation of genome organization concerning the family *Paramyxoviridae*.

The Nucleoprotein (N); Phosphoprotein/non-structural proteins (P, V & C); Matrix protein (M); Fusion protein (F); Hemagglutinin-neuraminidase protein (HN); RNA dependent RNA protein or large protein; SH=Small hydrophobic protein gene

Source: (Shahriari et al., 2016)

### 1.5 Geographical Distribution

For quite a while, it was considered that PPR is a West African disease after it was described for the first time in 1942 by Gargadennec and Lalanne in Cote d'Ivoire since the disease was endemic in Senegal (Gilbert and Monnier, 1962) and Nigeria (Durtnell, 1972). Yet, later it was shown that PPR has far more extensive geographic circulation. The disease has now been documented from whole Africa (Gibbs, 1981), Kenya, Uganda, Ethiopia (Roeder et al., 1994) and Sudan (El Hag and Taylor, 1984) and it showed up in the Middle East in 1987 and from that point forward has been reported from the Arabian peninsula, Egypt, Pakistan, Afghanistan, Bangladesh, India (Lefèvre and Diallo, 1990; Amjad et al., 1996; Nanda et al., 1996; Shaila et al., 1996) and Turkey (Özkul et al., 2002). The disease has escalated to central Asia as in Tajikistan (Kwiatek *et al.*, 2007) and documented reports based on serology affirms its existence in Kazakhstan (Lundervold et al., 2004). Reports have affirmed its presence in China too (Wang et al., 2009).

PPR has been categorized into four definite lineages based on the comparative analysis of F gene (Shaila et al., 1996; Dhar et al., 2002) and N gene (Wang *et al.*, 2009; Kwiatek *et al.*, 2007). The first two lineages (I and II) are restrained in African countries, lineage III is evidenced in Africa and Asia and lineage IV in Asia. But recent reports confirm the presence of all four lineages throughout Africa: the first two lineages are prevalent in West Africa, the third lineage in East Africa, and the last in West, North, East and Central Africa.

Some lineages have the potential to expend its range as in East Africa lineage III was prevalent initially until the appearance of Asian lineage (Lineage IV) in Ethiopia, Sudan, Uganda and Tanzania. Similarly, lineage I has been replaced by lineage II. In Senegal, PPR was last reported in 1994. After that, all studies were indicative of lineage II in West Africa. Recent report detected lineage IV in Niger, West Africa that was thought to be prevalent with lineage II (Tounkara et al., 2018).

## 1.6 Epidemiology

### 1.6.1 Transmission

The transmission of infection is possible from the infected to the susceptible animal through exhaled aerosol (Ocular or nasal discharges) or excretions (feces) (Parida et al., 2016) which contain plentiful virus (Abegunde and Adu, 1977). Animals in the febrile stage of infection may also be contagious (Kozat and Sepehrizadeh, 2017). It was noticed that PPRV shedding continued in fecal material of recovered goats for 12 weeks that would explain the carrier status in low and high incidence periods of the disease which can facilitate the new outbreaks of PPR (Ezeibe et al., 2008). The infection can also be transmitted indirectly for a short time through water, feed troughs and bedding (Kozat and Sepehrizadeh, 2017) as the virus cannot survive outside of its host for long time due to thermo-labile nature of virus (Parida et al., 2016).

### 1.6.2 Risk Factors for PPR

Animal movements, livestock markets and sharing of water and grazing points are considered as the main elements for disease spread. Various disease episodes resulted when agriculturists added recently acquired animals to their existing flocks (Taylor, 1984). The same water and grazing areas outside the towns additionally perform a role in the further spread of the ailment among the herds of same town or diverse towns (Taylor and Barrett, 2007). The transhumant animal and nomadic movements to faraway places serve as an imperative mode for PPRV transmission (Taylor and Barrett, 2007). In Pakistan, the lack of awareness, livestock markets, animal and nomad's movements were considered the key factors for PPRV persistence and transmission (Zahur et al., 2008).

### 1.6.3 Host Range

Sheep and goats are contemplated to be the natural hosts although susceptibility to disease vary by species. Goats are known to be more vulnerable to PPR than sheep (Kardjadj et al., 2015); (Kardjadj et al., 2015). But in sheep the recovery rate was found to be superior to goats (Gargadennec and Lalanne, 1942). Susceptibility may also be affected by breeds as Guinean breeds of goats were revealed to be more prone to PPR infection (Lefèvre and Diallo, 1990; Rony et al., 2017). However, there were reports of similar or higher infection

rate in sheep than goats (Taylor, 1979; Intisar et al., 2017). It has been estimated that breed and species might play a crucial part in epidemiological study of PPR (Dayhum et al., 2018).

The studies conducted on the prevalence of PPR antibodies in cattle and buffaloes (Baazizi et al., 2017), camels and pigs (Gopilo, 2005); (Nawathe and Taylor, 1979) in different countries were indicative of the presence of disease in these animals. Moreover, cattle are considered as the dead end hosts of PPR showing subclinical disease (El Hag Ali and Taylor, 1988). They resist the natural or experimental challenge with virulent RPV after developing a humoral immune response to PPR virus (Özkul et al., 2002).

PPRV has jumped from domestic to wild animal populations resulting in serious concerns for endangered species (Fournié et al., 2018). During 2014-2016, there were massive laboratory confirmed death cases of wild goats and sheep in national parks of Iran due to PPR (Marashi et al., 2017). In Tibet, China, two cases were reported in bharals which showed 99.7–100% nucleotide identity with isolated PPRV isolates from small ruminants (Bao et al., 2011). Another similar report of PPRV was documented in Saudi Arabian wild ungulates and Pakistani Sindh Ibex in a national park and American white deer (Abubakar et al., 2011); (Hamdy and Dardiri, 1976). It has also been described in antelope, deer, gazelle (Elzein et al., 2004) and other wild animals which include Nubian Ibex, gemsbok, dorcas gazelles, laristan sheep, and nigale. It has been estimated that sharing of pastures could be the reason for disease transmission to other wild species. It was concluded that controlling infection in small ruminants can control disease in wild species.

### 1.6.4 Disease Pattern

Goats are more susceptible to PPR than sheep. Different geographical areas and ecological systems affect the epidemiological pattern of the disease. Small ruminants are generally fed on shrubs pasture and forest in the subcontinent of India and Pakistan. During the dry season, animals travel to faraway places due to unavailability of these sources (Nanda et al., 1996). The patterns of PPRV activity was therefore, determined by movement of animals. In the damp zones, the disease appeared as epidemic with high morbidity (80-90%) and mortality (50-80%) but it occurs sub-clinically in arid and semi-arid areas with least mortality making animals vulnerable to other opportunistic infections (Lefèvre and

Diallo, 1990). In regions, where PPR is endemic, the mortality is below 20% (Roeder et al., 1999). Young animals between 3 to 4 months of age are more vulnerable to the disease due to decline in the maternal antibodies (Srinivas and Gopal, 1996) and with increasing age, the seropositivity for PPR also increases (Losos, 1986). The serological studies provided clear evidence that antibodies against PPR existed in all age groups showing a steady course of infection (Taylor, 1984). The expended frequency of disease occurs with an increase in susceptible young animals which is generally correlated with the season of parturition (Taylor *et al.,* 1990).

### 1.6.5 Seasonal Occurrence

PPR spread globally has been facilitated by loss of antibody cross protection between PPR and RP with the cessation of RP vaccine usage after its successful eradication thus exposing the small ruminants fully to PPR (Libeau *et al.,* 2011). The outbreaks occur throughout the year while the disease incidence is generally more during the peak winter in Pakistan (Zahur et al., 2008) and dry summer months in India (Singh et al., 2004). The disease occurred with high morbidity and mortality in West Africa during the wet rainy seasons in humid areas (Lefèvre and Diallo, 1990). Significant association between prevalence of PPR and winter season was reported in Sudan (Abdalla et al., 2012) while in Ethiopia, peak disease outbreak was found to be associated with seasonal movements in search of water and pasture during the dry seasons (Waret-Szkuta et al., 2008). Other factors like animal movements during the periods of nutritional deficiencies, cross border livestock trade, cultural festivals, changes in husbandry practices, climatic and environmental change and difference in PPRV virulence may alter the seasonal occurrence of disease and contribute to PPR spread (Taylor and Barrett, 2007).

## 1.7 Clinical Manifestation

Different breeds of goats and sheep show variations in sensitivity to PPR virus infection (Couacy-Hymann et al., 2007). Though they exhibit an identical, if slightly less severe, clinical picture to cattle infected with RPV (Baron et al., 2016). The disease may depict itself in any of 3 forms which includes acute, per-acute and sub-acute however PPR mainly occurs in acute form in sheep and goats.

### 1.7.1 Per-acute

It mainly occurs in youngsters who are 4 months or more whereupon maternal antibody level is diminished. It has shorter period of incubation mainly 2 to 3 days. The body temperature reaches up to 40-42°C. The infected animal shows depression and is reluctant to move and eat. Other symptoms include congestion of oral and nasal mucosae and conjunctivitis. Death of affected animal occurs within 4-6 days.

### 1.7.2 Acute

It is the most common form which commences after an incubation period of 4-6 days. Animals become dull, depressed and suffer from anorexia. The temperature of animal body suddenly increases reaching up to 40-41°C. One or two days after the onset of fever, there appears succulent release from nose and eyes which later develops into muco-purulent resulting in matting of eyelids and obstruction in nostrils making breathing difficult.

### 1.7.3 Sub-acute

This form shows mild clinical signs of PPR with longer disease duration. Inconsequential mortality is normally observed.

## 1.8 Pathology

### 1.8.1 Pathogenesis

PPRV like other members of morbillivirus is a lymphotropic and epitheliotropic virus and likewise its primary targets are lymphoid and epithelial tissues (Gibbs, 1981). PPRV enters its host via respiratory system and is restrained in the regional lymph nodes and tonsils which is the main consequence of lymphopenia in susceptible host. Viraemia develops after the virus enters into the bloodstream in 2 to 3 days after infection resulting in virus promulgation to mucosae of gastrointestinal and respiratory tract, spleen and visceral lymph nodes. This febrile stage persists until the $16^{th}$ day after infection and it is during this stage that the virus can easily be recovered from nasal secretions. Stomatitis and diarrhea result from virus entry into the gastrointestinal tract associated with abdominal pain and dehydration which culminates in death of animal (Bundza et al., 1988; Pawaiya et al., 2004).

Hematological parameters in affected animals showed marked differences like increase in total lymphocyte count and an overall decrease was observed in protein and albumin level (Bari et al., 2018). The PPRV infection results in severe immunosuppression including leucopenia in respective hosts (Rajak et al., 2005).

### 1.9 Diagnosis

One of the key elements of global strategy for PPR control by 2030 is diagnostics based on rapid, applicable, highly sensitive and specific assays. The disease can be diagnosed clinically based on the clinical signs and epidemiological aspects. Sometimes, the differentiation of disease from other similar diseases like hemorrhagic septicemia and caprine contagious pleuropneumonia becomes difficult in which case the confirmed diagnosis is imperative through various laboratory tests employing microbiological techniques (Sharma et al., 2015; Abubakar et al., 2016).

Laboratory tests for diagnosis of PPRV infection include virus isolation, antigen and antibody detection, nucleic acid detection and sequencing (Diallo et al., 1995).

#### 1.9.1 Virus Isolation

Gilbert and Monnier were the first to isolate PPRV in 1962 using cell culture system. After that, virus isolation studies have been reported from many countries including Ethiopia, Nigeria, Saudi Arabia, Sudan, Turkey, India and Pakistan (Taylor and Abegunde, 1979; El Hag Ali and Taylor, 1988; Abu-Elzein et al., 1990; Nanda et al., 1996; Özkul et al., 2002; Gopilo, 2005; Latif et al., 2018). Virus isolation was regarded as most veracious proof and a gold standard test for PPRV existence in a country (Taylor, 1984; Balamurugan et al., 2014). After the onset of fever, there is febrile phase of disease known to be crucial for successful virus recovery. Un-coagulated blood and swabs from eyes, nose and mouth secretions can be used for virus isolation under sterile conditions. For biopsies, lymph nodes and spleen tissue are ideal for virus recovery.

Primarily, primary cultures using ovine and bovine kidney and lung cells and Vero cells (African green monkey kidney) have been used for PPR virus isolation (Adombi et al., 2011). The major drawback with these cultures is that they require technical expertise, are time consuming and laborious. To resolve this issue, continuous cell lines, preferably Vero

cells were used for isolation of morbilliviruses (Sannat et al., 2014). But due to less probability of virus growth and requirements for multiple blind passages, these cells proved to be less efficient for PPR virus isolation (Albayrak and Alkan, 2009; Adombi et al., 2011). The fact that PPRV along with other morbilliviruses preferably use SLAM (Signaling lymphocyte activation molecule) as receptors for entry and attachment to the host cell proved these receptors important for virus isolation (Sannat et al., 2014).

Using different types of cells for PPRV isolation, it was revealed that time taken for appearance of visible CPEs and their morphological patterns is different for each cellular system. PPR virus in primary cell culture systems induced vacuolation, rounding of cells and syncytia formation (Anderson et al., 1996). In Vero cells, the onset of CPEs is delayed with the requirement for multiple blind passages. The CPEs developed as granular elongated cells which lead to cell depreciation and rounding. Whereas, in a newly developed BTS-34 cell line, PPRV induced clearly visible CPEs as early as 72 hours after infection. With the initiation of infection process, some cells became round and got detached from the surface. About 40-50% of CPEs were developed as rounded giant cells ultimately forming syncytia. The rounded cells increased in number and were seen as cluster of cells leaving behind very few intact cells on $4^{th}$ day post inoculation. This whole infection process took approximately 4 to 6 days on BTS-34 cell line (Latif et al., 2018).

Animal inoculation confirms the identity of cell culture isolated PPRV by producing clinical disease in goats and sheep. Cattle are not generally used for PPRV confirmation because of no obvious disease symptoms (Gibbs et al., 1979). However reference sera of both RP and PPRV proved effective for neutralization of PPRV (Taylor and Abegunde, 1979).

### 1.9.2 Methods for PPRV Antigen Detection

#### 1.9.2.1 AGID (Agar Gel Immunodiffusion)

Agar gel immune diffusion test is a simple, rapid, cheap and highly specific assay for detection of PPRV antigen (Munir et al., 2009).Both antibodies and antigen can be determined with the test and results can be obtained in 2-4 hours with RP hyper immune sera in comparison to PPR sera which gave results in 4-6 hours (Obi and Patrick, 1984).

Despite the highly specific diagnostic capacity (92%), the assay is unable to differentiate PPR and RP. Nevertheless, it may be used for everyday diagnosis and mass screening of disease in the absence of cELISA since RP has already been eradicated and offers no longer a differential diagnosis (Munir et al., 2009).

#### 1.9.2.2 Haemagglutination Assay

Among morbilliviruses, measles and PPRV have unique ability to agglutinate red blood cells of some animal species (Wosu, 1985; Ramachandran et al., 1993). PPR diagnosis in live animals can be accomplished employing haemagglutination (HA) test (Wosu, 1991). The test was developed for the rapid and cheap PPR diagnosis. Different assay conditions i.e. sensitivity of RBCs to different species, incubation temperature and agglutination buffer pH were standardized (Ezeibe et al., 2004). This simple rapid and inexpensive assay can easily be performed in any laboratory.

The assay was found non-specific for antigen detection using fecal samples (Latif et al., 2014), however persistence studies conducted for longer duration could be facilitated by using this assay as PPRV shedding was observed after 12 weeks in fecal samples of goats recovered from PPR (Ezeibe et al., 2008).

#### 1.9.2.3 Ic-ELISA (Immunocapture ELISA)

Another simple, rapid and specific test for routine diagnosis of PPRV in field samples is Immunocapture ELISA (Diallo et al., 1995) which is extensively used now a days. The assay utilizes two monoclonal antibodies (MAbs) targeting N protein (Libeau et al., 1995). Moreover, it can be performed in one step by adding test antigen, detecting MAbs and conjugate in a precoated ELISA plate with capture antibody and results can be obtained within 2 hours. The assay is able to differentiate between RP and PPR although with different capacities for detection because of difference in detecting antibody affinities for N proteins of different species. Another advantage of the test is that the assay can also be carried out on suspected PPRV samples which are not kept under appropriate conditions (Diallo et al., 1995).

### 1.9.3 Methods for PPRV Genome Detection

#### 1.9.3.1 cDNA Probe Technology

Specific cDNA probe based hybridization technique was developed initially for molecular detection of RP and PPRV in late 1980s (Shaila et al., 1989). For diagnosis of both viruses differentially, radio-labelled probes related to N gene of RP and PPRV were used without the need for virus isolation (Diallo et al., 1989). Although the test is highly sensitive yet cannot be employed for everyday diagnosis because of short lived radio-labelled isotopes, risks involved in handling radio-labelled substances and the requirement for fresh samples. To overcome this problem, non-radioactive labels like biotin or dioxin (Pandey et al., 1992; Diallo et al., 1995) were used although they provide less sensitive detection as compared to the use of labelled isotopes. PPRV presence was detected by probe hybridization in Ethiopia and India (Roeder et al., 1994); (Shaila et al., 1989).

#### 1.9.3.2 RT-PCR (Reverse Transcriptase Polymerase Reaction)

The crude techniques for PPRV diagnosis like AGID and differential diagnosis depending upon virus isolation are laborious, time consuming and require live virus in freshly collected sample (Forsyth and Barrett, 1995). The advancement made by development of PCR (polymerase chain reaction) has made sensitive and specific PPRV detection from indigently preserved sample having minimal quantities of nucleic acid (Couacy-Hymann et al., 2002). RNA viruses can also be detected through PCR by simple addition of a reverse transcription step. The technique is equally proficient and simple than nucleic acid hybridization and sensitive than ELISA for PPR detection (Forsyth and Barrett, 1995; Couacy-Hymann et al., 2019). Phylogenetic studies performed on data achieved by sequencing of PCR products can be used to study genetics of new viruses and their relationship with the previously isolated field strains from different regions (Shaila et al., 1996; Güler et al., 2014); (Shaila et al., 1996). RT-PCR appeared to be more sensitive test for early detection of PPRV genome compared to Ic-ELISA (Forsyth and Barrett, 1995). It was able to detect PPRV genome even before emergence of specific disease signs as early as 2 days after experimental infection (Couacy-Hymann et al., 2007). The test was 1000 fold more efficient in detecting N gene of PPR using RNA purification procedures on glass beads than titration on Vero cells (Couacy-Hymann et al., 2002). Specific amplification

using phosphoprotein (P) and fusion protein (F) based primers can differentiate between RP and PPR (Barrett et al., 1993).

### 1.9.4 Serological Diagnosis

Differential diagnosis of PPR from a closely related disease RP is essential for specific diagnosis of PPR. Serological tests like AGID and CIEP (Counter immune-electrophoresis) often fail to differentiate between two diseases hence are least preferred. Conventional virus neutralization (VNT) test and ELISA (Blocking and competitive) are able to differentiate between these diseases, however, ELISA is preferred over VNT where large sample size has to be screened.

#### 1.9.4.1 VNT (Virus Neutralization Test)

VNT is a highly sensitive and specific serological test used for detection of antibodies specific to PPRV (Rossiter, 1994). Primary culture or Vero cell line can be used to perform this assay. Although both viruses may be neutralized by serum against either PPR or RP but homologous virus would be neutralized with a high titer (Taylor and Abegunde, 1979). The assay is not practical due to its high cost and laborious nature.

#### 1.9.4.2 cELISA (Competitive ELISA)

The shortcoming in VNT has prompted looking for options in contrast to the VNT for RP and PPR sero-monitoring. The indirect ELISA used during Pan African Rinderpest Campaign (PARC) appeared effective for sero-monitoring after immunization but was unable to differentiate between RP and PPR (Anderson et al., 1982). Thus blocking ELISA or cELISA (competitive ELISA) utilizing antibodies which are monoclonal and specific for either H or N proteins (Anderson et al., 1991; Libeau et al., 1995) were developed to achieve this differentiation. In cELISA, the competition between detecting antibodies preferably monoclonal and antibody in the test sera is measured. The use of monoclonal antibodies as detecting antibody provides specificity to the assay (Libeau et al., 1995). Blocking ELISA specific to H protein was found to be 90.4% sensitive and 98.9% specific (Saliki et al., 1993). The results of ELISA were measured as percentage inhibition (PI) values and in recently developed cELISA, the sera showing less than fifty percent of percent inhibition values were considered as positive. Compared with VNT, the assay has

an overall sensitivity of 92.2% and specificity 98.4% (Singh et al., 2004). The H protein based cELISA has been used with great success for post vaccination sero-monitoring of rinderpest and expedited its global eradication.

### 1.10 Treatment and Control

In order to treat goat plague, no specific treatment is available. However, the affected sheep and goats having pneumonia, enteritis and stomatitis, can be given antibiotics which are broad spectrum to impede secondary bacterial infestations. To help animals counter diarrhea, remedial treatment based on fluid replacement might be used. The mortality rates can be reduced by the use of drugs in animals infected with bacterial and parasitic infections (Nawathe, 1984). Moreover the sick animals can be treated with hyper immune sera obtained from cattle raised against RP (Fentahun and Woldie, 2012).

#### 1.10.1 Sanitary Prophylaxis

The disease can be prevented by taking sanitary prophylactic measures which are rapid, inexpensive and effective (Taylor et al., 1995). These include

- Segregation of unsold animals till vaccination of entire stock
- Not introducing new stock from various sources
- Quarantine of diseased stock from apparently healthy animals of the infected flocks in case of an outbreak
- Slaughter the infected animals; proper disposal of offal and in case of mortality deep burial of dead animals.
- Movement control within infected premises

The successful eradication of closely related disease, rinderpest from Europe was made possible by slaughtering of all infected animals and stringent movement restriction (Taylor et al., 1995) which is difficult in developing countries with endemic disease status.

#### 1.10.2 Medical Prophylaxis

For PPR control, vaccination is the most efficient approach (Fentahun and Woldie, 2012). TCRV (Tissue culture rinderpest vaccine) was adopted effectively for quite a while to control PPR due to close antigenic association between two viruses (Diallo et al., 2007;

Aitken, 2008). Later its use was discontinued due to its hindrance in sero-surveillance of RP. The first homologous vaccine preparation was made by attenuation of a virulent isolate of PPRV (Nigeria 75/1) originally screened in 1975 from an ailing Nigerian goat (Diallo et al., 1989; Liu et al., 2014). The vaccine provides a lifelong immunity in small ruminants (Zahur et al., 2014) against all PPRV strains (Diallo et al., 2007). Another live attenuated vaccine Sungri-96 was developed in India which belongs to Lineage IV. But the major drawback with these vaccines is their sensitivity to high temperatures especially in areas with hot climatic conditions. In many developing countries including Pakistan, the vaccine loses its potency under field conditions due to poor infrastructure and inadequate cold chain maintenance resulting in vaccine failure (Yaqub et al., 2016). International agencies like Food and Agriculture Organization (FAO) of United Nations and the World Organization for Animal Health have already declared PPR as next eradicating target by 2030 (Baazizi et al., 2017). The control programs for PPR can be facilitated by the development of a thermostable vaccine to eliminate the cold chain related issues in different regions.

Another disadvantage of these live attenuated vaccines is that infected and vaccinated animals cannot be differentiated. Dual recombinant vaccines expressing H and F genes of PPRV to secure goats and sheep against two viral diseases (Capri pox and PPRV) were developed to overcome this problem (Diallo, 2003). However, due to the cost of vaccine and requirement for a booster, live attenuated vaccine under field conditions is routinely used for controlling PPR.

#### 1.10.2.1 Efforts to Make Thermotolerant PPR Vaccine

In an effort to contribute in the Global PPR Eradication Program, many researchers have started important initiatives to control this trans-boundary animal disease.

The modification of free-drying process and addition of trehalose as cryoprotectant has extended the PPR vaccine thermostability sufficiently (Worrall et al., 2000). Thermostability of newly developed vaccine was evaluated using four different stabilizers namely lactalbumin hydrolysate–sucrose (LS), trehalose dihydrate (TD), buffered gelatin-sorbitol (BUGS) and weybridge medium (WBM). LS was proved to be superior stabilizer with respect to half-life as compared to other stabilizers at 4°C, 25°C and 37°C. Additionally, three different diluents (water, 1M $MgSO_4$, 0.85% NaCl) were used for reconstitution of

vaccine, 1M $MgSO_4$ proved to be superior as it maintained the required virus titer with lowest titer loss (Sarkar et al., 2003).

Another study revealed that Weybridge medium (WBM) provided better stability to PPR vaccine than lactalbumin hydrolysate-sucrose and lactalbumin hydrolysate-manitol (Asim et al., 2008). PPR vaccine stability having Tris buffer was compared by addition of either sucrose or trehalose with the previously formulated vaccine having Weybridge medium as cryoprotectant at 4°C, 37°C and 45°C in liquid and lyophilized form. The results of the study were suggestive of using Tris/Tre for better stability of vaccine in lyophilized as well as liquid form. Vaccine prepared with this composition was stable after reconstitution for 21h at 37°C and 30 days at 4°C. The vaccine in lyophilized form maintained the titer of 1 x $10^4$ $TCID_{50}$/ml for 21 months at 4°C, 144h at 37°C and 120h at 45°C. Higher vaccine stability (2.6 fold) and 1 log increase in virus production was observed after addition of 25mM fructose compared to glucose. Moreover increasing NaCl concentrations facilitated the virus release (Silva et al., 2011).

The effect of two stabilizers namely stabilizer E and lactalbumin hydrolysate-sucrose (LS) was investigated in lyophilized state and two diluents (0.85% NaCl and 1 mol/L $MgSO_4$) in reconstituted form on the thermo-adapted (Ta) PPR vaccines. Both vaccines in lyophilized form maintained the infectious titer at 25°C, 37°C and 40°C for 24-26 days, 7-8 days and 3-4 days respectively. However, LS in combination with 0.85% NaCl diluent performed well at high temperatures (at 42°C the shelf-life of LS and stabilizer E was 44 h and 40 h respectively with a comparable half-life) (Riyesh et al., 2011).

PPR vaccine prepared with polyvinyl pyrrolidone-NZamine (PPNZ) stabilizer reported to exhibit an average titer loss of 0.4 log10 TCID50/ml and inducing higher neutralizing PPR antibody titers in goats was preferred over vaccine with Weybridge medium (WBM) which showed a total of 0.6 log10 TCID50/ml loss of titer during freeze drying process. Both vaccines when stored at 4°C and 25°C maintained sufficient virus titer for 12 and 9 months respectively. Whereas titer was lost by $19^{th}$ day of storage at 37°C (El-Bagoury et al., 2015).

Four different stabilizers namely TD, LS, BUGS and WBM in five formulations were tried to evaluate their efficacy on thermostability of PPR vaccine. The results revealed higher stability of PPR vaccine at 37°C with LS showing minimum virus titer loss (0.42

log10TCID50) and more shelf life (3.69 days) than commercially available PPR vaccine (1.23 days). At 4°C, WBM showed remarkable increase in shelf life of vaccine (304 days) while LS, 2.5 %TD and 5%TD were able to increase the vaccine shelf life up to 208, 206, 191 days. Maximum protection of viral titer in PPR vaccine was observed with LS and WBM (20 days) at 25°C in comparison to protection with commercial vaccine (12 days). BUGS and TD stabilizers (2.5% and 5%) resulted in complete loss of titer after 18 days at this temperature (Yaqub et al., 2016).

Tris/Trehalose stabilizer was tested in comparison with Weybridge medium. The vaccine lyophilized with Tris/Tre formulation maintained the required viral titer for 9 and 12 months when stored at 4 and -4°C respectively to protect goats from PPR. Even at high temperatures of 37°C and 45°C, Tris/Tre vaccine formulation proved to be effective for 5 and 2 days respectively (Khadr et al., 2017).

A thermostable PPR vaccine formulation was prepared by following Rinderpest lyophilization methods and Lactalbumin hydrolysate (LS) as stabilizer. The vaccine batches produced by this method were most stable having shelf life of 177.6, 105 and 148.9 days at 37°C and 13.7 days at 56°C with half-life of 1.3 years at 25°C. This adequately facilitated the vaccine delivery without dependence on cold chain for up to 30 days (Mariner et al., 2017).

Effect of different chemical stabilizers was evaluated on the activity of freeze dried PPR vaccine. The stabilizers used in the study (Weybridge medium, Goat skimmed milk, Lactalbumin hydrolysate sorbitol and Lactalbumin hydrolysate sucrose) were able to sustain the infectious titer for 12 hours. However, Tris, Trhalose-TT when reconstituted in Phosphate buffered saline and stored at 4°C could protect the vaccine titer for 24 hours (Latif *et al.,* 2018).

### 1.11 Livestock Production Systems in Pakistan

Pakistan lies between longitude 60$^{o}$ and 76$^{o}$E and latitude 23$^{o}$ and 37$^{o}$N. It has borders with India, Afghanistan, Iran and Arabian Sea in the southeast, northwest and south respectively. It has an area of 0.8 million sq. km with diverse geography. The computational population is 196.2 million with growth rate of 1.49%. Pakistan has five

federating units (Provinces), namely Punjab, Khyber Pakhtunkhwa (KP), Baluchistan, Sindh, Gilgit-Baltistan (GB) and one federally administered tribal area (FATA) and one autonomous region namely Azad Jammu & Kashmir (AJK). The two highly populated provinces are Punjab comprising of 56.5% and Sindh with 22.6 of total population.

In Pakistan, the common production systems for rearing of sheep and goats are extensive and traditional (Ishaque, 1993). These include:

### 1.11.1 Nomadic

The nomads change their place steadily round the year in pursuit of pastures and water. They have well established routes of migration usually based on the availability of grazing land and water. The flocks comprise of more than 100 animals. The lambing and kidding season is between January and April. At that time the flocks are usually at lowland pastures along the banks of rivers and canals. One year old males are sold; however, females are retained for breeding purpose. The nomadic flocks mostly derive their feed from rangelands and grazing is generally free. About 6% of the small ruminants in Pakistan are raised under nomadic system. The nomadic flocks are mostly found in parts of Sindh, Baluchistan, Cholistan and Thal areas of Punjab province.

### 1.11.2 Transhumant

It is a semi migratory system of small ruminants' production where the farmers utilize the regular seasonal fields situated at various zones. The stock movement occurs in months of October and November each year mainly from rain fed areas to low land pastures in canal irrigated areas. If there are no rains the movement may take place early. The farmers move along with their families to other grazing areas and they have a fixed base. They have fixed routes of migration. The flocks are usually large and comprise of more than 100 animals. The males are sold often at low weight and females are retained for breeding purpose. These flocks derive their feed by grazing on rangelands and crop residues. Sometimes farmers have to pay for grazing on crop residues. Approximately, 32% of sheep and goats are raised under this system. Such type of production system is prevailing in all the provinces, tribal areas of the country and in Azad state of Jammu Kashmir.

### 1.11.3 Sedentary

Under this type of production system the flocks during the day are taken out for pasturing but return to the village late in the evening. A common practice is that various flocks are pastured collectively by a shepherd retained for this purpose. Unlike nomadic and transhumant system the animals inhabit the same habitat round the year. The flocks are usually limited to 30-50 animals. About 40% of sheep and goats are raised under this system. These flocks derive their feed by grazing on road-sides, waste land, water logged areas, canal banks and rangelands.

### 1.11.4 Household

Rural households keep around 4-7 animals. These are kept restricted close to the houses and pastured on weeds, household scraps and proximate grazing. About 22% of the small ruminants in the country are being raised under this system.

## 1.12 Population and Distribution of PPR Susceptible Species:

Pakistan has a combined small ruminants' population of 107 million (Goats = 76.1 million and sheep = 30.9 million) (livestock census, 2018-19).

## 1.13 PPR in Pakistan

In Pakistan the disease was first identified in 1991 (Athar et al., 1995). During the same period rinderpest like disease affecting goats was reported in other parts of the country (Pervez et al., 1993; Ayaz et al., 1997). The preliminary communiques were based on epidemiological, clinical and postmortem findings. The true confirmation for PPR presence was made possible in 1994 when RT-PCR was employed for its detection in tissues and blood of PPR affected goats (Amjad et al., 1996). Primarily, TCRV (tissue culture rinderpest vaccine) was adopted effectively for quite a while to control PPR due to close antigenic association between two viruses (Diallo et al., 2007; Aitken, 2008). However, its use was discontinued due to hindrance in sero-surveillance of RP after its eradication in 2003. Serious efforts to review the disease epidemiology methodically in Pakistan were initiated in 2002. The PDS (participatory disease searching) activity was carried out in more than 10,000 villages during 2002-05 which revealed the PPRV presence in all provinces and regions of the country. The documented evidence based on serology for

PPRV existence in towns investigated by PDS teams was also reported (Zahur et al., 2008). Later workers demonstrated continuous PPRV activity in all regions.

PPR outbreaks in Pakistan during the last decade have expanded to a disturbing level evolving in newer territories (Hussain et al., 2008). A report provided details of the presence of PPRV based on confirmed outbreaks by 50 laboratories and documented a prevalence of 40.98 % in the country. It was concluded that the outbreaks were more frequently observed during winter with more positive cases (30–60 %) in north and south parts of the country than south-west and western parts (10–30 %) (Abubakar et al., 2008). In an outbreak of PPR in Afghan (Bulkhi) sheep, a higher morbidity (88%) was recorded with a negligible mortality rate of 1.2% in lambs.

Goats respond seriously to PPR infection in Pakistan like other global places where endemic disease status is prevailing. The reports revealed that sheep developed no disease signs although kept in the same locality with the sick goats, rather sero-conversion was observed in sheep (Amjad et al., 1996; Abubakar et al., 2008). Analysis of data from 62 outbreaks occurring among small ruminants' stock in Azad Jammu & Kashmir (AJK) and all five provinces of Pakistan revealed an overall mean cumulative morbidity, mortality and case fatality to be 65.37%, 26.51% and 40.40% respectively for sheep and goats. The species specific mean cumulative morbidity (68.80%), mortality (29.45%) and case fatality (42.75%) were significantly higher for goats than those for sheep i.e. 48.77%, 14.98% and of 26.16% (Zahur et al., 2014). These studies suggest that disease is epidemic in Pakistan.

According to a recent report of Food and Agriculture Organization (FAO) of United Nations, the number of lab confirmed PPR outbreaks in Pakistan during January 2014 to June 2017 is 2593.

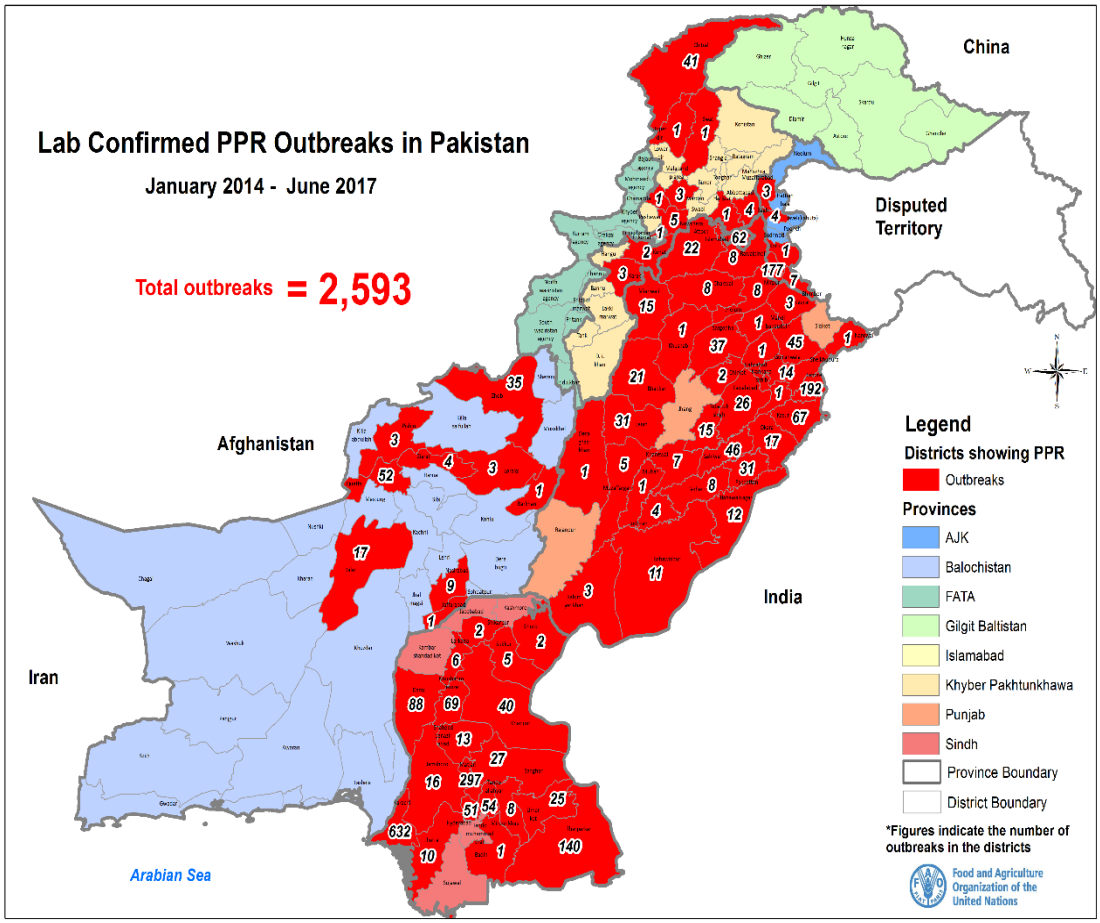



**Figure 1.2:** Lab confirmed PPR outbreaks during in Pakistan during January 2014 to June 2017

### 1.14 Sero-prevalence of PPR in Pakistan

It was estimated that there was 48.45% of PPR sero-prevalence (goats=52.89% and sheep=37.72%). AJK was found to have the highest overall sero-prevalence (55.17%) whereas Punjab province had the lowest (44.56%). It was found that 49.3 % of sheep and 65.9 % of goats more than two years of age experienced PPR. A linear relationship was found between seropositivity for PPR and age of the animal in goats (Zahur et al., 2011). Another study reported that in Punjab province an overall sero-prevalence in small ruminants were 43.33% (goats=39.02%, sheep 51.29%) (Khan et al., 2009). While during the same study in large ruminants of the same province, an overall sero prevalence of 59.09% was recorded with species specific estimates of 41.86% in cattle and 64.42% in buffaloes. In another study a prevalence of 44.15 % was recorded in goats than 54.9 % in sheep. The highest prevalence (55.10 %) was reported in small ruminants of Sindh province (Figure 1.3) (Abubakar et al., 2009).

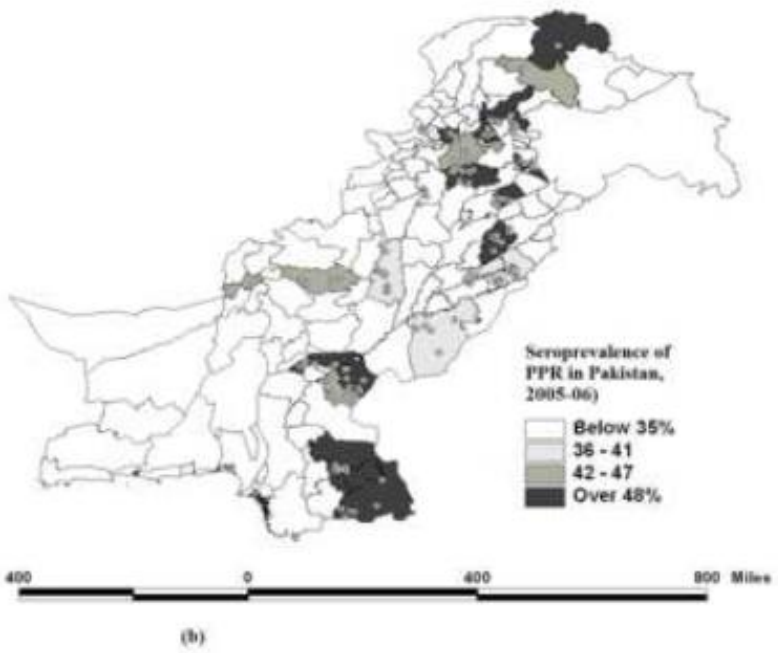



**Figure 1.3:** PPR Sero-prevalence in Pakistan (Source: Zahur et al., 2011/ Preventive Veterinary Medicine 102 (2011) 87–92)

## Aim and Objectives

The main aim of this study was to develop a thermostable live attenuated homologous lineage PPR vaccine. The objectives of the study were as following:

1. Isolation and characterization of Peste des Petits Ruminants virus (PPRV) from field outbreaks
2. Evaluation of thermostability potential of local isolates of PPRV
3. Attenuation of a thermo-tolerant strain of PPRV and pathogenicity analysis in goats
4. Development of a live attenuated thermo-tolerant PPR vaccine
5. Comparative efficacy of different chemical stabilizers on the thermostability of newly developed PPR vaccine

# Chapter 2

## Materials and Methods

# Chapter 2: Materials and Methods

## 2.1 Isolation and Characterization of Peste des Petits Ruminants Virus from field Outbreaks

### 2.1.1 Outbreak Investigation

During this study, suspected PPR outbreaks reported by resource persons designated in concerned areas were inspected by research team during the year 2012-2014. Sampling was conducted in different high risk tehsils within a livestock rich district in each province/region, selected with the consultation of provincial L&DD (Livestock and Dairy Development) departments as target areas of the project. These include Tehsil Darush of District Chitral in Khyber Pakhtunkhwa province, tehsil Umerkot in Sindh province, tehsil Chilas of district Diamer in province Giligit Baltistan and tehsil Barnala in district Bhimber AJK. The tehsil Liaqat Pur of district Rahimyar Khan was selected as project target area in Punjab province. The samples and field data was collected from the affected animals in different locations (Table 2.1).

### 2.1.2 Sample Collection

#### 2.1.2.1 Tissue, Swabs and Blood Samples

A total of 239 samples comprising of lymph nodes (mesenteric and bronchial), spleen and lungs were collected from dead goats and sheep at the time of necropsy. Swabs (Ocular and nasal) were collected from diseased goats and sheep. The swabs were dipped in 500µl of PBS (Phosphate buffered saline). Blood was collected from suspected sheep and goats by jugular vein puncture using sterile vacutainers to prevent clotting. The samples were marked with specific IDs (Identifiers) including date, place, age and sex. All the collected samples under aseptic conditions were shifted to laboratory in transfer boxes using frozen gel packs. The tissue samples were then placed at -70°C, swabs at -20°C and blood at 4°C till further use.

**Table 2.1:** Details of PPR suspected outbreaks confirmed during 2012-2014.

| Outbreak region | Frequency | Samples collected |
|---|---|---|
| KPK* | 13 | 79 |
| Gilgit Baltistan | 5 | 29 |
| Sindh | 4 | 18 |
| ICT** | 9 | 51 |
| Punjab | 8 | 48 |
| AJK*** | 3 | 16 |
| Baluchistan | 1 | 8 |
| **Total** | **43** | **239** |

*KPK: Khyber Pakhtunkhwa **ICT: Islamabad Capital Territory ***AJK: Azad Jammu and Kashmir

### 2.1.3 PPRV Antigen Detection in Clinically Suspect Sheep and Goats Using RT-PCR (Reverse Transcriptase Polymerase Chain Reaction)

The tissue samples (n=71) collected from dead animals at necropsy and swabs (n=168) from suspected goats and sheep were examined in the laboratory for the presence of PPRV antigen. One step RT-PCR kit (QIAGEN) was used for this purpose according to the manufacturer's instructions.

#### 2.1.3.1 Sample Processing

The infected tissue (lymph nodes, spleen and lungs) was taken (approximately 1 g) and processed by grinding in sterile mortar and pestle. The 10% homogenate was made in sterile PBS (0.01M, pH 7.4). Cotton swabs were squeezed into sterile 1.5ml eppendorf tubes.

#### 2.1.3.2 Extraction of RNA

An Invitrogen kit (Life Technologies) was used for RNA extraction following manufacturer's instructions. Briefly, 25µl of Proteinase K (provided with the kit) was added into a sterilized centrifuge tube. Tissue/swab sample (200 µl) was added into same

tube. After that, 200µl of lysis buffer (comprising of 5.6 µg carrier RNA) was added and contents were vortexed for approximately 15 seconds. After incubation for 15 minutes at 56°C, the tube was centrifuged briefly. At this point, 250 µl of 96-100% of ethanol was poured into the lysate tube and mixed by vortexing vigorously for 15 seconds. The tube was then incubated for 5 minutes at room temperature and briefly centrifuged. At this point, the tube contents were transferred onto the spin column and centrifuged for 1 minute at 6800 x g. The collection tube containing flow through was discarded and spin column was placed in another clean wash tube. The spin column was washed twice with 500 µl of wash buffer and centrifuged for 1 minute at 6800 x g. The wash tube was replaced with the new clean tube and spin column was centrifuged for 1 minute at maximum speed to dry the membrane completely. The wash tube was discarded again with the flow through. The spin column was then placed in a new sterile 1.5ml eppendorf tube and 40µl of RNase-free water was added exactly in the center of column for viral RNA recovery. The tube was incubated for 1 minute at room temperature and centrifuged at maximum speed for 1 minute. The spin column at this point was discarded and recovery tube (containing purified RNA) was stored at -70°C till further analysis.

**2.1.3.3 RT-PCR** (Reverse Transcription Polymerase Chain Reaction)

The RT-PCR employing amplification of N gene was standardized for PPR virus confirmation (Forsyth and Barrett, 1995). The assay was performed using QIAGEN One-step RT-PCR kit following manufacturer's instructions.

**2.1.3.3.1 Assay Protocol**

DNA was amplified with PPRV specific primers employing highly conserved N gene sequence. The primer sequences of N gene of PPRV are given in Table 2.3 (Couacy-Hymann et al., 2002). All the components in the kit including 5x QIAGEN one step RT-PCR buffer, dNTP mix, RNase free water (Table 2.2), forward and reverse primers and template RNA were thawed and vortexed before use and put on ice. The PCR was performed in clean 0.2ml capacity PCR tubes (Biologix) and final reaction volume was adjusted to 25µl for one reaction. Briefly, the master mix was prepared in a clean Eppendorf tube by adding all contents of PCR kit given in the Table 2.1 except template RNA and distributed equally into each PCR tube. In the last, 5µl of template RNA was added

separately in all tubes. The negative control tube contains an equal quantity of RNase free water. The tubes were then given a short spin after vortexing to mix the contents of the tubes. All tubes were then placed in thermal cycler (Veriti 96 well Thermal Cycler 9902 by Applied Biosystems, USA) and RT-PCR program was used as described in Table 2.4. Positive and negative controls were included in each test i.e. RNA extraction and RT-PCR. PPR vaccine (Nigeria 75/1) served as a positive control. A negative control (RNase free water) was used to trace any possible contamination by reagents.

**Table 2.2:** Contents of QIAGEN One step RT-PCR kit and composition of master mix

| **Kit reagents** | **One step QIAGEN Mix for 1 reaction** | **Final concentration** |
|---|---|---|
| Nuclease-free water | 15 | - |
| QIAGEN Buffer (5X) | 10 | 1x |
| dNTP mix (each of 10mM) | 2 | 400µM of each dNTP |
| NP3 10uM | 3 | 0.6µM |
| NP4 10uM | 3 | 0.6µM |
| QIAGEN Enzyme mix | 2 | - |
| Q solution | 10 | - |
| RNA | 5 | - |
| **Total** | **50µl** | - |

**Table 2.3:** PPRV specific primer details (N gene)

| **Primer ID** | **Forward/ Reverse** | **Sequence (5'----------3')** | **Position** | **Product size** |
|---|---|---|---|---|
| NP3 | F | GTCTCGGAAATCGCCTCACAGACT | 1232–1255 | 351bp |
| NP4 | R | CCTCCTCCTGGTCCTCCAGAATCT | 1583–1560 | |

F Forward (Genome sense) primers

R Reverse (anti genome sense) primer

**Table 2.4:** Thermal profile used for PCR amplification

| S.No. | Steps | | Temperature | Time |
|---|---|---|---|---|
| 1 | Reverse Transcription | | 50°C | 30 minutes |
| 2 | Initial denaturation | | 95°C | 15 minutes |
| 3 | Cyclic amplification (40 cycles) | Denaturation | 94°C | 30 seconds |
| | | Annealing | 60°C | 30 seconds |
| | | Extension | 72°C | 1 minute |
| 4 | Final extension | | 72°C | 10 minutes |
| Hold at 4°C till further analysis | | | | |

### 2.1.3.4 Analysis of PCR Amplified Product

The analysis of amplified DNA was carried out by gel electrophoresis using 1.5% agarose gel in 1x TBE buffer (Appendix-2) and stained with syber safe @ 7µl /100ml. Briefly, 3µl of 6x DNA loading dye (Thermo Scientific) was mixed with 10µl of amplified product and run in parallel with 100bp plus DNA ladder (Thermo Scientific) at a concentration of 0.1µg/µl and 80mA for 40 minutes. The gel was visualized for expected band size in gel documentation system (Syngene, DR4V3/1015).

### 2.1.4 Ic-ELISA (Immunocapture ELISA)

Ic-ELISA was performed using the kit produced jointly by BDSL (Biological Diagnostic Supplies Limited) with the collaboration of CIRAD, EMVT, France and Flow Laboratories following manufacturer's instructions. The absorbance was measured at 492nm wavelength. The EDI (ELISA Data Interchange) software was used to calculate percent positivity (PP) values (FAO/IAEA, Vienna, Austria). The following formula was used to convert optical densities to percentage positivity:

$$PP = \frac{100 - (\text{OD of control / test sample})}{\text{OD reference antigen}} \times 100$$

The sample with PP > 18% was considered as positive (Libeau et al., 1995).

## 2.1.5 PPRV Isolation and Propagation

### 2.1.5.1 Cell Lines

Two types of cell lines were used in this study for isolation of PPRV.

- Vero-76 (African green monkey kidney cells)
- BTS-34 (CV1 cells constitutively expressing bovine SLAM using the Flp-In system)

The tissue and swab samples declared positive by both Ic-ELISA and RT-PCR were attempted for virus isolation by inoculating onto these cell lines.

#### 2.1.5.1.1 Vero Cells

Low passaged Vero cells (Vero-76, ATCC Number: CRL-1587™) were maintained in tissue culture laboratory of Animal Health Program, Animal Sciences Institute, National Agriculture Research Center. These cells were cultivated in GMEM (Glasgow Minimum Essential Medium) augmented with 10% FBS (fetal bovine serum) produced by Sigma-Aldrich, 1% mixed antibiotics/antimycotic solution.

#### 2.1.5.1.2 BTS Cells

Low passaged BTS-34 cell line was received with the courtesy of CIRAD (Center for International Cooperation in Agricultural Research for Development), France. The cells were maintained in DMEM (Dulbecco's Modified Eagle Medium), 10% FBS 1% mixed antibiotic/ antimycotic solution supplemented with 600µg/ml of hygromycin B (Sigma-Aldrich).

### 2.1.5.2 Sub Culturing of Cells

A pre-cultured flask of Vero or BTS cells with 80-90% of confluency was trypsinized following split ratio method (Florence et al., 1992). Briefly, the media in the flask having confluent monolayer of cells was discarded and the cells were washed using phosphate buffered saline (PBS) by gently swirling the solution on the monolayer. After washing, the solution was discarded and 1ml Trypsin-EDTA solution (Versene 0.02%, trypsin 0.05%) was poured into the cell flask. The solution was discarded after about 1 minute and the

flask was incubated at 37ºC for 1 to 2 minutes. Monolayer in the flask was observed under the microscope. Once the cells became rounded and detached, 6 ml of growth media (Appendix-3) was added into the culture flask. Gentle pipetting of media in culture flask was carried out to break down the clumping of cells for making an even suspension. This cell suspension (1:3 split ratio) in the medium was distributed equally into three tissue culture flasks (25 $cm^2$) each obtaining 2ml of cell suspension. Then an additional 4ml of growth medium was poured into the flask and incubated at 37ºC with 5% $CO_2$ necessary for the growth of cell. The cells were observed under the microscope after 24 or 48 hours of incubation for cell attachment and confluency.

#### 2.1.5.3 Inoculum Preparation

A total of 80 tissue samples (spleen, lymph nodes and lungs) tested positive for PPR virus antigen by Ic-ELISA (Immuno-capture enzyme-linked immunosorbent assay) and RT-PCR (Reverse transcriptase polymerase chain reaction) were inoculated simultaneously onto Vero and BTS cell lines. Briefly, the inoculum was prepared by grinding approximately 1g of infected tissue using sterilized mortar and pestle. After grinding, 500µl of sterile PBS with antibiotic solution was added and a 10% suspension (W/V) was made. The homogenate was then transferred to a sterile eppendorf tube and clarified by centrifuging for 15 minutes at 1500 rpm. The supernatant was collected into another sterilized eppendorf tube. A further fivefold dilution was made by adding 2 ml of supernatant to 8 ml of sterile GMEM and filtered using a 0.2 µm filter (Minisart NML). The resultant filtrate was used as inoculum in Ic-ELISA, RT-PCR and virus isolation.

#### 2.1.5.4 Inoculation onto Vero cells

Briefly, a sub-confluent flask of Vero cells was washed with PBS. After thorough washing, 0.5 ml of prepared inoculum of suspected samples was added into the flasks after filtration with 0.2µ filter. The control flask was inoculated with 500µl of sterile GMEM only. The flasks were incubated for 1 hour at 37°C to adsorb the virus. The inoculum was discarded and 6 ml of growth media was poured into the respective flasks. Each flask was labeled and placed at 37ºC in an incubator. The media was changed on alternate days. Flasks were observed daily until the CPEs became visible. The flasks showing 80% of CPEs were harvested. However, for the cultures showing no CPEs, a blind passage was made using

freeze/thaw method. The sample was considered negative if no CPEs were observed till 3 successive blind passages. The positive culture flasks were stored at -80°C till further analysis.

#### 2.1.5.5 Identification of PPR Virus

The PPR virus was identified by:

a. Characteristic CPEs produced by the isolates on Vero cells
b. Testing of cell culture supernatant for confirmation of virus using RT-PCR as described in section 2.1.3.

#### 2.1.5.6 Comparative Pattern of PPRV Growth on Vero and BTS Cell Lines

Tenfold serial dilutions of eighteen PPR virus local isolates were made and added in micro-titration plates seeded with Vero and BTS cells at a density of $0.01x10^6$. The plate was incubated for 24 hours at 37°C. The CPEs were observed and recorded. The tissue culture infective dose fifty (TCID50) was calculated following Reed and Munch method (Reed and Muench, 1938).

## 2.1.6 Genetic Characterization of PPRV

### 2.1.6.1 PCR Purification

Purification of amplified PCR products was done by QIAGEN PCR purification kit (QIAquick) following manufacturer's instructions. Briefly, buffer PB (5 volumes) was added to the PCR product (1 volume) and mixed gently. The mixture was added to the spin column which was placed onto a 2ml collection tube to bind DNA and centrifuged at 13000rpm for one minute in super speed refrigerated centrifuge (Sanyo, MSE, Hawk 15/05, UK). The spin column was placed onto the same collection tube after discarding the flow through. For washing DNA, buffer PE (750µl) was added to the spin column and centrifuged at 13000rpm for one minute. The flow through was again discarded and spin column was placed onto the same collection tube. The column with the collection tube was centrifuged at 13000rpm for one minute to remove completely the remaining ethanol from buffer PE. The spin column was then placed in a sterile 1.5ml eppendorf. For DNA elution, 40µl of water (pH 7-8.5) was added central to the QIAquick column. The column was placed at room temperature for one minute and then centrifuged at 13000rpm for an additional one minute. The spin column at this point was discarded and the eppendorf tube containing purified DNA was then stored at -20°C till further analysis.

### 2.1.6.2 Sequencing of Purified PCR Products

Sequencing of purified PCR products was conducted using Beckman Coulter Dye Terminator Cycle sequencing Quick start kit following manufacturer's instructions. Briefly, 5µl of PCR water was added in 0.2ml capacity clean PCR tubes. Then 3µl of DTCS (Dye terminator cycle sequencing) mix, 1 µl of forward primer and 1µl of purified PCR product was added in each PCR tube. The contents of PCR tubes were mixed thoroughly by centrifugation for short time. The tubes were then placed in Thermal cycler (Swift maxi, ESCO, USA) set at desired thermal profile given in Table 2.5.

**Table 2.5: Thermal profile used for sequencing**

| S.No. | Steps | | Temperature | Time |
|---|---|---|---|---|
| 1 | Cyclic amplification (35 cycles) | Denaturation | 94°C | 30 seconds |
| | | Annealing | 60°C | 30 seconds |
| | | Extension | 72°C | 1 minute |
| 2 | Final extension | | 72°C | 5 minutes |
| Hold the reaction at 4°C till further use | | | | |

### 2.1.6.3 Ethanol Precipitation

The sequencing reaction was stopped by pouring 5µl of freshly prepared stop solution (2µl of 3M sodium acetate at pH 5.2, 2µl of 100mM, EDTA disodium salt at pH 8, and 1µl of 20mg/ml of glycogen) in PCR tube. All contents of tube were transferred in another labelled eppendorf and mixed completely. Then 60µl of cold ethanol 95% (v/v) was added into the tube and mixed thoroughly. The tube was centrifuged for 15 minutes at 14000 rpm in a refrigerated centrifuge. Using a micropipette, the supernatant was removed carefully and pellet was washed twice with 200µl of 70% cold ethanol and centrifuged twice after each wash for 2 minutes at 14000 rpm. The supernatant was again removed carefully and pellet was vacuum dried for 10 minutes. Finally, a 40µl of sample loading solution (Dimethyl sulphoxide) was used for re-suspension of sample.

### 2.1.6.4 Loading of Samples in Sequencer

The re-suspended samples were poured in respective wells of sequencer plate. The wells were then overlaid with mineral oil (1 drop). The plate was loaded in sequencer (GeXP, Beckman Coulter, USA) for desired sequencing program. Specific primers (Forward and reverse) were used to sequence both strands of PPRV.

### 2.1.6.5 Sequence Data Analysis

The sequence data for forward and reverse sequences was assembled and edited using ApE a plasmid editor Version 2.0.50. The corrected nucleotide sequences from both strands were complimentary aligned with PPRV N gene sequences already available in Gene Bank using MEGA 7 and a consensus sequence from each strain was generated. The final sequences were submitted in the Gene Bank.

### 2.1.6.6 Phylogenetic Analysis

The sequences were aligned using ClustalW algorithm in the BioEdit and were trimmed to obtain uniform lengths. The phylogenetic analysis was executed by constructing phylogenetic tree using Kimura two-parameter model in Neighbor-joining method implemented in Molecular Evolutionary Genetics Analysis (MEGA) Program, version 7. The significance of generated tree was evaluated by bootstrap of 1000 replicates (Kumar et al., 2014).

## 2.1.7 Full Genome Sequencing of PPRV

Two sequencing methods were used for sequencing of PPRV genome.

1. Sequencing by random amplification
2. Sequencing by specific amplification

### 2.1.7.1 Sequence-Independent Single-Primer Amplification (SISPA) or Random Amplification Sequencing

#### 2.1.7.1.1 Study Samples

Three PPRV isolates (Table 2.6) were used for full genome sequencing. These isolates were previously isolated using Vero-76 and BTS-34 cells and tested positive by RT-PCR using N gene specific primers (NP3/NP4) for PPRV. The representative isolates selected for sequencing belonged to different areas to oversee the changes in circulating strains of PPRV in the country (Table 2.6).

**Table 2.6:** Representative isolates used for full genome sequencing

| S. No. | IDs of samples | Sample area | Year of isolation | Lineage |
|---|---|---|---|---|
| **1** | PAK*-LRS-13/NARC* | Taxila (Punjab) | 2013 | IV |
| **2** | PAK-ICT*-12/NARC | ICT | 2012 | IV |
| **3** | PAK-MZD*-13/NARC | Muzaffarabad (AJK) | 2013 | IV |

*PAK=Pakistan, MZD=Muzaffarabad, ICT=Islamabad Capital Territory, NARC=National Agricultural Research Centre

#### 2.1.7.1.2 Extraction of RNA

RNA extraction was performed using an Invitrogen kit (Life Technologies) as described in section 2.1.3.2. Viral RNA was reverse-transcribed using universal and tagged primers (Victoria et al., 2009) (Table 2.9). Each sample was assigned a tag (Sequence of 20 bp), allowing several samples to be grouped on the same sequencing run.

### 2.1.7.1.3 cDNA Synthesis for Next Generation Sequencing (NGS) Illumina MiSeq (Part 1)

First strand cDNA synthesis kit RevertAid (Fischer Scientific) was used following manufacturer's instructions. A set of universal and tagged primers were used (Table 2.9). Briefly, 7.5 µl of RNA was mixed with 5 µl of primer (tagged) (20µM) and incubated for 5 min at 65°C. To this mixture, following reaction mixture was prepared and added (Table 2.7).

**Table 2.7:** Contents of RevertAid (Fischer Scientific) kit and composition of master mix

| **Kit reagents** | **RevertAid Mix for 1 reaction (**µl) |
|---|---|
| Buffer (5X) | 4 |
| dNTP mix (each of 10mM) | 2 |
| Rnase out | 0.5 |
| RevertAid enzyme | 1 |
| **Total** | **7.5µl** |

A 7.5 µl of RevertAid mixture was added in tube containing 7.5 µl of RNA and 5 µl of primer previously heated. The negative control tube had an equal quantity of nuclease free water. The tubes were then given a short spin after vortexing to mix the contents of the tubes. All tubes were then placed in thermal cycler (Veriti 96 well Thermal Cycler 9902, Applied Biosystems, USA) and cDNA synthesis program was used as described in Table 2.8.

PPR vaccine (Nigeria 75/1) strain served as a positive control. A negative control (Nuclease free water) was used to trace any possible contamination by reagents.

**Table 2.8:** Thermal profile used for first strand cDNA synthesis

| S.No. | Steps | Temperature | Time |
|---|---|---|---|
| 1 | Heating | 25°C | 10 minutes |
| 2 | Reverse transcription | 42°C | 60 minutes |
| 3 | Reaction termination | 70°C | 10 minutes |
| Hold at 4°C till further use | | | |

**Table 2.9:** Sequences of the tagged primers and primers for extremities

| Primer tagged | Sequence | |
|---|---|---|
| A | ATCGTCGTCGTAGGCTGCTC | |
| B | GTATCGCTGGACACTGGACC | |
| C | CGCATTGGTCGGCACTTGGT | |
| D | CGTAGATAAGCGGTCGGCTC | |
| E | CGCAGGACCTCTGATACAGG | |
| F | CGCAGGACCTCTGATACAGG | |
| I | CCGAGGTTCAAGCGAGGTTG | |
| J | ACGGTGTGTTACCGACGTCC | |
| **Primers to be used in combination with TAG E/A to get the terminal regions of the genome** | | |
| N-terminal region | PPRV-315R | CATGCTGATCATCACGCCG |
| C-terminal region | PPRV-15619F | GTCTTGCTATATTCTGATGA |

### 2.1.7.1.4 cDNA Synthesis for NGS Illumina MiSeq (Part II)

Second strand cDNA synthesis kit DNA polymerase I, large (klenow) Fragment (Fischer (Fischer scientific) was used following manufacturer's instructions. Briefly, added 10 µl cDNA prepared previously (Part 1) to 2.5 µl of same previously used primer tagged (20 µM) in each tube. The reaction mixture was prepared (Table 2.10) and added in this tube

**Table 2.10:** Contents of double strand cDNA Klenow kit and composition of master mix

| Kit reagents | RevertAid Mix for 1 reaction (µl) |
|---|---|
| Buffer (10X) | 3 |
| dNTP mix (0.5mM) | 1 |
| DNA pol1 (0.5U/µl) | 1 |
| Water | 12.5 |
| **Total** | **17.5** |

A 17.5 µl of RevertAid mixture was added in tube containing 10 µl of cDNA and 2.5 µl of primer tagged previously heated. The negative control tube had an equal quantity of nuclease free water. The tubes were then given a short spin after vortexing to mix the contents of the tubes. The tubes were placed at room temperature (25°C) for 60 minutes.

PPR vaccine strain (Nigeria 75/1) served as a positive control. A negative control (Nuclease free water) was used to trace any possible contamination by reagents.

**2.1.7.1.5 Polymerase Chain Reaction (PCR)**

The assay was performed using Phusion-High Fidelity DNA Polymerase kit (Thermo Scientific) following manufacturer's instructions. Briefly, all the components in the kit including Phusion 10X buffer, dNTP mix, nuclease free water (Table 2.11) and template DNA were thawed and vortexed before use and put on ice. The PCR was performed in clean 0.2ml capacity PCR tubes (Biologix) and final reaction volume was adjusted to 50µl for one reaction. Briefly, the master mix was prepared in a clean Eppendorf tube by adding all contents of PCR kit given in the Table 2.11 except template DNA and distributed equally into each PCR tube. In the last, 5µl of template DNA was added separately in all tubes. The negative control tube contains an equal quantity of nuclease free water. The tubes were then given a short spin after vortexing to mix the contents of the tubes. All tubes were then placed in thermal cycler (Veriti 96 well Thermal Cycler 9902, Applied Biosystems, USA) and PCR program was used as described in Table 2.12

PPR vaccine strain (Nigeria 75/1) served as a positive control. A negative control (Nuclease free water) was used to trace any possible contamination by reagents.

**Table 2.11:** Contents of Phusion-High Fidelity DNA Polymerase kit and composition of master mix

| Kit reagents | Phusion Mix for 1 reaction (µl) |
|---|---|
| Nuclease-free water | 31.5 |
| Buffer (10X) | 10 |
| dNTP mix (each of 10mM) | 1 |
| Tagged primers (5uM) | 2 |
| Phusion polymerase | 0.5 |
| DNA | 5 |
| **Total** | **50** |

**Table 2.12:** Thermal profile used for PCR amplification

| S.No. | Steps | | Temperature | Time |
|---|---|---|---|---|
| 1 | Initial denaturation | | 98°C | 30 seconds |
| 2 | Cyclic amplification (40 cycles) | Denaturation | 98°C | 10 seconds |
| | | Annealing | 65°C | 20 seconds |
| | | Extension | 72°C | 30 seconds |
| 3 | Final extension | | 72°C | 10 minutes |
| Hold at 4°C till further analysis | | | | |

### 2.1.7.1.6 Analysis of PCR Amplified Product

The analysis of amplified DNA was carried out by gel electrophoresis as described in section 2.1.3.4.

### 2.1.7.1.7 Gel extraction of PCR products

The 300bp bands were cut from the agarose gel and gel bands were then purified using QIAquick Gel Extraction Kit (Qiagen) following manufacturer's instructions. The PCR product was then eluted in 20 µl of nuclease free water. The Qubit fluorometer was used to determine the concentrations of RNA and DNA in PCR products.

#### 2.1.7.1.8 Sequencing and Bioinformatics analysis

The PCR products were then sent to Macrogen Inc. for full genome sequencing. The company first prepared the sequencing libraries with the TruSeq kit and then performed the sequencing on a HiSeq 2500 in 250bp paired-end mode. The forward and reverse reads (small sequences generated during sequencing) obtained were de-multiplexed and sorted according to the tag used during the PCR. The sequences corresponding to each PPR sample were grouped together in a single file in fasta format. The tags were removed from the reads and the forward and reverse reads were assembled. The contigs thus obtained were aligned on a complete reference genome (Vaccine strain Nigeria 75/1). All the data manipulations were carried out using the tools available on the SAMtools (Li et al., 2009) and bwa (Li and Durbin, 2009) software accessible on the Genetool plateform (CIRAD). The results of the final assembly obtained were visualized with IGV software (Robinson et al., 2011). The final (full or partial) genome consensus sequence was obtained with Geneious v. 2020.2.

### 2.1.7.2 Sequencing by specific amplification

This technique is based on the PCR amplification of the long fragments of viral genome using specific primers covering the whole genome of PPRV. Sequencing was done from the PCR products obtained. A total of five pairs of primers (forward and reverse) namely F1, F2, F3, F4 and F5 were synthesized (Table 2.13) employing conserved regions of PPRV genomes from alignments of 48 complete genomes (GenBank and unpublished CIRAD genomes). These primers were used to amplify 3000 to 4000bp fragments and overlap by about 300 to 400bp (Figure 2.1).

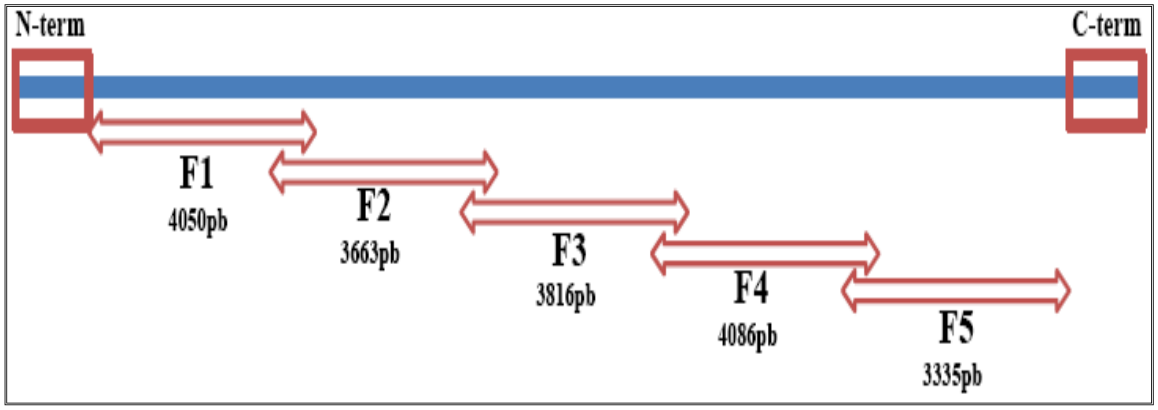



**Figure 2.1:** Specific primers designed for full genome sequencing of PPRV with product sizes

**Table 2.13:** Primers for amplification of long overlapping PPRV fragments used for full genome sequencing of PPRV

| Primer ID | Forward/Reverse | Sequence | Position | Product size |
|---|---|---|---|---|
| Fragment F1 | PPRV-3F | CAAACAAAGTTGGGTAAGGA | 3-4053 | 4050bp |
| | PPRV-4053R | TTCTCCCATGAGCCGACTATGTA | | |
| Fragment F2 | PPRV-3394F | TATTACAAAAAACTTAGGAGCAAGGG | 3394-7057 | 3663bp |
| | PPRV-7057R | CAGCAGCATATTAATGTGACAAG | | |
| Fragment F3 | PPR-6025F | CTTGCAAATAAGGAGACCATACT | 6025-9841 | 3816bp |
| | PPRV-9841R | GGCATATATGGGACTGTTCTTT | | |
| Fragment F4 | PPRV-9145F | ATAGAGGTAACATGCAATCCAG | 9247-13066 | 3800bp |
| | PPRV-13231R | TCATTGGAGATGGTTGTGTA | | |
| Fragment F5 | PPR-12607F | CGCCTTTCCAATTATGATTA | 12607-15942 | 3335bp |
| | PPRV-15942R | GAGACAAAGCTGGGAATAGA | | |

F Forward (Genome sense) primers

R Reverse (anti genome sense) primer

#### 2.1.7.2.1 Study samples

The isolates attempted for SISPA were also used in specific amplification. The details of isolates used are given in Table 2.6.

#### 2.1.7.2.2 cDNA synthesis using five pairs of specific primers

Viral RNA was extracted using an Invitrogen kit (Life Technologies) as described in section 2.1.3.2. The extracted RNA was reverse-transcribed into cDNA using 5 pairs of specific primers (F1 to F5) (Table 2.13). For reverse transcription, 2µl of RNA from each sample was mixed with 2 µl each of forward and reverse primers (10µM) and 10µl of nuclease free water. The mix was incubated at 65°C for 5 minutes and then kept on ice. To this mixture, 4µl of 5X buffer, 1µl of RNaseOut, 2µl of d NTPs (10 mM) and 1µl of RevertAid reverse transcriptase from RevertAid first strand cDNA synthesis kit (Fischer scientific) were added. The reaction was carried out at 42°C for 60 minutes. The reaction was then terminated by final heating at 70°C for 5 minutes. The resulting cDNA was stored at -20°C till PCR amplification.

#### 2.1.7.2.3 Amplification

Two types of PCR kits were used for amplification, the Kapa (Biosystems) and Phusion-High Fidelity DNA Polymerase kit (Thermo Scientific). Both of these kits use DNA polymerase enzymes for efficient polymerization activity and with less error rate due to their exonuclease activity. Standardization of kit protocols were done for effectively amplifying PPRV strains with specific primers.

##### 2.1.7.2.3.1 Amplification using Phusion kit

PCR amplification was carried out using same primers as those for reverse transcription. Briefly, 5 µl of cDNA was added to the following PCR mix using Phusion High-Fidelity DNA Polymerase kit.

**Table 2.14:** Contents of Phusion-High Fidelity DNA Polymerase kit and composition of master mix

| Kit reagents | Phusion Mix for 1 reaction (µl) |
|---|---|
| Nuclease-free water | 29.5 |
| Buffer (10X) | 10 |
| dNTP mix (each of 10mM) | 1 |
| Forward primer (20uM) | 2 |
| Reverse primer (20uM) | 2 |
| Phusion polymerase | 0.5 |
| DNA | 5 |
| **Total** | **50** |

PCR was performed according to program depending upon the primer pair used as for each pair of primers, a different hybridization temperature was required for a specific elongation time. An initial denaturation step was carried out at 95°C for 1 minute. Then 40 cycles of denaturation at 98°C for 20 seconds, annealing for 20 seconds (Table 2.15), and extension 72°C (Table 2.15) and a final extension step at 72°C for 10 minutes

**Table 2.15:** Different PCR conditions (annealing temperature and elongation time) for each set of specific primers

| Fragment | Annealing temperature | Extension time |
|---|---|---|
| F1 | 60°C | 5 minutes |
| F2, F4, F5 | 55°C | 5 minutes |
| F3 | 62°C | 3 minutes |

#### 2.1.7.2.3.2 Amplification using Kapa kit

Briefly, 5 µl of cDNA was added to the following PCR mix using Phusion High-Fidelity DNA Polymerase kit.

**Table 2.16:** Contents of Kapa kit and composition of master mix

| Kit reagents | Phusion Mix for 1 reaction |
|---|---|
| Nuclease-free water | 29.5 |
| Buffer (10X) | 10 |
| dNTP mix (each of 10mM) | 1.5 |
| Forward primer (20uM) | 1.5 |
| Reverse primer (20uM) | 1.5 |
| Kapa enzyme | 1 |
| DNA | 5 |
| **Total** | **50µl** |

PCR was performed according to the following program for F2, F3, F4 and F5. An initial denaturation step was carried out at 95°C for 3 minute. Then 10 cycles of denaturation at 98°C for 20 seconds, annealing at 50°C for 15 seconds and extension at 72°C for 5 minutes then 25 cycles of denaturation at 98°C for 20 seconds, annealing at 55.2°C for 15 seconds and extension at 72°C for 5 minutes and final extension at 72°C for 4 minutes.

For the set of F1 primers, PCR was carried out according to the following program. An initial denaturation step was carried out at 95°C for 3 minute. Then 10 cycles of denaturation at 98°C for 20 seconds, annealing at 55°C for 15 seconds and extension at 72°C for 4 minutes then 25 cycles of denaturation at 98°C for 20 seconds, annealing at 58.9°C for 15 seconds and extension at 72°C for 4 minutes and final extension at 72°C for 4 minutes.

The amplification products obtained with both amplification were separated by electrophoresis on 1.5 % agarose gel. The bands (between 3000bp and 4000bp) were cut from the gel and were then purified using QIAquick Gel Extraction Kit (Qiagen) following manufacturer's instructions. The PCR products were eluted in 42µl of nuclease free water.

The Qubit fluorometer was used to determine the concentrations of RNA and DNA in PCR products.

#### 2.1.7.2.4 Preparation of sequencing libraries

Sequencing libraries were prepared for the sequencing of complete genome by following the Nextera XT DNA protocol. It consists of three essential steps. The first step is DNA tagmentation that fragments DNA and then labels DNA with adapter sequences in one step. The second step is amplification of the libraries which amplifies the tagged DNA by adding the index 1 and index 2 adapters required for cluster formation. The third step allows the libraries to be cleaned by using AMPure XP beads to purify the DNA from the library and selects based on the size of the fragments. This step removes small fragments from the library.

#### 2.1.7.2.5 Library quality control

A library verification step is necessary before sequencing. First, a quantitative PCR (Q-PCR) (Library quantification kit, Takara Clonetech) assesses the exact amount of material for each library. Then, analysis with the bioanalyzer (Agilent 2100 Bioanalyzer, Agilent High Sensitivity DNA kit, Agilent Technologies) makes it possible to evaluate the size distribution of the fragments within each library. The quality and average size of each library were used to equimolarly pool the libraries to be sequenced in the same run on the Illumina platform.

#### 2.1.7.2.6 Sequencing and bioinformatics analysis

The data obtained was subjected to the bioinformatics processing.

#### 2.1.7.2.7 Phylogenetic and molecular data analysis from complete and incomplete genomes

Molecular data analysis was performed on a sequence alignment of complete or near complete PPR genomes (without 1-5 nucleotides at the end) including the sequences obtained during this study as well as complete genomes from Genbank. The same analysis was performed on an alignment including incomplete genomes (>90% coverage) at the baseline alignment. Geneious Prime v 2020.2 software was used to carry out the alignments

with the MAFFT (v7.450) method (Katoh and Standley, 2013). From the alignments obtained, the phylogentic tree was constructed using the Maximum Likelihood method with 1000 bootstraps on MEGA 7 (Kumar et al., 2016). A comparative analysis of complete genome sequences of 3 present study isolates and one Nigerian strain (Accession No. HQ197753.1) retrieved from database of GenBank was also performed using Genious Prime v 2020.2.

## 2.2 Evaluation of Thermostability of Local Isolates of Peste des Petits Ruminants (PPR) Virus

### 2.2.1 Cell Stocks

Vero cell line derived from American Type Culture Collection (ATCC) was used in this experiment. The cells were maintained in Glasgow Minimum Essential Medium (GMEM) supplemented with 10% fetal bovine serum (Sigma Aldrich) and 10% tryptose phosphate broth at $37^0$C in an incubator.

### 2.2.2 PPR Virus Isolates

A total of seven local isolates of PPRV were used to select most thermo-tolerant strain of PPR virus on the basis of genotyping and immunogenicity.

These isolates were previously isolated from outbreak samples (Table 2.1), lyophilized and stored at repository of Animal Health Program of Animal Sciences Institute of NARC, Islamabad and were revived on Vero cells. The lyophilized viruses were reconstituted in phosphate buffered saline (PBS) solution and inoculated onto semi-confluent Vero cells after thorough washing with PBS. The viruses were given one hour of adsorption at 37°C. After that 10% GMEM was added to each flask and incubated for 24 hours at 37°C. When the cytopathic effects were 70-80%, the virus was harvested and centrifuged at 2000rpm for 5 min for clarification. The cell free supernatant was stored at $-80^0$C till further use. The infectious titer of virus stocks was determined by calculating 50% tissue culture infective dose ($TCID_{50}$) as given in section 4.2.1. The initial titers of seven different isolates of PPR virus (undiluted) are given in Table 4.3. For experimental purpose, the initial titers of all the isolates were homogenized by diluting the viruses having more initial titers (Table 4.3).

### 2.2.3 Viral Stability Assay

PPRV stock with an initial titer of $10^{4.9}$ TCID50/ml was dispensed into 1.5ml micro-centrifuge tubes (100ul in each tube) and incubated at 37°C, RT (25 ± 2°C) and 4°C. One of the aliquots at 37°C was removed every 3h, while those incubated at RT or 4°C were

removed every day or every 2 days, respectively, and stored at -70°C until virus titration on Vero cells. The stability experiment description is given in Table 4.4.

### 2.2.4 Heat Treatments

PPRV stock with an initial titer of $10^{4.9}$ TCID50/ml was dispensed into 1.5ml micro-centrifuge tubes (100ul in each tube) and incubated in water baths with temperatures of 56°C, 60°C, and 65°C, respectively. At designated time points, aliquots were removed, transferred immediately into ice blocks and then subjected to TCID50 assay for virus titration.

### 2.2.5 UV-C (Ultraviolet-C) Light Irradiation

Aliquots containing two hundred microliter of a PPRV stock ($10^{4.9}$ TCID50/ml) were placed in 48-well plates to give a volume depth of about 0.2 cm and then exposed to continuous UVC (Ultraviolet-C) light 30 cm beneath the longitudinal midpoint of a UV-C lamp (model: ZSZ20D, wavelength = 253.7 nm, Beijing Haidian Konghou High Temperature Composite Material Factory, China). At the distance of 30 cm, the radiant intensity of the UV-C lamp was 450 μW/cm2 (where μW = 10-6 J/sec), as specified by the manufacturer. After varying lengths of exposure, aliquots (200μl) were removed, and their residual infectivity was titrated onto Vero cells immediately. Control samples were set up in parallel and incubated for the same time period but protected from UV-C light.

### 2.2.6 Determination of Infectious Titer

#### 2.2.6.1 Virus Titration

##### 2.2.6.1.1 Preparation of Virus Dilution

The virus titration was carried out in a 96 wells sterile flat bottom tissue culture plate (Burleson et al, 1992). Briefly, 180μl of the infectious media (GMEM without FBS and TPB) was added in each well. In all wells of the first column, 20μl of each virus suspension was added and 10 fold serial dilutions were made by transferring 20μl from first well of each column to the next by using multichannel pipette. The last column was taken as negative control. The dilution plate was stored at 4°C till further used.

#### 2.2.6.1.2 Infection in 96 Wells Micro-titer Plate

50 µl of each virus suspension was dispensed in respective wells of cell culture plate keeping the last column as un-infected control. A fully confluent layer of Vero cells was sub cultured as described in section 2.1.5.2. The cell suspension in growth medium was mixed gently to make even suspension of the cells and was dispensed in 96 well flat bottom micro-titer plate (100µl of cell suspension/well). The plate was covered with lid and incubated at 37ºC and was observed under inverted microscope for the development of CPE after 48 hours in an incubator supplying 5% $CO_2$ to the cells. The cells were examined microscopically. The infectious titers in terms of 50% tissue culture infective dose per ml ($TCID_{50}$/ml) were determined following Reed and Muench method (1938) as described below.

**Proportionate distance (PD)** = %CPE at dilution above 50% - (50) / %CPE at dilution above 50% - %CPE at dilution below 50%

**Log $TCID_{50}$**= (log dilution above 50%) + [(PD) x (log dilution factor)]

### 2.2.7 Testing of Thermo-stability

In order to check the thermo-stability potential of PPR virus, aliquots of each isolate were incubated at different temperatures. One aliquot incubated at 37°C was removed every 3 hours while those placed at RT (22°C) and 4°C were removed every day and after every 2 days respectively (Table 4.4)

### 2.2.8 Testing of Heat Sensitivity

All the seven isolates of PPR virus were placed at four increasing temperatures, 56 °C, 60°C and 62°C. For each time point, samples were removed after every 5, 15, 30, 45, 60 and 120 minutes (Table 4.4)

### 2.2.9 Calculation of Titer

Tissue culture infective dose (TCID50) of all treated samples was calculated again to determine the infectious titers using Vero cells by Reed and Muench method (Reed and Muench, 1938).

## 2.3 Attenuation and Pathogenicity in Goats of a Thermo-tolerant Strain of Peste des Petits Ruminants (PPR) Virus

### 2.3.1 Attenuation of a Thermo-tolerant Strain of Peste des Petits Ruminants (PPR) Virus

#### 2.3.1.1 Virulent Virus

A field isolate of PPRV designated as PAK-LRS-13/NARC was used for attenuation onto Vero cells. The virus was initially recovered during an outbreak in a commercial goat farm of Islamabad in September, 2013. The morbidity and mortality rates were 87% to 34% respectively. The main cause of this outbreak could have been introduction of new animals from Fateh Jhang city in Attock district of Punjab province. The affected animals acquired fever up to 106°F. The animals were dull and depressed with oculo-nasal discharges and mouth lesions. Some affected animals got diarrhea before death within 10-14 days of onset of clinical disease. The outbreak was confirmed by RT-PCR and PPR virus was isolated from mesenteric lymph-node tissue from a dead goat. The virus was propagated onto Vero cells as described in 2.1.5.4 and it was declared positive by RT-PCR after second blind passage on cells.

#### 2.3.1.2 Titration of Virus

The titration of virulent virus was carried out in 96 wells tissue culture plates following the technique described by Burleson *et al* in 1992. Briefly, the Vero cells were sub-cultured in 10% GMEM as described in section 3.1.4.2. The cell suspension was pipetted up and down gently with a micropipette to make an even suspension. 200µl of this cell suspension was added in each well of 96 wells tissue culture plate. 10µl of virulent virus (PAK-LRS-13/NARC) was added in the first wells (A-F) of column 1. The virus was serially diluted ($10^{-1}$ to $10^{-8)}$ by adding 10µl from each well of column 1 to columns 2 till 19. Columns 10 and 11 were taken as cell control in which 100µl of growth media was added. The plate was covered with the lid (provided with the plate) and incubated at 37°C and observed under inverted microscope for development of CPEs after 24-48 hours of incubation. The wells showing characteristic CPEs of PPR was considered positive and infectious titer was calculated according to Reed and Munch method (1938) described in section 4.2.1.

#### 2.3.1.3 Attenuation of Virus

The virus was attenuated by successive serial passages on Vero cells as follows:

The sub-confluent monolayers of Vero cells were inoculated with 0.5ml of virulent virus (PAK-LRS-13/NARC) at $10^{6.9}$ $TCID_{50}$/ml as described in section 2.1.5.4. A 0.5ml of infectious media was also added to the culture flask to prevent dehydration of cells. The virus was harvested two times when the CPEs reached 50-60%. The collected harvests were mixed together and all the flask contents (Media+cells) were subjected to 2 freeze/thaw cycles. The flask contents were transferred into a 15ml falcon tube (Corning, Inc.) and the supernatants were clarified by centrifuging at 700rpm for 5 minutes and distributed equally in 1.5ml eppendorf tubes and stored in the freezer at -80°C. The following passage was performed using 0.5ml of this viral suspension.

#### 2.3.1.4 Confirmation of Passage by RT-PCR

RT-PCR was accomplished to confirm the PPR virus at each passage level. RNA extraction was performed as described in section 2.1.3.2. RT-PCR was then conducted using One-Step RT-PCR kit (Thermo Scientific) as described in section 2.1.3.3.

### 2.3.2 Evaluation of Pathogenicity in Goats of a Thermo-tolerant, Attenuated Strain of Peste des Petits Ruminants (PPR) Virus

#### 2.3.2.1 Ethical Approval

Due permission was taken from Ethical Committee of NARC, Islamabad (Approval date: 18-5-2016, Approval number: 1191) to perform clinical trials on animals (goats).

#### 2.3.2.2 Experimental Animals

The pathogenicity trial was conducted in a bio-secure animal house at Animal Sciences Institute of National Agricultural Research Center, Pakistan. A total of twelve crossbred (Beetal × Local hairy) goats were used in this study. These animals aged between 4-5 months were purchased from a local village near Rawalpindi district to test the pathogenicity of an attenuated strain of virus at different cell passages. Animals were stall fed with green fodder and concentrates and their body weight was calculated and recorded. Each animal was identified by an ear tag. Prior to infection, the goats were kept under observation for 1 week to monitor their general health conditions. The animals which did not show any clinical sign of a disease were used further for experiment. The animals were de-wormed with an anthelmintic (Levamizoles 15-17ml / 15kg) bolus at a dose rate of 7.5 mg/ Kg. Their sera were screened negative for PPRV antibodies determined by using competitive Enzyme Linked Immuno-Sorbent Assay (c-ELISA) (Libeau et al., 1995), manufactured by Innovative Diagnostics (ID-Vet), CIRAD, France according to the manufacturer instructions.

#### 2.3.2.3 Virulent Virus

A field isolate of PPR virus (PAK-LRS-13/NARC) was used for pathogenicity trial experiment. The description of virus is given in section 2.3.1.1. The virus was propagated and serially attenuated upto 60 passages on Vero cells as described in section 2.3.1.3. The virus at passage level 20, 40 & 60 stored at $-80^0$C was revived onto sub confluent layers of Vero cells for inoculation into animals.

### 2.3.2.4 Experimental Design and Virus Inoculation

Twelve goats were randomly allocated to four groups (Groups A, B, C and D) each containing 3 animals as described in Table 2.17. Two goats in groups A, group B and group C were inoculated subcutaneously through a syringe with 2ml of $10^{4.5}$ $TCID_{50}$/ml of attenuated viral suspension at passage 20, 40 and 60 respectively. One animal in each group (Groups A, B, C) was kept as transmission control. Group D was taken as placebo control and two goats in this control group were inoculated subcutaneously with 2ml of sterile GMEM without FBS. Each group was kept in separate room.

**Table 2.17: The experimental design and virus inoculation details**

| **Group A** | | **Group B** | | **Group C** | | **Group D** |
|---|---|---|---|---|---|---|
| Infected animals | Transmission control | Infected animals | Transmission control | Infected animals | Transmission control | Sterile GMEM without PBS (3 goats) |
| Attenuated PPRV ($20^{th}$ passage) (2 goats) | (1 goat) | Attenuated PPRV ($40^{th}$ passage) (2 goats) | (1 goat) | Attenuated PPRV ($60^{th}$ passage) (2 goats) | (1 goat) | |

### 2.3.2.5 Clinical Examination

The experimental animals in each group were examined twice a day for a period of 22 days post inoculation to oversee the appearance of clinical signs and outcome of disease. All the observations were recorded using a proforma given in Appendix-1.

### 2.3.2.6 Necropsy Examination

The necropsy examination was executed soon after the death of animal in any of 4 groups. The pathological lesions in dead animals were recorded.

#### 2.3.2.7 Sample Collection

The swabs (Ocular, nasal and oral) were collected from animals in each group from day 0 to 9 post infection and dipped in 0.5ml of PBS in sterile Eppendorf tubes. These swabs were processed immediately for PPR virus detection by RT-PCR.

The blood was collected daily from all animals in experimental and control groups from day 0 to 21 post exposure by jugular vein puncture. It was left to clot under cold conditions. The sera tubes were centrifuged to remove any blood cells and transferred into clean, labelled cryovials. The vials were stored at -20°C till further analysis.

The tissue samples (lungs, spleen, lymph nodes) were also collected at necropsy under aseptic conditions and stored at -80°C till processed.

#### 2.3.2.8 Detection of PPR Virus by RT-PCR

All swabs were squeezed in 1.5ml capacity sterile eppendorf tubes. The samples were subjected to RT-PCR (Reverse transcriptase polymerase chain reaction).

##### 2.3.2.8.1 RNA Extraction

RNA extraction was carried out from swab and tissue samples as described 2.1.3.2.

##### 2.3.2.8.2 Reverse Transcription and PCR

RT-PCR was conducted as described in section 2.1.3.3. Briefly, PPRV specific primers targeting N gene of PPR (Table 2.3) and QIAGEN One-step RT-PCR Enzyme mix were used for cDNA synthesis and amplification following Forsyth and Barrett, 1995 (Forsyth and Barrett, 1995). The resulting PCR products were subjected to gel electrophoresis as described in section 2.1.3.4.

#### 2.3.2.9 PPRV Isolation

Vero cells were used for isolation of PPRV (section 2.1.5.1.1). The cells were cultivated as described in section 2.1.5.2. Tissue samples including lungs, spleen and lymph nodes were processed as described in section 2.1.5.3 and inoculated in Vero cells as described in section 2.1.5.4.

#### 2.3.2.10 Seroconversion in Experimentally Infected Animals

A Competitive ELISA (cELISA) using anti-N MAbs was used for the detection of PPRV antibodies in serum samples. The assay was performed using the kit manufactured by BDSL, with the collaboration of Flow Laboratories and Institute for animal health, Pirbright, Surrey, England following kit instructions.

##### 2.3.2.10.1 Reagents Preparation

The reagents were prepared as under:

**PPRV Antigen**

The lyophilized antigen vial (provided with the kit) was reconstituted by adding 1ml of distilled water and the contents were mixed gently. The vial was stored at -20°C till further use.

Before experiment, a further 1:100 dilution of antigen was made in 1X PBS solution.

**Monoclonal Antibody (Mab)**

The lyophilized vial (provided with the kit) was reconstituted by adding 1ml of distilled water and the contents were mixed gently. The vial was stored at -20°C till further use.

**Anti-mouse HRPO-Conjugate**

The conjugate was prepared by making a 1:100 dilution in blocking buffer.

**Serum Controls**

The three serum controls (strong positive, weak positive and negative serum) were prepared by dissolving the lyophilized contents of vials in distilled water. All control vials were stored at -20°C till further use.

All prepared vials including antigen, Mab and control sera were placed on ice during the experiment.

#### 2.3.2.10.2 Preparation of Diluents

The diluents were prepared as under:

**Phosphate Buffer Saline (PBS)**

The PBS (provided with the kit) was dissolved in one liter of distilled water and stored at 4°C.

**Blocking Buffer**

It was prepared in PBS solution by adding 0.1% Tween-20 and 0.5% negative serum.

**Chromogen-Substrate Solution**

It was prepared by dissolving one tablet (30mg) of OPD (Orthophenylenediamine) in 75ml of distilled water and stored at 20°C till further use. A 3% $H_2O_2$ solution was added just before use at 4µl of $H_2O_2$ /ml of OPD solution.

**Stop Solution (Sulphuric acid)**

One normal (1N) sulphuric acid acting as stop solution was prepared by slowly adding 5.45ml of concentrated sulphuric acid in a dark colored bottle containing 94.5ml of distilled water.

#### 2.3.2.10.3 Assay Protocol

A 50µl of prepared PPRV antigen was dispensed in all wells of ELISA plates. The plate was tapped gently, covered with a lid and incubated at 37°C for 60 minutes on a shaker at 300rpm. The contents in the plate were discarded and it was washed three times with 300µl of washing buffer. The plate after each discard was tapped robustly over a clean towel. Then 40µl of blocking buffer was added in all wells of plate. A further 20µl and 60µl of blocking buffer was added in control wells designated for monoclonal antibody and conjugate respectively. Then 20µl of test serum was dispensed (in duplicate) in wells labelled for test sera. After adding the test serum samples, 20µl each of strong positive (C++), weak positive (C+) and negative (C-) control sera were added in four designated wells of plate. Then the monoclonal antibody (40µl) was added in each well excluding the conjugate control wells (Cc).

The contents of the plate were mixed by tapping the sides of plate gently. The plate was covered with a lid and incubated at 37°C for 60 minutes on a shaker at 300rpm. The contents in the plate were discarded and it was washed three times again with 300µl of washing buffer. The plate after each discard was tapped robustly over a clean towel. After washing, 50µl of anti-mouse conjugate was added in all wells of plate. The contents of the plate were mixed by tapping the sides of plate gently. The plate was covered with a lid and incubated at 37°C for 60 minutes on a shaker at 300rpm. The plate was again washed three times by PBS as described earlier.

A 50µl of freshly prepared OPD-substrate solution was added in all wells of plate and it was incubated at 25°C for 10 minutes without shaking. The reaction was stopped by adding 50µl of stop solution to all wells of the plate. The contents in the plate were mixed by gentle tapping and read in ELISA plate reader (BDSL Immunoskan MS, Type 355) at 492nm using EDI software.

The absorbance recorded were converted to percent inhibition (PI) using the formula:

PI = 100 – (Absorbance of the test wells/ Absorbance of the Mab control wells) x 100

**2.3.2.10.4 Interpretation of Results**

The results were interpreted as under:

The test sera giving more than 50 percent inhibition calculated by the formula given above were taken as positive which could be seen by the colorless wells of the plate.

The percent inhibition of controls must fall within the range as under:

Strong positive control (C++) = 81-100 %

Weak positive control (C+) = 51-80%

Negative control (C-) = -25-25%

Conjugate control (Cc) = 91-105%

### 2.3.3 Development of a Thermostable Vaccine against PPR

#### 2.3.3.1 Virus Stock

The working seed for PPR vaccine formulation was produced from the first passage of PPRV isolate PAK-LRS-13/NARC. The virus details are given in section 2.3.1.1. The virus was serially passaged onto Vero cells up to 60$^{th}$ passage as described in the section 2.3.1.3. This passage was used for thermostable vaccine preparation.

#### 2.3.3.2 Vaccine Stabilizers

Three different stabilizers were used in this study to evaluate the thermostability of newly developed PPR vaccine (Silva et al., 2011).

a. Lactalbumin Hydrolysate-Sucrose (LAH)
b. Weybridge medium (WBM)
c. Trehalose dehydrate (TD)

100ml of each stabilizer was prepared as under:

##### 2.3.3.2.1 Preparation of 5% LAH

The LAH sucrose stabilizer was prepared by adding 5% Lactalbuminhydrolysate (LAH) and 10% sucrose (Appendix-4) in Hank's balanced salt solution (HBSS) (Appendix-7) maintained at pH 7.2.

##### 2.3.3.2.2 Preparation of WBM

The WBM stabilizer was prepared by adding 5% sucrose, 1% sodium glutamate and 2.5% LAH (Appendix-5) in HBSS maintained at pH 7.2.

##### 2.3.3.2.3 Preparation of TD

The TD stabilizer was prepared by adding 5% (initial conc.) of TD (Appendix-6) in distilled water. The final concentration after stabilizer and virus harvest mixing was 2.5%.

All prepared stabilizers were incubated at 37°C for 48 hours and then store at 4°C till use.

### 2.3.3.3 Preparation of PPR Vaccine

Vero cells seeded in 162cm$^2$ tissue culture flasks at a concentration of 0.4 x 10$^6$ cells /ml in 30ml of GMEM supplemented with 10% FBS and 1% mixed antibiotics/antimycotic solution. The cells were infected when they attained 60 to 70% confluency with PPR vaccine at multiplicity of infection of approximately 0.01 virus/cells. The flasks were incubated at 37°C for 48 hours or till CPEs development. The virus was harvested when 80% CPEs were visible under inverted microscope after 3 days of inoculation. The flasks were subjected to two free/thaw cycles to detach the cells adhered to the monolayer. The flasks were stored at -80°C till further use. Equal volumes of virus stock and stabilizers were mixed and 200ml of mixture was dispensed in 5ml capacity sterilized vaccine vials (1ml / vial) and capped with sterilized rubber stoppers (Figure 2.2). Before putting them in lyophilizer, the vials were placed overnight at -80°C.

### 2.3.3.4 Lyophilization of Vaccine

Lyophilization of vaccine was performed using Telstar, LyoBeta, automatic freeze drier (Figure 2.3) at Lyophilization section of Veterinary Research Institute (VRI), Lahore.

**Figure 2.2:** Addition of three different stabilizers to the vaccine vials under biosafety cabinet 2.


**Figure 2.3:** Automatic freeze drier (Telstar) for vaccine lyophilization

#### 2.3.3.4.1 Lyophilization Protocol

The lyophilization was executed following the method described by Mariner (Mariner et al., 2017) with some modifications. The protocol was as under:

The vaccine vials were first chilled at -45°C for 2 hours. The temperature was maintained for an additional 2 hours at the same temperature. The condenser preparation was done at 600 µbar for 10 minutes. The shelf temperature was reduced to -30°C for primary drying at 173µbar (microbar) for 1 hour. Primary drying was executed at the same temperature and pressure for an additional 16 hours. The temperature was then raised to 0°C at the same pressure for 8 hours. The shelf temperature was sustained for 18 more hours at 33µbar pressure which was then maintained for the rest of the lyophilization cycle. The temperature was then raised to 25°C for 8 hours which was then maintained for an additional 18 hours. Finally, the temperature was raised to 35°C for 2 hours and it was sustained to carry out the secondary drying step for 4 hours (Figure 2.4). The vaccine vials were stoppered under vacuum.

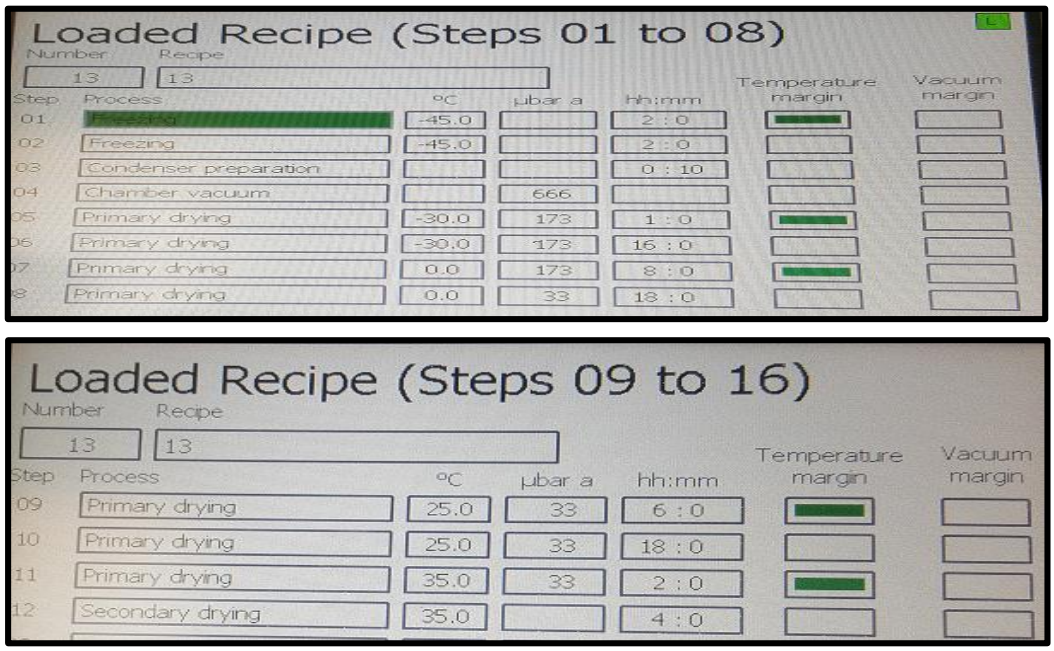



**Figure 2.4:** Loaded recipe of lyophilization process

## 2.4 Comparative Efficacy of Different Chemical Stabilizers on the Thermostability of Newly Developed PPR Vaccine

### 2.4.1Thermostability Testing of Newly Prepared Vaccine

To evaluate the efficacy of each stabilizer, the freeze dried vaccine vials with an initial titer of $10^{4.5}$ TCID50/ml stabilized with three different stabilizers were subjected to thermo-stability testing. Briefly, the vials were placed at four different temperatures (25°C, 37°C, 45°C and 56°C) for defined time periods. For testing the vials at 25°C and 37°C, the vials were placed in an incubator while for testing at 45°C and 56°C, a water bath was used and temperature was monitored at regular intervals and it was found consistent over the experiment period. One of the vial placed at 25°C was removed after every 3 days within the range of 60 days. While one of the vial placed at 37°C was removed every 2 days for a week. One vial each at 45°C and 56°C was removed and tested after every 12 and 6 hours up to 144 and 48 hours respectively. All the treated vials were placed at -80°C until virus titration.

### 2.4.2 Virus Titration

Treated vaccine vials were reconstituted in sterilized water and titrated in 96 well tissue culture plates and their infectious titer was calculated using Reed and Munch method as described in the section 2.2.6.1.2.

## 2.5 Vaccine Trial of a Newly Developed Peste des Petits Ruminants Vaccine

### 2.5.1 Ethical Approval

Due permission was taken from ethical committee of NARC, Islamabad (Approval date: 18-5-2016, Approval number: 1191) to perform clinical trials on animals (goats).

### 2.5.2 Experimental Animals

The vaccine trial was conducted in a bio-secure animal house at Animal Sciences Institute of National Agricultural Research Center, Pakistan. A total of six crossbred (Beetal × Local hairy) goats were used in this study. These animals aged between 4-5 months were purchased from a local village in Rawalpindi district. Animals were retained following the protocol as described in section 2.3.2.2.

### 2.5.3 Vaccines

PPR vaccine (Nigeria 75/1) and PPR vaccine (Pak-LRS-13/NARC) were used for conducting vaccine trail.

### 2.5.4 Experimental Design and Virus Inoculation

Six goats were randomly allocated to 3 groups (Groups A, B and C) each containing 2 animals (Table 2.18). Two goats in group A were inoculated subcutaneously through a syringe with 2ml of $10^{4.5}$ $TCID_{50}$/ml of PPR vaccine (Nigeria 75/1) and Two goats in group B were inoculated with 2ml of $10^{4.5}$ $TCID_{50}$/ml of PPR vaccine (Pak-LRS-13/NARC). Group C was taken as placebo control and two goats in this control group were inoculated subcutaneously with 2ml of sterile PBS. Each group was kept in separate room.

**Table 2.18:** The experimental design and vaccine trial details

| Group A | Group B | Group C |
|---|---|---|
| PPR vaccine (Nigeria 75/1) | PPR vaccine (Pak-LRS-13/NARC) | Placebo control |
| (2 goats) | (2 goats) | (2 goats) |

### 2.5.5 Clinical Examination

The experimental animals in each group were examined twice a day for a period of 22 days post inoculation to oversee the appearance of clinical signs and outcome of vaccine. All the observations were recorded (Appendix-1).

### 2.5.6 Sample Collection

The blood was collected daily from all animals by jugular vein puncture on day 0 and thereafter daily for 7 days. After 7 days, samples were taken on day 9, 11, 13, 15, 17, 19, 21 post immunization. It was left to clot under cold conditions. The sera tubes were centrifuged to remove any blood cells and transferred into clean, labelled cryovials. The vials were stored at -20°C till further analysis.

### 2.5.7 Sample Analysis

#### 2.5.7.1 Humoral Immune Response in Challenged Animals

The humoral immune response was determined by executing Competitive ELISA (cELISA) as described in section 2.3.2.10.

### 2.5.8 Challenge Protection Studies

The challenge protection studies were carried out at in a bio-secure animal house at Animal Sciences Institute of National Agricultural Research Center, Islamabad.

A virulent field isolate catalogued as PAK-Fjg-14/NARC (Un-attenuated) stored in Animal Health repository, NARC was used in challenge protection studies. The virus was propagated on Vero cells as described in section 2.1.5.4. A second passage of virus was used after virus titration as described in section 2.2.6.1.

The animals in groups A and B were previously inoculated with PPR vaccine (Strain-Nigeria 75/1) and PPR vaccine (Strain-Pak-LRS-13/NARC) were challenged subcutaneously with 2ml of $10^{4.5}$ $TCID_{50}$/ml of un-attenuated viral suspension and two animals in control group were inoculated with 2ml of PBS (Table 2.7).

#### 2.5.8.1 Clinical Examination

The challenged animals in each group were examined twice a day for a period of 10 days to monitor the clinical signs and observations were recorded (Appendix-1).

#### 2.5.8.2 Sample Collection

The swabs (ocular, nasal and oral) were collected from challenged animals from day 0 to 9 post challenge to find out the evidence for virus shedding in challenged animals. These swabs were dipped in 0.5ml of PBS in sterile eppendorf tubes. These swabs were processed immediately for PPR virus detection by RT-PCR.

The blood was collected daily from all challenged animals by jugular vein puncture prior to challenge on day 0 and thereafter daily for 10. It was left to clot under cold conditions. The sera tubes were centrifuged to remove any blood cells and transferred into clean, labelled cryovials. The vials were stored at -20°C till further analysis.

#### 2.5.8.3 Sample Analysis

##### 2.5.8.3.1 Detection of PPR Virus Shedding by RT-PCR

All swabs were squeezed in 1.5ml capacity sterile eppendorf tubes. The samples were subjected to RT-PCR following Forsyth and Barrett, 1995.

#### 2.5.8.3.1.1 RNA Extraction

RNA extraction was carried out from swab and tissue samples as described 2.1.3.2.

#### 2.5.8.3.1.2 RT-PCR

RT-PCR was conducted as described in section 2.1.3.3. The resulting PCR products were subjected to gel electrophoresis as described in section 2.1.3.4.

### 2.5.8.3.2 Humoral Immune Response in Challenged Animals

The humoral immune response was determined by executing Competitive ELISA (cELISA) as described in section 2.3.2.10.

# Chapter 3

## Isolation and Characterization of Peste des Petits Ruminants Virus

# Chapter 3: Isolation and Characterization of Peste des Petits Ruminants Virus from Field Outbreaks

## 3.1 Introduction

PPR virus exists as one serotype but has been classified into four genetically distinct lineages based on the molecular characterization (Banyard et al., 2010; Clarke et al., 2017). Lineage I is mainly confined in Western Africa, Lineage II in central Africa, Lineage III in eastern Africa and Southern parts of Middle East and Lineage IV is predominant in Middle East and Asia (Kwiatek et al., 2011) although co-circulation of Lineages II, III and IV in Eastern Africa has also been reported (Kwiatek et al., 2011; Muniraju et al., 2014; Woma et al., 2015; Zhu et al., 2016). A comparative analysis of F and N genes revealed that N protein gene tends to be more pertinent for segregating between closely related but geographically distinct PPR viruses (Kwiatek et al., 2007).

In Pakistan, the disease was first reported in 1991 and the diagnosis was based on the clinical picture (Athar et al., 1995). However with the commencement of molecular techniques, it was confirmed in laboratory in 1994 (Amjad et al., 1996). Since then, PPR has ingrained as an endemic infection in Pakistan (Zahur et al., 2014) despite the use of homologous lineage tissue culture PPR vaccine.

Generally small ruminants are raised in unorganized farms mostly in rural areas of the country. Prevailing production systems for their rearing are extensive involving lot of stock movement. There are no physical, political and economic barriers regarding the livestock movement both within the country and nomadic movement from Afghanistan. There are no specific points for the control of movement of animals or animal products within the country. Although there are two border inspection posts at Peshawar (Khyber Pakhtunkhwa) and Chaman (Baluchistan) however; the border between Pakistan and Afghanistan is porous and Afghan nomads follow their traditional routes to reach target areas in Pakistan.

Due to poor surveillance monitoring, the true disease epidemiology cannot be ascertained making its control difficult. Hence studying the circulating PPRV strains in different

localities of the country and improvement in current disease diagnostic techniques will be helpful for devising control strategies for this deleterious disease.

The infection caused by members of genus morbillivirus results in severe immunosuppression including leucopenia in respective hosts; since lymphoid cells are a major target of morbilliviruses (Rajak et al., 2005). The immunosuppression triggers secondary opportunistic bacterial and parasitic infections which can add to the severity of disease (Seki et al., 2003). This response is associated with the presence of a cell surface protein receptor CD150, the signaling lymphocyte activation molecule (SLAM) preferentially used by wild-type morbilliviruses for attachment to the host cell (Adombi et al., 2011; Baazizi et al., 2017). All morbilliviruses including PPR virus are capable of utilizing two receptors, CD150 (Tatsuo et al., 2001) and CD46. CD150 is a glycoprotein belonging to the CD2 subset of the immunoglobulin super family and is expressed on the surface of a proportion of primary B cells, Epstein-Barr virus (EBV) transformed B cells (B95a cells), T cell clones, memory T cells, activated T cells, immature thymocytes, mature dendritic cells, and activated monocytes (Tatsuo et al., 2001) whereas, CD46 is a complement regulatory protein expressed on all cells except red blood cells (Sannat et al., 2014).

For PPR virus isolation, primary cultures of ovine and bovine kidney, African green monkey kidney (vero cells), and lung cells have been used (Adombi et al., 2011). However, isolation using primary cultures is laborious, time consuming and needs technical expertise. To overcome this problem, continuous cell lines are used for isolation and cultivation of morbilliviruses. The most commonly used cell line is Vero cell line (Sannat et al., 2014) but these cells do not favor efficient PPR virus isolation due to less probability of virus growth and require numerous blind passages for the development of visible cytopathic effects (CPEs) (Adombi et al., 2011); (Albayrak and Alkan, 2009). The identification of SLAM receptors used preferably by wild-type PPR virus strains and other members of morbillivirus as a mode of entry and attachment revealed the importance of these receptors for isolation of virus.

In this chapter, the circulating PPRV strains reported during prodigious outbreaks in different parts of the country were characterized and the efficiency of BTS-34 cell line

(CV1 cells constitutively expressing bovine SLAM receptors) in comparison with Vero-76 (African green monkey kidney cells) is presented.

## 3.2 Results

### 3.2.1 Outbreak Confirmation:

A total of 43 suspected field outbreaks of PPR reported by resource person in each of the L&DD (Livestock and dairy department) throughout the country were confirmed in the Animal Health laboratory during 2012-2014. The details of outbreaks are given in Table 3.1. Samples from the affected and dead animals were collected from different locations (Table 3.1). The outbreaks were confirmed by RT-PCR and Ic-ELISA. Figure 3.1 depicts the locations of PPR outbreaks in different parts of the country.


**Figure 3.1:** Location and number of PPR outbreaks in different provinces of the country during 2012-2014.

**Table 3.1:** Details of PPR suspected outbreaks confirmed during 2012-2014.

| Outbreak region | Frequency (%) | Samples collected |
|---|---|---|
| KPK* | 13 (30.23) | 79 |
| Gilgit Baltistan | 5 (11.63) | 29 |
| Sindh | 4 (9.30) | 18 |
| ICT** | 9 (20.93) | 51 |
| Punjab | 8 (18.60) | 48 |
| AJK*** | 3 (7.0) | 16 |
| Baluchistan | 1 (2.32) | 8 |
| **Total** | **43 (100)** | **239** |

*KPK: Khyber Pakhtunkhwa **ICT: Islamabad Capital Territory ***AJK: Azad Jammu and Kashmir

### 3.2.2 Detection of PPRV Antigen

A total of 239 samples comprising of lymph nodes (mesenteric and bronchial), spleen and lungs collected from dead goats and sheep at the time of necropsy and swabs (Ocular and nasal) and blood were collected from diseased goats and sheep from each of the 43 outbreaks. These were tested using RT-PCR where 174 (70.17%) were found positive for PPRV antigen and 151 samples (61.58%) were found positive by Ic-ELISA as shown in Table 3.2.

**Table 3.2:** Clinical samples collected from suspected outbreaks and tested by Ic-ELISA and RT-PCR for PPRV

| Outbreak region | Samples tested | Ic-ELISA | | RT-PCR | |
|---|---|---|---|---|---|
| | | Positive (n) | Percent | Positive (n) | Percent |
| KPK* | 79 | 43 | 54.43 | 54 | 68.35 |
| Gilgit Baltistan | 29 | 16 | 55.17 | 20 | 68.96 |
| Sindh | 18 | 11 | 61.11 | 12 | 66.66 |
| ICT** | 51 | 34 | 66.66 | 36 | 70.58 |
| Punjab | 48 | 33 | 68.75 | 35 | 72.91 |
| AJK*** | 16 | 10 | 62.50 | 11 | 68.75 |
| Baluchistan | 8 | 5 | 62.50 | 6 | 75.00 |
| **Total** | **239** | **151** | **61.58** | **174** | **70.17** |

### 3.2.3 Virus Isolation and Identification

Only those tissue and swab samples which were transported under refrigerated conditions and were positive by both Ic-ELISA and RT-PCR (n=80) were attempted for virus isolation by propagation in two different cell lines (Vero-76 and BTS-34). The CPEs were observed after 48 hours and onward of virus inoculation. Both cell lines revealed different patterns of CPEs in terms of morphology as well as time taken for their development (Tables 3.3 and 3.4).

The Vero cell line showed a delayed onset of CPEs with 1 to 2 blind passages from Vero to Vero cell line (Table 3.5). The CPEs in this cell line developed as elongated granular cells followed by cell shrinkage and rounding. At the third day post infection, a significant number of vacuolated round and refractive cells (approx. 90-100%) were prominent with very few intact cells (Figure 3.2).

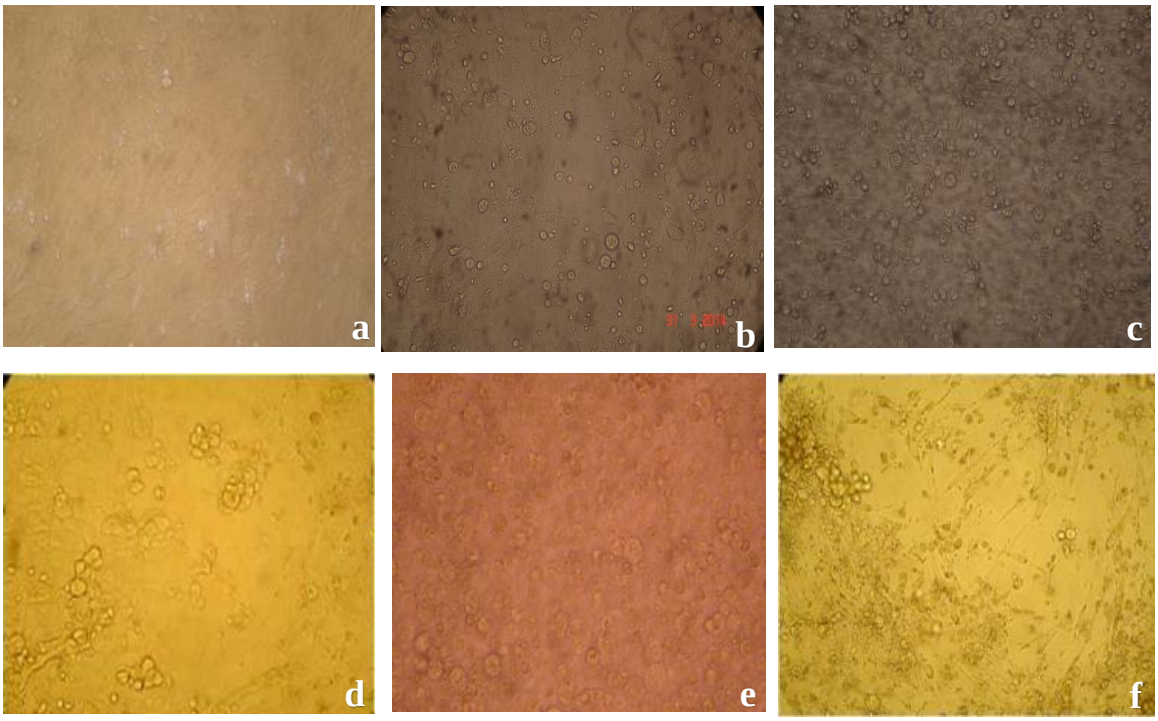



**Figure 3.2** Characteristic CPEs on Vero-76 cell line **(a):** Normal Vero cells, **(b):** CPEs-Initiation of CPEs development **(c):** CPEs- Rounding of Vero cells (40-50%), **(d):** Clumping of Vero cells, **(e):** Rounding of Vero cells (80-90%), **(f):** Vero cells with elongated processes

In BTS-34 cell line, the PPR virus revealed visible CPEs from 72 hours post infection. About 40-50% of CPEs were rounded giant cells followed by formation of syncytia and layer detachment. On fourth day post inoculation, 80-90% CPEs were observed with an increased number of round cells, large cluster of cells and with very few intact cells (Figure 3.3). The complete process of PPRV infection on BTS-34 cell line took approximately 4 to 6 days (Table 3.5).

PPRV was harvested as soon as 80% of the cells showed characteristic CPEs. The monolayers in the control flasks remained intact and did not show any CPE during the observation period.

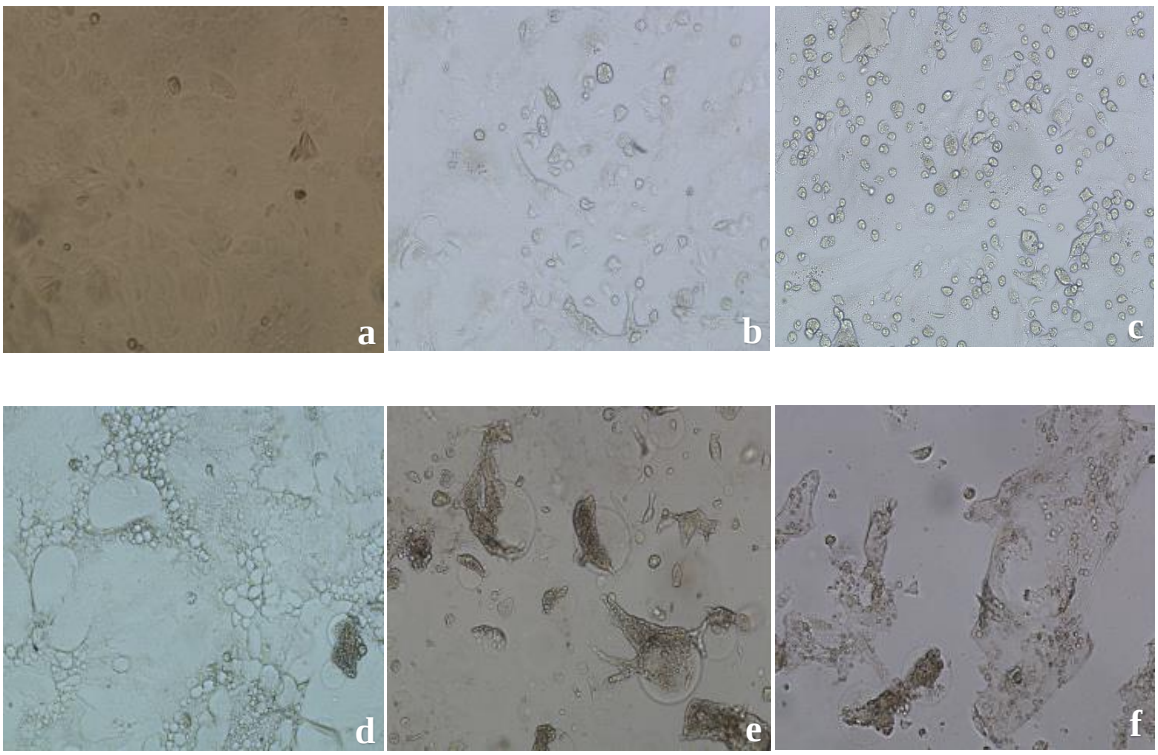



**Figure 3.3** Characteristic CPEs on BTS-34 cell line **(a):** Normal BTS-34 cells, **(b)** Initiation of CPEs development **(c):** Rounding of BTS cells **(d):** Initiation of syncytia formation, **(e):** Enlargement of syncytia (3 days post inoculation), **(f):** Detachment of cell monolayer (4 days post inoculation)

Out of a total 80 samples inoculated onto Vero and BTS cell lines, 18 PPRV isolates were recovered (Table 3.3).

**Table 3.3:** Tissue and swab samples collected from suspected outbreaks and inoculated onto Vero76 and BTS-34 cell lines

| Outbreak Province | Outbreak Area | Samples attempted for virus isolation | Virus recovery | Total virus recovered from each province |
|---|---|---|---|---|
| Sindh | Karachi | 8 | 2 | 3 |
| | Thar | 3 | 1 | |
| ICT* | Islamabad | 20 | 6 | 6 |
| Punjab | Taxila | 8 | 1 | 6 |
| | Rawalpindi | 8 | 2 | |
| | Murree | 3 | 1 | |
| | Faisalabad | 8 | 1 | |
| | Bhakkar | 6 | 1 | |
| AJK** | Muzaffarabad | 6 | 1 | 2 |
| | Bhimber | 4 | 1 | |
| Gilgit Baltistan | Gilgit Baltistan | 6 | 1 | 1 |
| **Total** | | **80** | **18** | **18** |

*ICT: Islamabad Capital Territory **AJK: Azad Jammu and Kashmir

**Table 3.4:** Visible CPEs by different isolates onto Vero and BTS cells

| PPRV Isolates used | Characteristics of PPR CPEs on Vero and BTS cells | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cell type for PPRV isolation | | Cell rounding | | Detachment from surface | | Vacuolation | | Syncytia formation | | Clumping | | Cells with elongated processes | |
| | | | Vero | BTS | Vero | BTS | Vero | BTS | Vero | BTS | Vero | BTS | Vero | BTS |
| Pak-VEH-12/NARC | Vero | BTS | + | + | + | + | + | + | + | + | - | - | - | - |
| Pak-ICT-12/NARC | Vero | BTS | + | + | + | + | + | + | + | + | - | - | - | - |
| Pak-KP-12/NARC | Vero | BTS | + | + | + | + | - | - | - | + | + | + | + | + |
| Pak-FGJ-14/NARC | Vero | BTS | + | + | + | + | - | - | - | + | + | + | + | - |
| Pak-ATT-13/NARC | Vero | BTS | + | + | + | + | - | - | - | + | + | + | + | - |
| Pak-MZD-13/NARC | Vero | BTS | + | + | + | + | - | - | - | + | + | + | + | - |
| Pak-LRS-13/NARC | Vero | BTS | + | + | + | + | - | - | - | + | + | + | + | - |
| PAK-FSD-14/NARC | Vero | BTS | + | + | + | + | - | - | - | + | + | + | + | + |
| PAK-ICT-14-1/NARC | Vero | BTS | + | + | + | + | - | - | - | + | + | + | + | + |
| PAK-BKR-12/NARC | Vero | BTS | + | + | + | + | - | - | - | + | + | + | + | + |
| PAK-BRNL-13/NARC | Vero | BTS | + | + | + | + | - | - | - | + | + | + | + | - |
| PAK-HAFZ-14/NARC | Vero | BTS | + | + | + | + | - | - | - | + | + | + | + | - |
| PAK-RWP-13/NARC | Vero | BTS | + | + | + | + | - | - | - | + | + | + | + | - |
| PAK-THAR-14/NARC | Vero | BTS | + | + | + | + | - | - | - | + | + | + | + | + |
| PAK-CHLS-13/NARC | Vero | BTS | + | + | + | + | - | - | - | + | + | + | + | + |
| PAK-ICT-14-2/NARC | Vero | BTS | + | + | + | + | - | - | - | + | + | + | + | - |
| PAK-MAYI-12/NARC | Vero | BTS | + | + | + | + | - | - | - | + | + | + | + | - |
| PAK-RWP-14/NARC | Vero | BTS | + | + | + | + | - | - | - | + | + | + | + | + |

*PAK=Pakistan, Veh= Vehari, ICT=Islamabad Capital Territory, KP= Khairpur, FJG= Fateh Jang, ATT= Attock, MZD=Muzaffarabad, LRS= Livestock Research Institute, FSD=Faisalabad, BKR=Bhakar, BRNL=Barnala, HAFZ= Hafizabad, RWP= Rawalpindi, CHLS= Chilas, NARC=National Agricultural Research Centre

**Table 3.5:** Time taken for both cell lines (BTS & Vero) to develop characteristic CPEs and number of blind passages required for PPRV isolation

| Isolate IDs | Days taken for appearance of CPEs | | Blind passages | |
|---|---|---|---|---|
| | Vero cells | BTS cells | Vero cells | BTS cells |
| Pak-VEH-12/NARC | 16 | 5 | 1 | - |
| Pak-ICT-12/NARC | 8 | 5 | - | - |
| Pak-KP-12/NARC | 16 | 5 | 1 | - |
| Pak-FGJ-14/NARC | 8 | 7 | - | - |
| Pak-ATT-13/NARC | 8 | 6 | - | - |
| Pak-MZD-13/NARC | 21 | 8 | 2 | - |
| Pak-LRS-13/NARC | 16 | 5 | 1 | - |
| PAK-FSD-14/NARC | 21 | 8 | 2 | - |
| PAK-ICT-14-1/NARC | 8 | 5 | - | - |
| PAK-BKR-12/NARC | 8 | 6 | - | - |
| PAK-BRNL-13/NARC | 21 | 8 | 2 | - |
| PAK-HAFZ-14/NARC | 21 | 6 | 2 | - |
| PAK-RWP-13/NARC | 16 | 5 | 1 | - |
| PAK-THAR-14/NARC | 21 | 8 | 2 | - |
| PAK-CHLS-13/NARC | 21 | 8 | 2 | - |
| PAK-ICT-14-2/NARC | 21 | 5 | 2 | - |
| PAK-MAYI-12/NARC | 21 | 5 | 2 | - |
| PAK-RWP-14/NARC | 21 | 8 | 2 | - |
| **Mean** | 16.27778 | 6.277778 | 1.22 | - |
| ***p*-value** | 1.9E-08 | | | |

### 3.2.4 Comparative Pattern of PPRV Growth on Vero and BTS Cell Lines

Titers of eighteen isolates of PPRV were compared separately using both cell lines (Vero-76 and BTS-34) by making tenfold serial dilutions and calculation of tissue culture infective dose fifty (TCID50). It was found that there was approximately one log increase in viral titer in case of BTS cells as depicted in Figure 3.4 which suggested that BTS cells produced high titer of virus as compared to Vero cells.

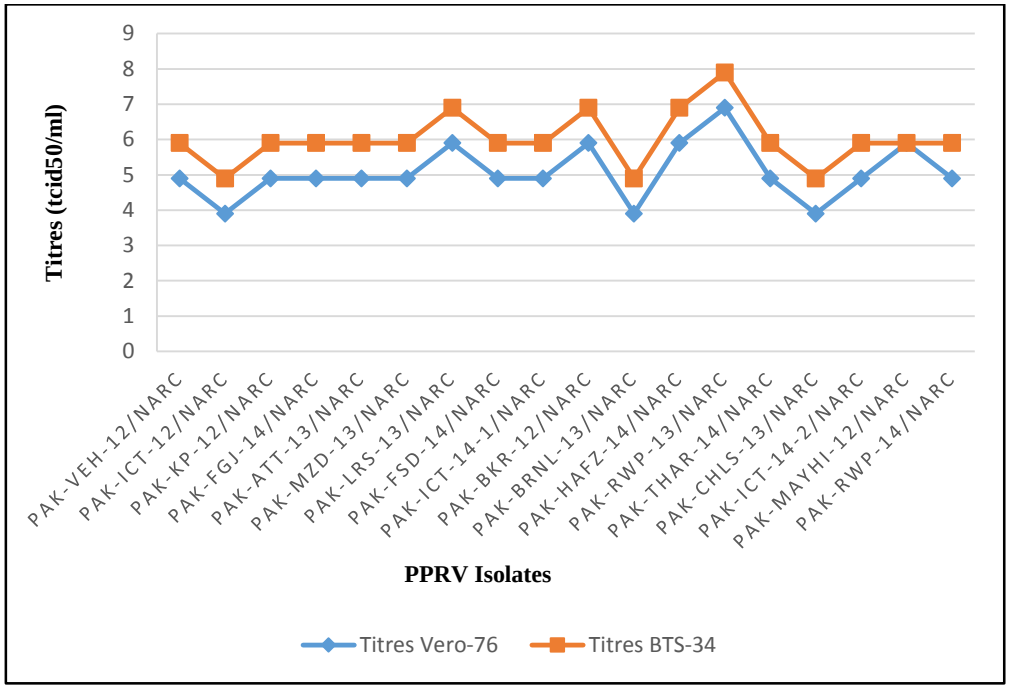



**Figure 3.4:** Comparison of PPRV titers propagated onto Vero and BTS cells

### 3.2.5 Confirmation of Virus Recovery by Ic-ELISA and RT-PCR

A total of 18 PPR virus isolates recovered on both cell lines were then confirmed by Ic-ELISA and RT-PCR. Of 18, 13 viruses were confirmed by RT-PCR (72.22%) (Figure 3.5) while Ic-ELISA confirmed 6 PPR virus isolates (33.33%) as shown in Table 3.6. The positive cell cultures confirmed that CPEs were produced by PPRV in both cell lines. The recovered isolates were lyophilized and catalogued on the basis of outbreak location, year and place of isolation and stored at -20°C in Animal Health laboratory repository for future analysis.

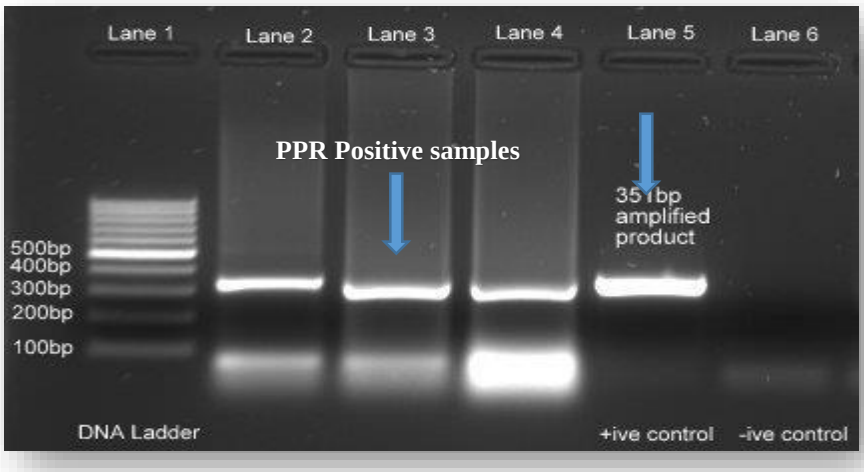



**Figure 3.5:** A representative gel image showing N gene based RT-PCR amplification with product size of 351bp (from left to right). Lane 1: DNA ladder (100bp); Lanes 2-4: PPR +ive samples; Lane 5: +ive control; Lane 6: -ive control.

**Table 3.6:** Confirmation of virus recovery by Ic-ELISA and RT-PCR

| **Area** | **Virus recovery** | **Confirmation of virus recovery by** | | | |
|---|---|---|---|---|---|
| | | **Ic-ELISA** | | **RT-PCR** | |
| | | **Positive** | **%** | **Positive** | **%** |
| Karachi | 2 | 1 | 50 | 2 | 100 |
| Islamabad | 6 | 2 | 33.33 | 4 | 66.67 |
| Taxila | 1 | 1 | 100 | 1 | 100 |
| Rawalpindi | 2 | - | 0 | 2 | 100 |
| Muzzafarabad | 1 | 1 | 100 | 1 | 100 |
| Bhimber | 1 | - | 0 | 1 | 100 |
| Faisalabad | 1 | 1 | 100 | 1 | 100 |
| Gilgit Baltistan | 1 | - | 0 | - | 0 |
| Murree | 1 | - | 0 | - | 0 |
| Bhakkar | 1 | - | 0 | - | 0 |
| Thar | 1 | - | 0 | 1 | 100 |
| **Total** | **18** | **6** | **33.33** | **13** | **72.22** |

## 3.2.6 Genetic Characterization of Selected PPRV Isolates

### 3.2.6.1 Partial (N gene) sequencing

Amplification of PPRV-N gene by RT-PCR using primers NP3/NP4 (Details are given in Table 2.2, section 2) gave expected amplicons of 351bp. A total of 9 representative samples (Tissues=4 Isolates=5) selected based on the geographical distribution from different regions of Pakistan were sequenced and compared with previous sequences of PPRV submitted in the gene bank to establish genetic relationship. One attenuated isolate at Passage level 10 and passage 20 was also included to compare the mutational changes in the N gene after attenuation. The details of isolates used are given in Table 3.7.

**Table 3.7:** Representative isolates with their identification IDs and sample type

| S. No. | IDs of samples | Sample type |
|---|---|---|
| **1** | PAK-VEH*-12/NARC | Isolate |
| **2** | PAK-FJG*-14/NARC | Isolate |
| **3** | PAK-TAX*10-14/NARC | Isolate (Attenuated) |
| **4** | PAK-TAX20-14/NARC | Isolate (Attenuated) |
| **5** | PAK-ATT*-13/NARC | Isolate |
| **6** | PAK-MZD*-13/NARC | Isolate |
| **7** | PAK-BKR*-12/NARC | Tissue |
| **8** | PAK-BRNL*-13/NARC | Tissue |
| **9** | PAK-FSD*-14/NARC | Tissue |
| **10** | PAK-FSD-14LN*/NARC | Tissue |
| **11** | PAK-ICT*-14/NARC | Isolate |

*PAK=Pakistan, Veh= Vehari, FJG= Fateh Jang, TAX=Taxila, Att= Attock, MZD=Muzaffarabad, BKR=Bhakar, BRNL=Barnala, FSD=Faisalabad, ICT=Islamabad Capital Territory, LN=Lymph-node, NARC=National Agricultural Research Centre

### 3.2.6.2 Phylogenetic Analysis

As shown in Figure 3.6, all the present study PPRV isolates belonged to lineage IV which is the Asian lineage of PPRV. The nucleotide sequence analysis of the 351bp N gene of 9 PPRV isolates and 1 attenuated isolate of present study revealed a 97% to 99% identity with previously identified Pakistani isolates and identity to the Chinese, Indian, Iranian and Kurdistan PPRV isolates ranged from 95% to 99%. The phylogenetic analysis revealed that present study isolates were genetically closely related to other PPRV isolates from Pakistan, China, Iran, India and Turkey.

The constructed phylogenetic tree using 351bp N gene sequences showed that all study isolates and other Asian isolates from Pakistan, China, Iran, India and Turkey were clustered into a separate branch from Nigerian strains. All isolates from Asian origin fall into lineage IV of PPRV while Nigerian in lineage 1 and Cote d' Ivoire in lineage II (Figure 3.6). Furthermore, the distribution of Pakistani strains was more diverse as five present study isolates clustered together with other Pakistani and Chinese isolates while two isolates had sequence homology with Indian and Iranian isolates.

The N gene sequences of one of the local isolate of PPRV attenuated up to 20 serial passages using Vero cells (PAK-TAX10-07/NARC and PAK-TAX20-07/NARC attenuated up to 10 and 20 serial passages respectively) were also included while constructing phylogenetic tree. The details of attenuation are discussed in Chapter 5. The analysis revealed that both the attenuated serotypes were clustered into a separate branch than the rest of the study isolates due to mutational changes in the nucleotides of N gene undergoing attenuation.

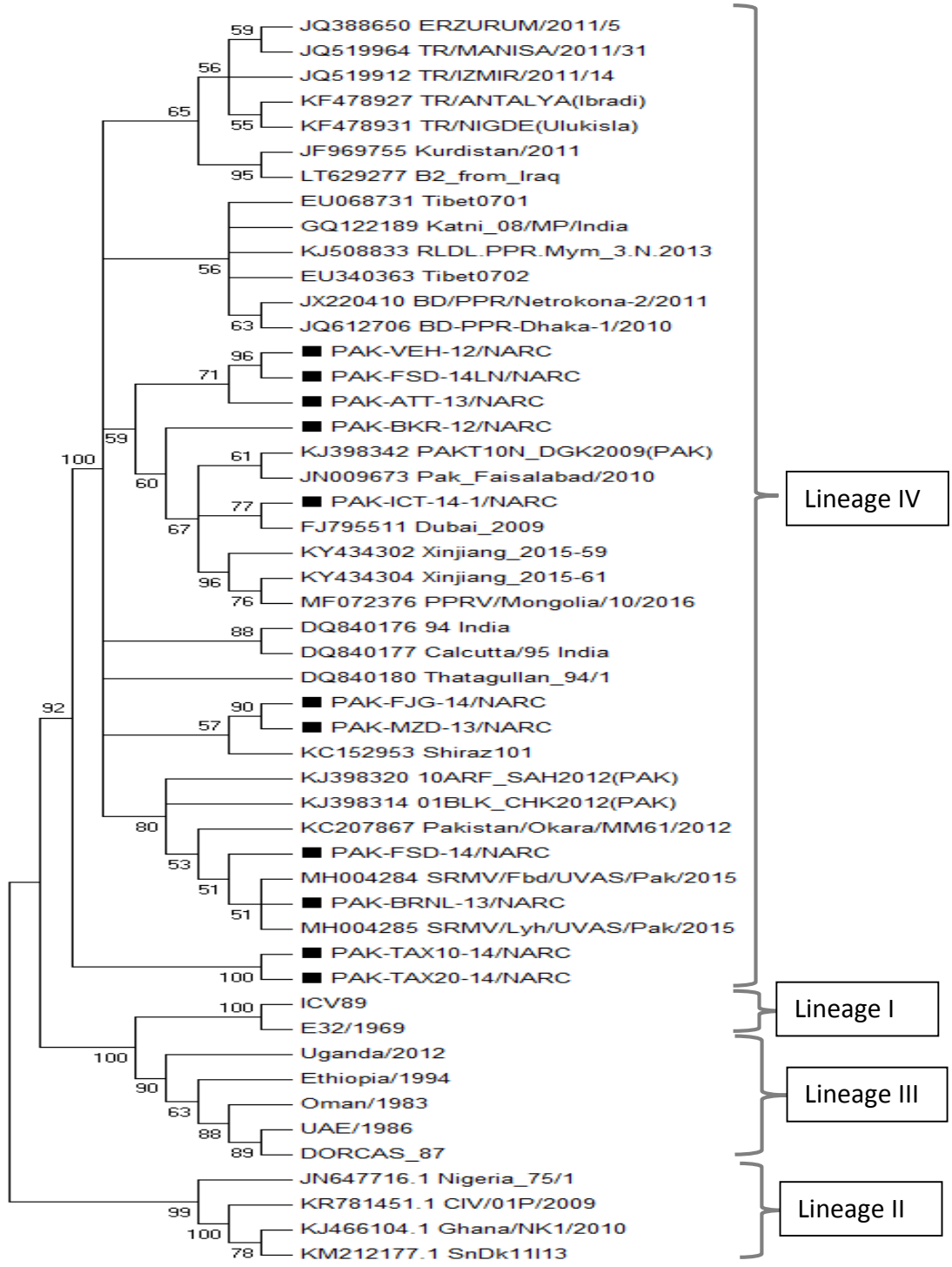



**Figure 3.6.** Unrooted NJ phylogenetic tree based on 351bp partial sequences of N gene of PPRV detected in Pakistan and other countries. Bootstrap values are indicated on the branches.

■ Shows present study isolates

### 3.2.7 Full genome sequencing of PPRV Isolates

Amplification of PPRV genome by Next generation sequencing (NGS) was done and full genome of PPRV was amplified using a set of primers. A total of 3 isolates were sequenced completely and compared with previously submitted sequences of PPRV in Genbank. A comparative analysis of full genome from these 3 Pakistani isolates and one Nigerian strain 75/1 was also performed. The details of isolates used are given in Table 3.8.

**Table 3.8:** Representative isolates with their identification IDs

| S. No. | IDs of samples | Sample type |
|---|---|---|
| **1** | PAK*-LRS-13/NARC* | Isolate |
| **2** | PAK-ICT*-12/NARC | Isolate |
| **3** | PAK-MZD*-13/NARC | Isolate |
| **4** | Nigeria 75/1 (HQ197753.1) | Strain |

*PAK=Pakistan, NARC=National Agricultural Research Centre, ICT=Islamabad Capital Territory, MZD=Muzaffarabad

#### 3.2.7.1 Phylogenetic analysis of complete genomes of PPRV

The phylogenetic analysis was performed using MEGA (Version 7.0.21). As shown in Figure 3.7, the 3 present study isolates belonged to lineage IV which is the Asian lineage of PPRV. The analysis revealed that these isolates were genetically closely related to other PPRV isolates from Pakistan, China, India and Bangladesh. The nucleotide sequence analysis of these isolates revealed 98% identity with previously identified Pakistani isolates and identity to the Indian, Chinese and Bangladesh PPRV isolates ranged from 98% to 97%.

The constructed phylogenetic tree of complete genome sequences of PPRV using neighbor joining method showed that all study isolates and other Asian isolates from Pakistan, India and China were clustered into a separate branch from Nigerian strains. All isolates from Asian origin fall into lineage IV of PPRV while Nigerian in lineage II (Figure 3.7). Furthermore, the distribution of Pakistani strains was more diverse as one present study isolates clustered together with other Pakistani and Chinese isolates while two isolate had some sequence homology with lineage II of PPRV.

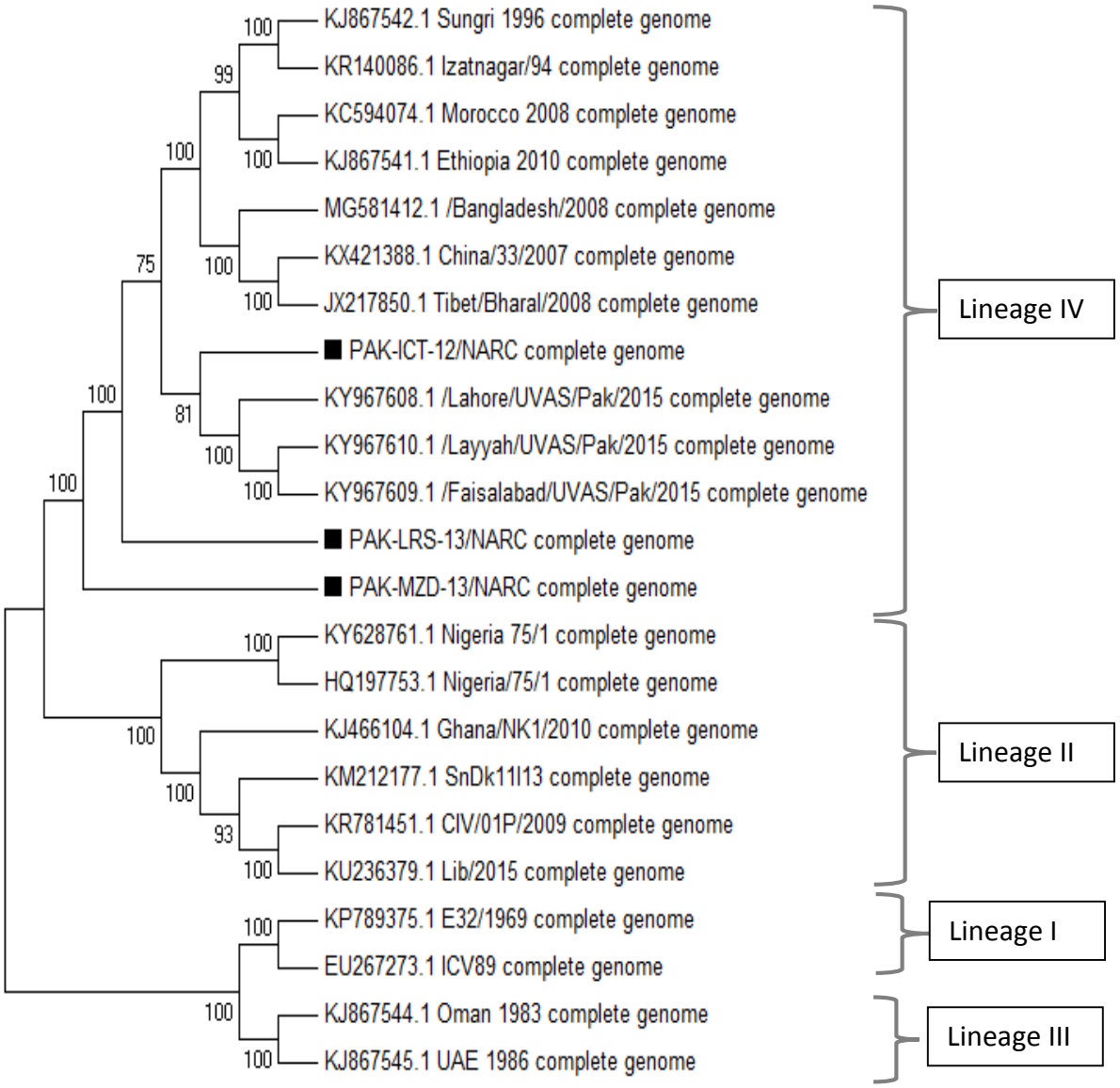



**Figure 3.7:** Unrooted NJ phylogenetic tree based on complete genome sequences of PPRV detected in Pakistan and other countries. Bootstrap values are indicated on the branches.

### 3.2.7.2 Comparative analysis

Full genome sequencing of three Pakistani strains (PAK-LRS-13/NARC, PAK-ICT-12/NARC and PAK-MZD-13/NARC) generated 15,948 bp of sequence which was same for all other PPRV genomes sequenced to date. The genome organization of these three Pakistani strains was also same and is in agreement with "rule of six" for Paramyxoviruses. There is genome promotor region comprising of 107 nucleotides (nt) at the 3′ end and anti-genome promotor at 5′ end. In between these two regions, the transcriptional units for structural (N, P, M, F, H) and nonstructural proteins (P and V) are present (Figure 3.8).

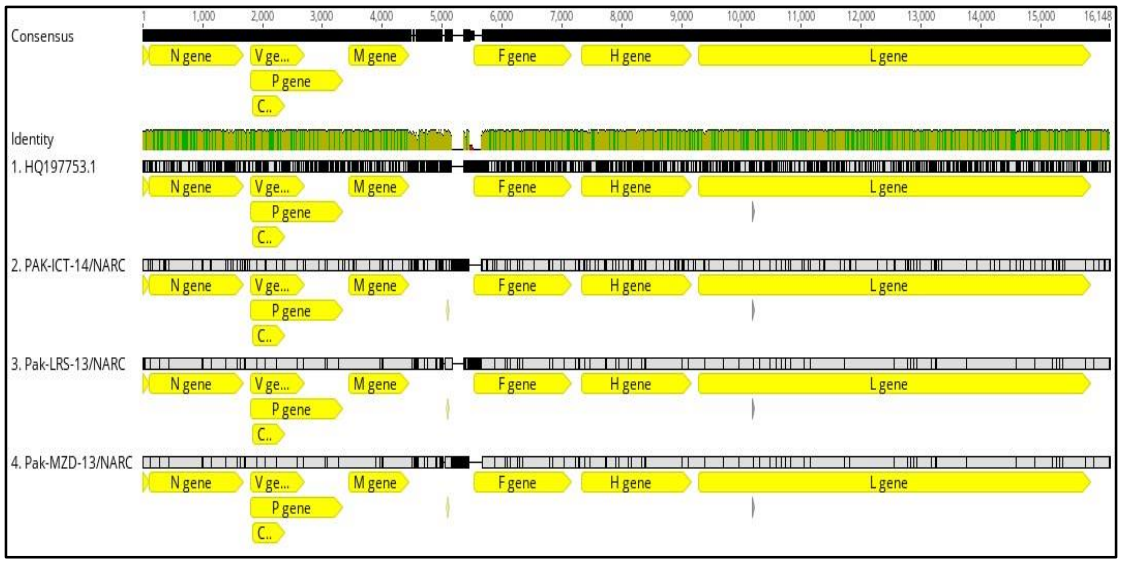



**Figure 3.8:** Genome organization of Nigerian strain (Accession No. HQ197753.1) and three local PPRV strains (PAK-ICT-12/NARC, PAK-LRS-13/NARC, and PAK-MZD-13/NARC).

A comparative analysis of complete genome sequences from 3 present study isolates and one Nigerian strain (Accession No. HQ197753.1) retrieved from database of GenBank was also performed using Geneious Prime (Version 2020.2).

The molecular analysis revealed that there is a well conserved genome promotor with 107 nucleotides at 3´ end of genome followed by N gene comprising of 1578 nucleotides. It showed the highest identity (98.73%) with Pakistani isolates and lowest (89.57% and 92.7%) with the Lineage I isolate (ICV-89) and Lineage II isolate (Nigeria 75/1) respectively (Table 3.9).

**Table 3.9:** Percent identities and differences in bases /residues of N gene of PPRV

| | **HQ197753.1** | | **Pak-MZD-13/NARC** | | **Pak-LRS-13/NARC** | | **PAK-ICT-14/NARC** | |
|---|---|---|---|---|---|---|---|---|
| | **Non-identical bases** | **% identities** | **Non-identical bases** | **% identities** | **Non-identical bases** | **% identities** | **Non-identical bases** | **% identities** |
| **HQ197753.1** | | | 92 | 94.17 | 92 | 94.17 | 94 | 94.04 |
| **Pak MZD-13/NARC** | 92 | 94.17 | | | 0 | 100 | 22 | 98.61 |
| **Pak-LRS-13/NARC** | 92 | 94.17 | 0 | 100 | | | 22 | 98.61 |
| **PAK ICT-14/NARC** | 94 | 94.04 | 22 | 98.61 | 22 | 98.61 | | |

The N gene of three Pakistani strains was more conserved in comparison with Nigerian strain, however, single nucleotide polymorphisms (SNPs) were evidenced at different positions (Figure 3.9).

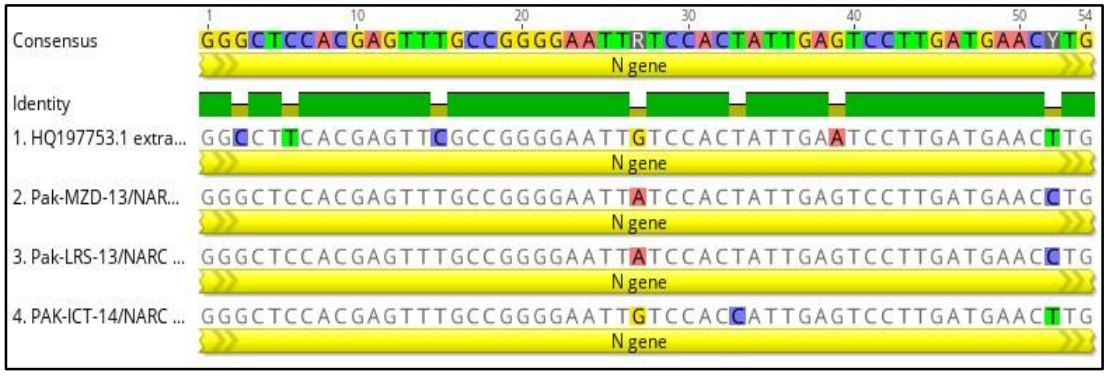



**Figure 3.9 (a)**

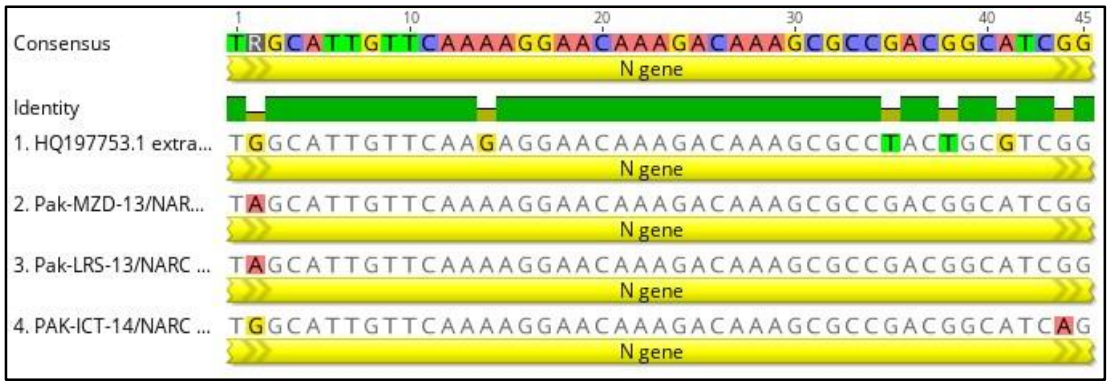



**Figure 3.9 (b)**

**Figure 3.9 (a & b):** Single nucleotide polymorphism (SNP) shown by N gene of one Nigerian strain (HQ197753.1) and three Pakistani isolates (PAK-MZD-13/NARC, PAK-LRS-13/NARC and PAK-ICT-12/NARC)

The P gene is 1529 nucleotides long. The isolate (PAK-ICT-14/NARC) showed the highest identity (99.08%, 93.27%) with two other present study isolates (Pak-LRS-13/NARC, Pak-MZD-13/NARC) respectively and lowest (93.40%) with Lineage II isolate (Nigeria 75/1). The differences in P gene of Nigerian strain and three Pakistani isolates of PPRV ranged from 102 to 103 bases/residues, however, among Pakistani isolates, the maximum difference was of 14 bases (Table 3.10).

**Table 3.10:** Percent identities and differences in bases /residues of P gene of PPRV

| | HQ197753.1 | | PAK-ICT-14/NARC | | Pak-LRS-13/NARC | | Pak-MZD-13/NARC | |
|---|---|---|---|---|---|---|---|---|
| | **Non-identical bases** | **% identities** | **Non-identical bases** | **% identities** | **Non-identical bases** | **% identities** | **Non-identical bases** | **% identities** |
| **HQ197753.1** | | | 101 | 93.40 | 103 | 93.27 | 103 | 93.27 |
| **PAK ICT-14/NARC** | 101 | 93.40 | | | 14 | 99.08 | 14 | 99.08 |
| **Pak-LRS-13/NARC** | 103 | 93.27 | 14 | 99.08 | | | 0 | 100 |
| **Pak MZD-13/NARC** | 103 | 93.27 | 14 | 99.08 | 0 | 100 | | |

The N-terminus of P protein of present study isolates showed high variability with PPRV strains from other countries and there was a comparatively conserved C-terminus. The 16 amino acid motif (EQAYHVNKGLECIKSL) referred to as soyuz 1 sequence was also shown at N termini of P protein of Pakistani strains as well as Nigerian strain of PPRV (Figure 3.10). It is evident from studies that this sequence prevents the self-assembly of N protein by binding to its active site (Karlin and Belshaw, 2012).

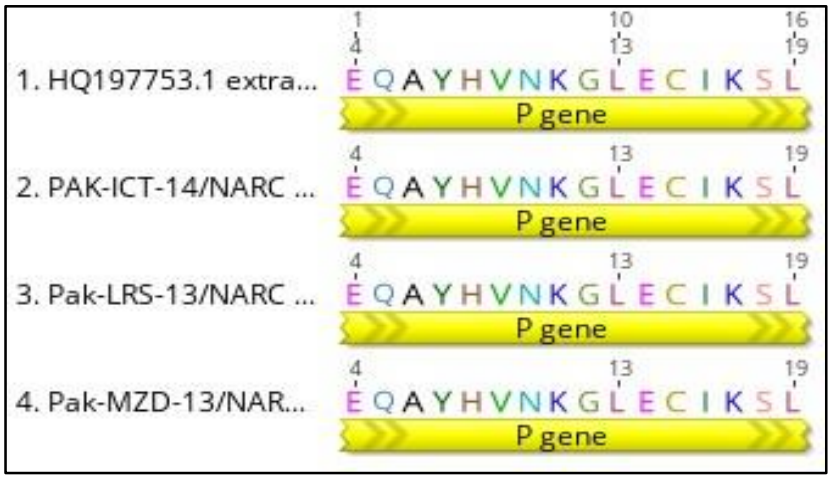



**Figure 3.10:** Soyuz 1 sequence in P protein shown by Nigerian strain (HQ197753.1) and three PPRV strains (PAK-ICT-12/NARC, PAK-LRS-13/NARC, and PAK-MZD-13/NARC)

The M protein is considered highly conserved among different lineages of PPRV although the comparative analysis of Nigerian strain with three Pakistani strains revealed that the strain PAK-ICT-12/NARC showed an amino acid change from threonine (Thr) to isoleucine (Ile) at position 24 while the strain PAK-MZD-13/NARC showed lysine (Lys) instead of glutamine (Gln) at position 153. The M protein of Nigerian strain showed 7 amino acid changes from Pakistani strains (histidine (His) at position 34, valine (Val) at position 51, leucine at position 107, arginine at position 126, threonine (Thr) at position 131 (Figure 3.11 a), serine (Ser) at position 203, lysine (Lys) at position 230 (Figure 3.11 b) while three Pakistani strains showed glutamine (Gln), methionine (Met), valine (Val), lysine (Lys), alanine (Aln), lysine (Lys), glutamine at the same positions) (Figure 3.11 a & b).

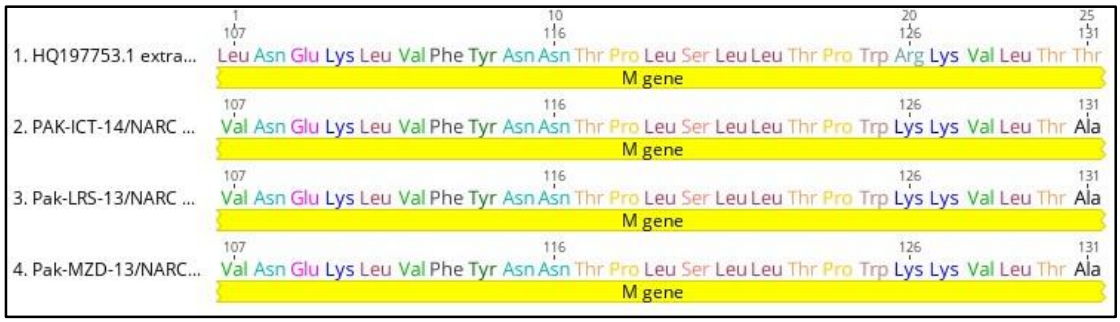



**Figure 3.11 (a)**

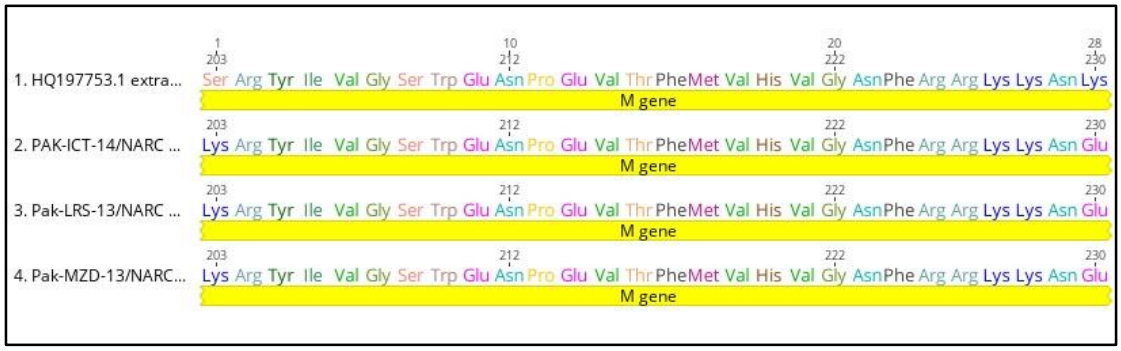



**Figure 3.11 (b)**

**Figure 3.11 (a & b):** Amino acid mutations in M protein shown by Nigerian strain (HQ197753.1) and three PPRV strains (PAK-ICT-12/NARC, PAK-LRS-13/NARC, and PAK-MZD-13/NARC)

The F gene of PPRV was 1640 nucleotides long. The amino acid motif GRRTRR of this protein which was described by Meyer and Diallo (1995) as cleavage site where F0 (inactive F protein) is cleaved by host cell proteases into two active sub units (F and F2) was only found in PAK-ICT-12/NARC and PAK-MZD-13/NARC at position 127 (Figure 3.12 a). The three glycosylation sites (NLS, NIT and NCT) shown by F2 protein which are considered as highly conserved in all morbilliviruses were also found only in PAK-ICT-12/NARC and PAK-MZD-13/NARC at positions 49, 81 and 87) (Figure 3.12 b, c & d).

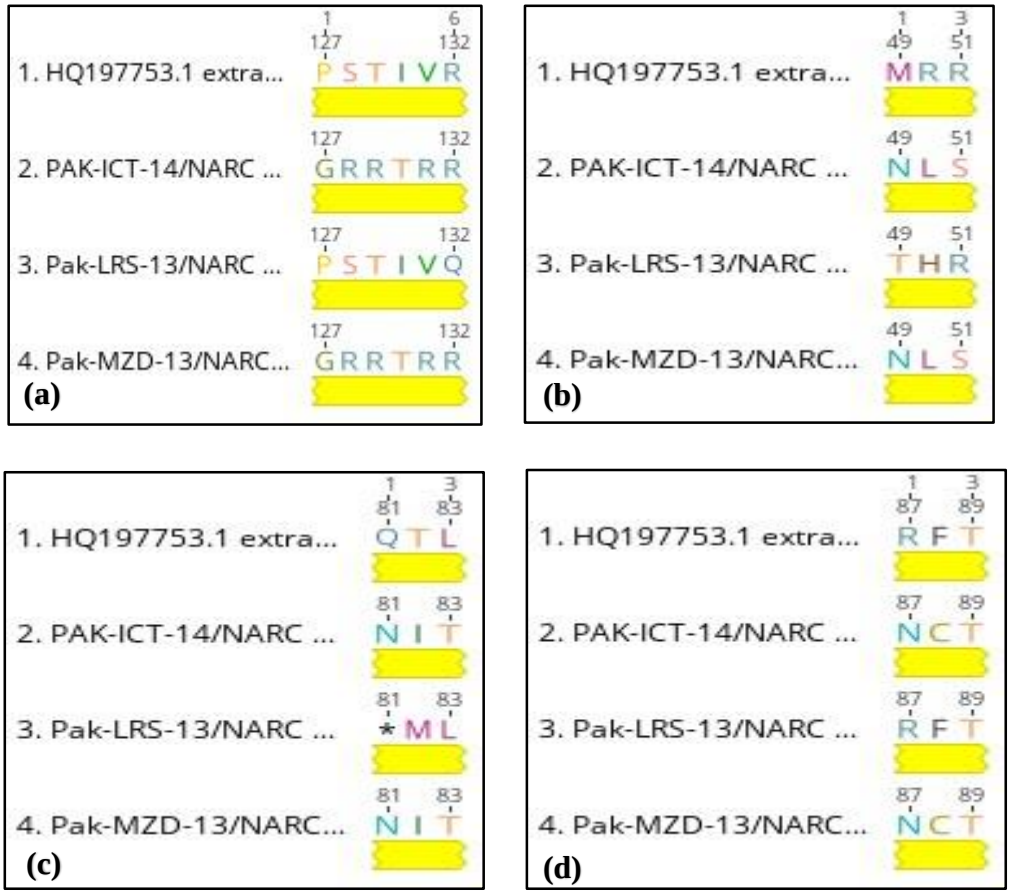



**Figure 3.12 (a, b, c & d):** Amino acid mutations in M protein shown by Nigerian strain (HQ197753.1) and three PPRV strains (PAK-ICT-12/NARC, PAK-LRS-13/NARC, and PAK-MZD-13/NARC)

The H gene was found 1829 nucleotides long. It was considered the least conserved gene among morbilliviruses. The Binding of MV to SLAM receptors on host cells was mediated by asparagine residues at position 481 but histidine was found at this position in most H proteins of PPRV submitted to Genbank. But recent analysis of H protein of present study isolates revealed that glycine residue was present at this position instead of histidine (Figure 3.13).

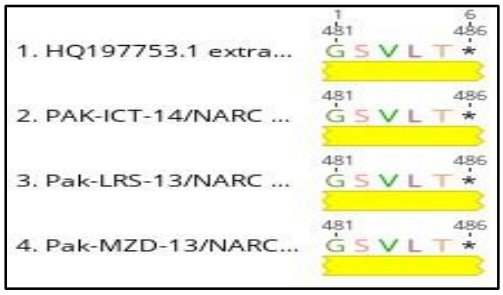



**Figure 3.13:** Amino acid glycine at position 481 in H protein of Nigerian strain (HQ197753.1) and three PPRV strains (PAK-ICT-12/NARC, PAK-LRS-13/NARC, and PAK-MZD-13/NARC)

The largest among all the proteins of PPRV is L protein consisting of 6552 nucleotide and 2183 amino acids (Figure 3.14). The previously identified conserved motifs QGDNQ and GDDD known for RNA polymerase activity at positions 771 and 1464 in lineage II PPRV strain (Ghana/ NK 1/2010) was not found in present study isolates (Lineage IV) as well as Nigerian strain (Lineage II).

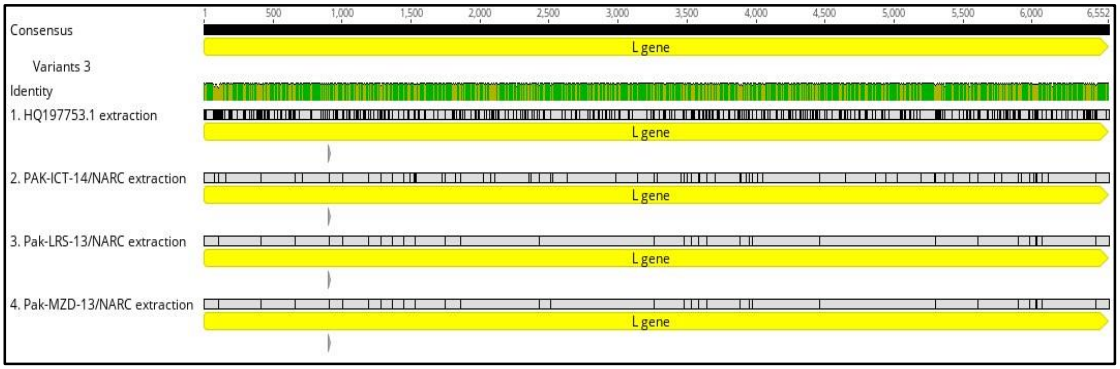



**Figure 3.14:** The large (L) protein of Nigerian strain (HQ197753.1) and three Pakistani PPRV strains (PAK-ICT-12/NARC, PAK-LRS-13/NARC, and PAK-MZD-13/NARC)

### 3.3 Discussion

Peste des Petits Ruminants (PPR) is considered as an important trans-boundary disease of small ruminants causing huge economic losses. In the present study, a total of 43 outbreaks reported by resource persons during 2012-2014 in targeted areas of the country were confirmed. Out of 239 samples collected during this period, a total of 174 and 151 were found positive by RT-PCR and Ic-ELISA respectively.

RT-PCR was found to be an effective test for PPRV identification from field samples as well as cell culture derived supernatant. The study revealed an appraisal of 70.17% of positive samples by RT-PCR which is high in comparison with Ic-ELISA which declared 61.58% of samples positive for PPR antigen (Table 3.2). The overall assessment of PPR positive samples (65.87%) was suggestive of proper sampling and disease investigation during this period. The results were in agreement with a study executed by Zahur and his colleagues where similar proportion (65%) of positive samples was found (Zahur et al., 2008). Recently, in another study, 61.15% of positive samples were found which is somewhat lower than the present study (Abubakar et al., 2018). This could be due to the difference in the targeted sampling areas of the country.

Despite the fact that the disease was not fully anticipated among the majority yet there is substantially more diligence in recent years (Abubakar et al., 2011). The highest outbreak frequency was reported by KPK (Khyber Pakhtunkhwa) followed by ICT (Islamabad capital territory) and Punjab province due to the fact that these provinces have more nomadic movement in comparison to others (Zahur et al., 2011). According to a recent study, more outbreaks were reported from Punjab province due to advancement in veterinary infrastructure along with excellent reporting system in this province (Abubakar et al., 2018). PPR has also been reported from Punjab province previously (Khan et al., 2007; Abubakar et al., 2008).

The highest numbers of positive samples were also found from Punjab province which indicates the well recognition of disease and the role of nomads in this region. Within this province, southern Punjab showed high disease incidence as compared to central and northern Punjab. In the northern Punjab, the arid areas showed more disease outbreaks which can be explained in terms of more disease prevalence during rainy season. Fewer outbreaks were reported from AJK while only one outbreak was reported

from Baluchistan which might be due to less understanding of disease and security situation in Baluchistan province (Abubakar et al., 2018).

As RNA viruses undergo variability in their genomic sequences (Anderson et al., 2006; Taylor and Barrett, 2007), the exigency to undertake the molecular epidemiological studies become a requisite (Zahur et al., 2014). The phylogenetic analysis consorted all the present study isolates in lineage IV which is the largest lineage of PPRV prevalent throughout Asia (Wang et al., 2009). It is evident in the current study that Pakistani isolates are more diverse which is indicative of new virus influx from various sources. Most isolates were found to be closely associated to Chinese and other Pakistani isolates. Two isolates clustered together with Indian isolates and one of the isolates showed similarities with Iranian isolate. The most understandable reason is the cross border animal movement which takes place among these countries. Another possibility for this alliance is the trade by nomads who sell their animals on the religious occasion of Eid-ul-Adha. These findings are in accordance with another study conducted by Abu Bakar and his colleagues who reported that Pakistani isolates were more dispersed based on phylogenetic analysis of F and N gene. The isolates were mainly fell in two groups. One group was clustered with previous Pakistani isolates and the other had similarities with Indian and Iranian isolates (Abubakar et al., 2018). In a similar study based on phylogenetic analysis of N gene, the authors revealed that the studied isolates from Pakistan were mostly clustered together with Tajikistani PPRV (Anees et al., 2013). One report, while studying the sequence variability of F gene found that the studied PPRV isolates were monophyletic being closely related to Indian isolates (Zahur et al., 2014). The reason suggested for PPRV transmission was also the sharing of border and massive trade by nomads with Tajikistan and India respectively.

In another similar study, a parallel comparison between N and F gene was conducted concluding that N gene of Pakistani isolates had similarities with Chinese, Iranian and Tajikistani isolates representing the true geographical trend for PPRV transmission whereas F gene revealed a different finding for PPRV isolates which had more similarities with Middle Eastern isolates (Munir et al., 2012).

In contrast to the present study, the phylogenetic analysis of a PPRV isolate (Nkp1/2012) from an outbreak in India showed that the isolate was not closely related to Pakistani PPR virus (Kumar et al., 2014).

In order to sequence the complete genome of PPRV, two methods were used which were standardized at CIRAD, France. First is the random sequencing method or SISPA and second is amplification with specific primers. The same samples were used in both sequencing techniques for comparative analysis.

The results obtained with SISPA showed a low PPRV specific reads as compared to the amplification using specific primers which does not cover the entire PPRV genome. Only one complete genome of PPRV was obtained with this technique. These low rates can be explained by the fact that non-specific amplification was used with random primers. Although the time required for carrying out this method was less but it is much expensive compared with other technique.

On the other hand, the amplification with specific primers resulted in high PPRV specific reads (96.7%). Two strains (PAK-LRS-13/NARC, PAK-ICT-12/NARC) were completely sequenced using this technique. The specific primers used in this technique were designed employing sufficient numbers of complete genomes retrieved from Genbank. Also the primers were drawn from highly conserved regions of different lineages of PPRV. The main disadvantage with this technique was time required for sample preparation. It is necessary to make a specific amplification for each of the pairs of primers under specific conditions. However, unlike the SISPA technique, it is less expensive. The technique also needs good quality sample as one sample (PAK-MZD-13/NARC) was difficult to amplify with all primer sets. This might be due to the poor quality of sample. As in Pakistan, the average temperature during summers reaches up to 40°C in hot climatic regions. The sample was not appropriately stored due to poor infrastructure from the area where it was originally collected. The sample was also used for multiple manipulations and several freeze thaw cycles had an effect on quality of sample. However, considering the cost of amplification and number of reads obtained with the method, sequencing with specific primers is highly suggested.

Complete PPRV genomes were used for phylogenetic analysis by Neighbor joining method. The phylogenetic tree thus obtained was compared with the tree obtained using partial N gene sequences of PPR strains isolated from different areas in Pakistan. The results revealed that phylogeographic distribution of different PPRV strains related to specific lineages were similar regardless of the type of sequencing used: complete, incomplete or partial sequence of specific protein (N) of PPRV. This suggested that

phylogenetic analysis even from partial sequence of N gene is very informative for a preliminary investigation of origin of a strain.

The present study PPRV isolates and those from neighboring countries (Lineage IV) were found closely related to each other though different subgroups can be observed. The phylogenetic analysis showed great diversity in PPRV strains belonging to lineage IV which might be due to cross border PPRV transmission related to animal trade which takes place among these countries.

Three present study PPRV strains (PAK-LRS-13/NARC, PAK-ICT-12/NARC and PAK-MZD-13/NARC) were sequenced completely using NGS technique. The sequencing generated 15,948 bp of sequence which was same for all other PPRV genomes sequenced to date. The genome organization of these three Pakistani strains was also same and is in agreement with "rule of six" for Paramyxoviruses. There was a genome promotor region comprising of 107 nucleotides (nt) at the 3′ end and anti-genome promotor at 5′ end. In between these two regions, the transcriptional units for structural (N, P, M, F, H) and nonstructural proteins (P and V) were present. The results were in agreement with the previous studies in which sequencing of complete genome of PPRV resulted in same genomic organization (Zhu et al., 2016; Dundon et al., 2018)

Comparative analysis of these strains with each other and with Nigerian strain 75/1 (HQ197753.1) revealed that N gene comprising of 1578 nucleotides was more conserved in comparison with Nigerian strain which showed highest identity (98.73%) with Pakistani isolates and lowest (89.57% and 92.7%) with the Lineage I isolate (ICV-89) and Lineage II isolate (Nigeria 75/1) respectively. However, single nucleotide polymorphisms (SNPs) were evidenced at different positions. The same mutations were seen within N gene in another study reported by Bao and his co-authors while studying the complete genome of PPRV from wild bharal in Tibet, China (Bao et al., 2012).

The P gene of Nigerian strain showed differences in 102 to 103 bases/residues from three Pakistani isolates of PPRV however, among Pakistani isolates, the maximum difference was of 14 bases. The 16 amino acid motif (EQAYHVNKGLECIKSL) referred to as soyuz 1sequence was also shown at N termini of P protein of Pakistani strains as well as Nigerian strain of PPRV. The conserved motif was observed for the first time by Karlin and Belshaw while studying conserved motifs in N termini of

Mononegavirales P proteins (Karlin and Belshaw, 2012). In another study, the same motif was found in P gene of Ghana/NK 1/2010 strain of PPRV (Dundon et al., 2014).

The M protein is considered highly conserved among different lineages of PPRV although the comparative analysis of Nigerian strain with three Pakistani strains revealed that an amino acid change was observed within Pakistani strains while the M protein of Nigerian strain showed 7 amino acid changes from Pakistani strains. The differences in different regions within M gene of a novel mutant of PPRV were also reported by Zhu and his coauthors (Zhu et al., 2016).

The amino acid motif GRRTRR found in F protein, described by Meyer and Diallo as cleavage site (Meyer and Diallo, 1995) was only found in PAK-ICT-12/NARC and PAK-MZD-13/NARC at position 127. The three glycosylation sites (NLS, NIT and NCT) shown by F2 protein which are considered as highly conserved in all morbilliviruses were also found only in PAK-ICT-12/NARC and PAK-MZD-13/NARC at positions 49, 81 and 87 (Dundon et al., 2014).

The H gene was found 1829 nucleotides long. It was considered the least conserved gene among morbilliviruses. Recent analysis of H protein of present study isolates revealed that glycine residue was present instead of histidine at position 481. The binding of MV to SLAM receptors on host cells was mediated by asparagine residues at this position. The study was in contrast with another study (Dundon et al., 2014) which revealed that histidine was found at this position in H proteins of most PPRV strains submitted to date.

The largest among all the proteins of PPRV is L protein consisting of 6552 nucleotide and 2183 amino acids (Figure 3.14). The previously identified conserved motifs QGDNQ and GDDD known for RNA polymerase activity at positions 771 and 1464 in lineage II PPRV strain (Ghana/ NK 1/2010) (Dundon et al., 2014) were missing in present study isolates (Lineage IV) as well as Nigerian strain (Lineage II). The L protein of PPRV was also studied by Zhu and his co-authors and it was found to be highly conserved at amino acid and nucleotide level (Zhu et al., 2016).

Improvement in current vaccine formulations and development of effective, inexpensive, and sensitive diagnostic techniques can facilitate the control strategy (Fakri et al., 2016). PPR virus isolation employing cell culture techniques is a gold standard test for better understanding of pathogen attributes and to investigate the disease pattern globally

(Hematian et al., 2016). Primary cultures of mammalian kidney and lungs have been used for many years for isolation of morbilliviruses (Diallo et al., 1989; Taylor et al., 1990) but the difficulty in maintaining these cultures and batch to batch variations makes their use less frequent. Therefore, continuous cell lines like Vero, BHK-21, CV1, and CHS-20 are preferred for cultivation of PPR virus and BTS cells are also used (Adombi et al., 2011). The present study revealed that PPR virus like other morbilliviruses also uses SLAM protein of their host species as a cellular receptor. A new cell line referred as BTS-34 was used in this study in comparison with the Vero cells for PPR virus isolation. This cell line constitutively expresses bovine SLAM receptors which aids in attachment and entry of virus into the host cells. Alternatively, Vero cells are also used for propagation of PPR virus as the cells are accessible and can easily be maintained and grown for long time *in-vitro*. However, as the origin of Vero cells is not bovine, PPR virus isolation in these cells is inefficient due to the absence of virus specific receptors on their surface. Pathological samples were grown on two different cell lines for PPR virus recovery. BTS-34 cell line developed visible cytopathic effects within 4 days post infection (Figure 3.3) while in Vero cells, 2 or 3 blind passages (Vero to Vero) were needed to isolate viruses successfully (Figure 3.2). Both cell lines exhibited different forms of changes *in-vitro*. In a similar experiment, the susceptibility of PPR virus towards BHK and Vero cell line was compared revealing that the cytopathic effects and their pattern was earlier and more obvious in BHK cells than in Vero cells (Emikpe et al., 2009).

The infected cells experienced variations in their morphology as the infection progressed. They became well defined, rounded, swollen giant cells with increased refractivity. In Vero cells typical rounding was observed on 21 days post infection. In the late stages of infection, the cells became refractive. Large cluster of cells were seen which later formed clumps, eventually leading to cell disintegration and detachment whereas, BTS cells presented a drastic change in morphology prior to the development of visible CPEs. Small polykaryons with many intercellular spaces were observed that became more prominent and larger the next day ultimately transforming into big syncytia. Finally, the cells detached from glass surface and were seen floating in the culture media.

The SLAM protein present on the cell surface aids in the attachment of virus with the host cell facilitating efficient PPRV isolation. This is supported by a similar work in which goat SLAM receptors were introduced in monkey CV1 cells referred to as CHS-20 cells, facilitating PPR virus growth and isolation from clinical samples. The study

revealed that the CPEs appeared after 11 to 12 weeks post infection in Vero cells while in infected CHS-20 cells syncytia produced within 1 to 2 days following infection (Adombi et al., 2011). It was also proposed that Vero cells expressing canine SLAMs were extremely susceptible to infection with canine distemper virus (CDV) in pathological specimens (Seki et al., 2003). In another study scientists compared PPR virus infection in Vero cells expressing canine SLAM (Vero Dog SLAM) with Vero cells expressing Nectin-4 (VeroNectin-4) depicting VeroNectin-4 cells as more competent than Vero Dog SLAM cells (Fakri et al., 2016). Recently a new suspension cell line (F9 lymphoid cells) has been used for PPRV isolation and it was suggested to be preferable over Vero cells in terms of high titer and could be used to propagate PPR virus for vaccine production (Mofrad et al., 2016).

The study shows that Vero cell line infected with PPRV developed CPEs after 2 to 3 blind passages while the same PPRV strains showed visible infection after 3 to 4 days post infection in susceptible BTS cells suggesting that PPR virus adapted to the BTS-34 cell line to a greater extent as compared to the Vero cells. This high affinity of PPRV towards BTS cells could be due to the presence of SLAM protein being used as cellular receptor. Nevertheless, un-attenuated PPRV strains may possibly use other receptors for entry into the host cells but less competently (Takeda et al., 2007; Tahara et al., 2008).

# Chapter 4

## Evaluation of Thermostability and Inactivation of Peste des Petits Ruminants Virus Isolates

# Chapter 4: Evaluation of Thermostability of Local Isolates of Peste des Petits Ruminants Virus

## 4.1 Introduction

Vaccines are available for controlling PPR disease but efficiency of these vaccines depends upon the maintenance of cold chain in areas of extreme hot weather. To date, no work has been reported related to the difference of thermal stability among PPRV. Similar work has been performed on many other viruses like Hepatitis C virus (HCV) (Song et al., 2010), Hepatitis E viruses (HEV) (Emerson et al., 2005) and Newcastle disease virus (NDV) (Omony et al., 2016).

In the present study, thermostability potential of 7 local isolates of PPR virus was evaluated and virus inactivation procedures were conducted to investigate the effects of heat and UV irradiation on the activity of PPRV.

## 4.2 Results

Out of a total of eighteen PPR virus isolates recovered on both cell lines and confirmed by RT-PCR and Ic-ELISA, one representative isolate from each province was selected based on its genotyping and immunogenicity. These isolates (n=7) were subjected to thermostability testing (Table 4.1).

**Table 4.1:** Description of PPRV isolates with respect to their source and location

| Sample ID | Animal species | Tissue / Organ | Year | Location |
|---|---|---|---|---|
| Pak-VEH-12/NARC | Goat | lymph node | 2012 | Vehari, Punjab |
| Pak-ICT-12/NARC | Goat | lymph node | 2012 | Islamabad |
| Pak-KP-12/NARC | Goat | lymph node | 2012 | Khairpur, Sindh |
| Pak-FGJ-14/NARC | Goat | lymph node | 2014 | Taxila, Punjab |
| Pak-ATT-13/NARC | Goat | lymph node | 2013 | Dhok Chodrian, Punjab |
| Pak-MZD-13/NARC | Goat | lymph node | 2013 | Muzzafarabad, AJK |
| Pak-LRS-13/NARC | Sheep | lymph node | 2013 | Islamabad |

### 4.2.1 Infectious Titers of Isolates

The infectious titers of PPRV isolates were calculated using Reed and Munch method (Reed and Muench, 1938). Table 4.2 shows the calculation of titer of one of the isolate with lowest infectious titer in terms of $TCID_{50}$.

**Table 4.2:** Reed and Munch method for calculation of infectious titers

| Log of virus dilution | Infected wells | Non-infected wells | Cumulative infected wells (X) | Cumulative non-infected wells (Y) | Ratio=X/(X+Y) | Percent (%) Infected |
|---|---|---|---|---|---|---|
| **-2** | 5/5 | 0/5 | 12 | 0 | 12/12=1 | 100% |
| **-3** | 4/5 | 1/5 | 7 | 1 | 7/8=0.875 | 87.5% |
| **-4** | 2/5 | 3/5 | 3 | 4 | 3/7=0.43 | 42.9% |
| **-5** | 1/5 | 4/5 | 1 | 8 | 1/9=0.11 | 11.1% |
| **-6** | 0/5 | 5/5 | 0 | 13 | 0/13=0 | 0% |

The following formula was used to calculate infectious titers ($TCID_{50}$)

**Log $TCID_{50}$**= (log dilution above 50%) + (PD x log dilution factor)

The PD or proportionate distance was calculated as under:

**Proportionate distance (PD)** = $\frac{\%CPE \text{ at dilution above } 50\% - (50)}{\%CPE \text{ at dilution above } 50\% - \%CPE \text{ at dilution below } 50\%}$

= (87.5-50) / (87.5-42.9) = 0.84

**Log $TCID_{50}$**= (log dilution above 50%) + (PD x log dilution factor)

Where dilution factor is 10 and log of 10 is 1

Log TCID50= (-3) + (0.84 x 1)

Log TCID50= -3.84

Log TCID50= $10^{3.84}$ / 0.1ml

So

$TCID_{50}$ / ml = 10 x $10^{3.84}$ = $10^{4.84}$

$TCID_{50}$ / ml = 4.9

### 4.2.2 Virus Titration

As the initial titers were different for different isolates, the isolates with more initial titers were diluted in sterile GMEM without serum to homogenize the titers as shown in Table 4.3.

**Table 4.3:** Initial titers of seven isolates of PPR virus used in this study

| Local Isolates used | Initial Titers (TCID50/ml) | Homogenized Initial Titers |
|---|---|---|
| Pak-VEH-12/NARC | $10^{5.9}$ | $10^{4.9}$ |
| Pak-ICT-12/NARC | $10^{4.9}$ | $10^{4.9}$ |
| Pak-KP-12/NARC | $10^{5.9}$ | $10^{4.9}$ |
| Pak-FGJ-14/NARC | $10^{5.9}$ | $10^{4.9}$ |
| Pak-ATT-13/NARC | $10^{5.9}$ | $10^{4.9}$ |
| Pak-MZD-13/NARC | $10^{5.9}$ | $10^{4.9}$ |
| Pak-LRS-13/NARC | $10^{6.9}$ | $10^{4.9}$ |

### 4.2.3 PPRV Stability at Different Temperatures

In order to investigate PPRV stability at different temperatures, the reduction of viral titers of isolates was determined at 37°C, 25°C and 4°C. The isolates were aliquoted in sterile 1.5ml Eppendorf tubes and these aliquots were placed at different temperatures. One aliquot from each isolate placed at 4°C was removed after every 2 days till 30 days. The aliquot placed at 25°C was removed every day for 1 week while one aliquot at 37°C was removed after every 3 hours for a total of 30 hours. Similarly the thermal inactivation of PPRV isolates was determined at defined time periods and temperatures. The isolates were placed in a water bath at 56°C, 60°C and 65°C and sampling was performed after every 15 minutes for 2 hours. All the treated isolates were placed at -70°C till virus titration. The experimental design is given in Table 4.4.

**Table 4.4:** The experiment design concerning thermal stability and inactivation of PPRV isolates at different temperatures and time points

| No. of days | Temperatures | | | | | |
|---|---|---|---|---|---|---|
| | **4°C (In days)** | **22°C (In days)** | **37°C (In hours)** | **56°C (In minutes)** | **60°C (In minutes)** | **65°C (In minutes)** |
| | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2 | 1 | 3 | 15 | 15 | 15 |
| | 4 | 2 | 6 | 30 | 30 | 30 |
| | 6 | 3 | 9 | 45 | 45 | 45 |
| | 8 ⇓ 30 | 4 ⇓ 7 | 12 ⇓ 30 | 60 ⇓ 120 | 60 ⇓ 120 | 60 ⇓ 120 |

### 4.2.3.1 Reduction of Titers at 4°C

PPRV isolates were relatively stable at 4°C, and titer loss was less frequent. PAK-ICT-12/NARC and PAK-MZD-13/NARC were considered most stable isolates at this temperature as they maintained their original titer for 2 weeks with no loss of infectivity. The least stable isolate was PAK-LRS-13/NARC which lost its complete titer on 13$^{th}$ day post infection. PAK-VEH-12/NARC and PAK-FGJ-07/NARC were considered moderately stable isolates as loss in infectious titer was less frequent. PAK-KP-12/NARC and PAK-ATT-13/NARC started to lose their titer by 2$^{nd}$ day post infection but the titer loss was gradual and they were still detectable on 28$^{th}$ day post infection. The gradual loss in infectious titers of isolates is given in Table 4.5.

**Table 4.5:** Loss in infectious titers of PPRV isolates at 4°C over 30 days

| Days | PAK-VEH-12/NARC | PAK-ICT-12/NARC | PAK-KP-12/NARC | PAK-FGJ-14/NARC | PAK-ATT-13/NARC | PAK-MZD-13/NARC | PAK-LRS-13/NARC |
|---|---|---|---|---|---|---|---|
| **0** | 4.9 | 4.9 | 4.9 | 4.9 | 4.9 | 4.9 | 4.9 |
| **2** | 4.9 | 4.9 | 3.9 | 4.9 | 3.9 | 4.9 | 2.9 |
| **4** | 3.9 | 4.9 | 3.9 | 4.9 | 3.9 | 4.9 | 2.9 |
| **6** | 3.9 | 4.9 | 3.9 | 3.9 | 3.9 | 4.9 | 2.9 |
| **8** | 3.9 | 4.9 | 3.9 | 3.9 | 3.9 | 4.9 | 1.9 |
| **10** | 2.9 | 4.9 | 3.9 | 3.9 | 3.9 | 4.9 | 1.9 |
| **12** | 2.9 | 4.9 | 3.9 | 2.9 | 3.9 | 4.9 | 1.9 |
| **14** | 2.9 | 4.9 | 3.9 | 2.9 | 3.9 | 4.9 | 0 |
| **16** | 2.9 | 4.9 | 3.9 | 2.9 | 2.9 | 4.9 | 0 |
| **18** | 1.9 | 3.9 | 2.9 | 1.9 | 2.9 | 3.9 | 0 |
| **20** | 1.9 | 3.9 | 2.9 | 1.9 | 2.9 | 3.9 | 0 |
| **22** | 1.9 | 3.9 | 2.9 | 1.9 | 2.9 | 3.9 | 0 |
| **24** | 0 | 2.9 | 1.9 | 0 | 1.9 | 2.9 | 0 |
| **26** | 0 | 2.9 | 1.9 | 0 | 1.9 | 2.9 | 0 |
| **28** | 0 | 2.9 | 1.9 | 0 | 1.9 | 2.9 | 0 |
| **30** | 0 | 0 | 0 | 0 | 0 | 1.9 | 0 |

The mean titer losses of all PPRV isolates at 4°C were calculated at defined time periods and are represented in Figure 4.1.

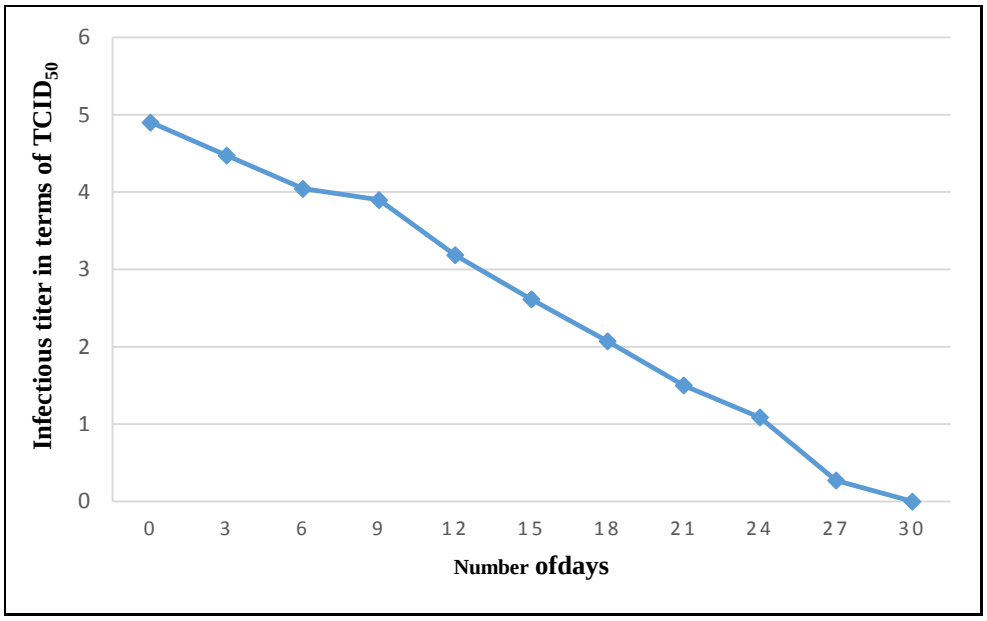



**Figure 4.1:** Mean titer losses of all PPRV isolates at 4°C over 30 days

#### 4.2.3.2 Reduction of Titers at 25°C

At 25°C, the decline in infectious titer of PPRV isolates was slow and moderate. The most stable isolate at RT was PAK-ICT-12/NARC that did not lose its titer up to the 4$^{th}$ day. After that there was one log decrease in titer up to one week. PAK-MZD-13/NARC and PAK-KP-12/NARC maintained their original titers for 2 and 1 day respectively. Then there was a gradual reduction in titer that was still detectable till 7$^{th}$ day for PAK-MZD-13/NARC and PAK-KP-12/NARC.

There was also gradual reduction in titers of PAK-VEH-12/NARC, PAK-ATT-13/NARC and PAK-LRS-13/NARC up to 6$^{th}$ day, after which their titers were undetectable by this assay.

The most fragile isolate of PPRV was PAK-FGJ-14/NARC which lost its complete titer at 5$^{th}$ day. The details of titers of all isolates at 25°C ae given in Table 4.6.

**Table 4.6:** Loss in infectious titers of PPRV isolates at 25 °C over 7 days

| Days | PAK-VEH-12/NARC | PAK-ICT-12/NARC | PAK-KP-12/NARC | PAK-FGJ-14/NARC | PAK-ATT-13/NARC | PAK-MZD-13/NARC | PAK-LRS-13/NARC |
|---|---|---|---|---|---|---|---|
| **0** | 4.9 | 4.9 | 4.9 | 4.9 | 4.9 | 4.9 | 4.9 |
| **1** | 3.9 | 4.9 | 4.9 | 3.9 | 3.9 | 4.9 | 4.9 |
| **2** | 2.9 | 4.9 | 3.9 | 2.9 | 2.9 | 4.9 | 3.9 |
| **3** | 2.9 | 4.9 | 3.9 | 2.9 | 2.9 | 3.9 | 3.9 |
| **4** | 1.9 | 4.9 | 2.9 | 1.9 | 2.9 | 3.9 | 2.9 |
| **5** | 1.9 | 3.9 | 2.9 | 0 | 1.9 | 2.9 | 2.9 |
| **6** | 0 | 3.9 | 1.9 | 0 | 1.9 | 1.9 | 1.9 |
| 7 | 0 | 3.9 | 1.9 | 0 | 0 | 1.9 | 0 |

The mean titer losses of all PPRV isolates at 25°C were calculated at defined time periods and are represented in Figure 4.2.

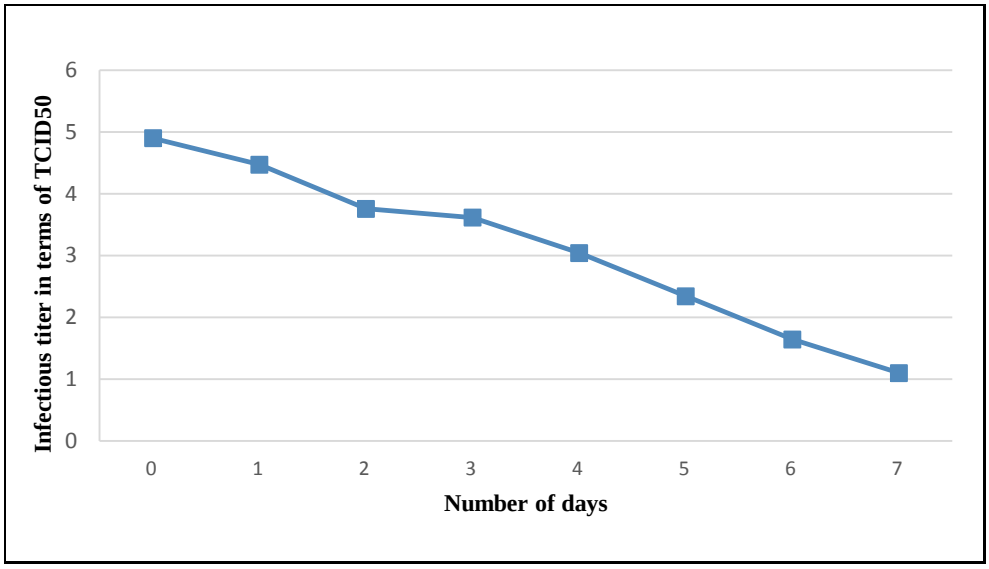



**Figure 4.2:** Mean titer losses of all PPRV isolates at 25°C over 7 days

#### 4.2.3.3 Reduction of Titers at 37°C

All PPRV isolates lost their infectivity after incubation at 37°C for 30 hours after which the assay became negative with no residual titer. Most of the viruses showed maximum titer loss in first 21 hours when the titer dropped from $10^{\mathbf{4.9}}$ to $10^{1.9}$ while in the next 6 hours, the titer dropped to the maximum detectable limit and became negative after 30 hours incubation at 37°C.

PAK-ICT-12/NARC and PAK-MZD-13/NARC were considered the most stable isolates at $37^{O}$C as no reduction in titers was observed in the first twelve hours for PAK-ICT-12/NARC, after that there was one log decrease in titer till 21 hours. Similarly, PAK-MZD-13/NARC did not lose its infectious titer for the first nine hours at $37^{o}$C but showed a gradual reduction in titer after nine hours (20.4% after twelve hours, 40.81% after 15 and 18 hours and 61% after 21 and 24 hours).

PAK-KP-12/NARC, PAK-FGJ-14/NARC and PAK-LRS-13/NARC were considered as moderately stable isolates. They showed a gradual reduction in titers after three hours as shown in Table 4.7. PAK-VEH-12/NARC and PAK-ATT-13/NARC were least stable isolates as they maintained their infectious titers till fifteen hours. After that the assay became negative for these isolates.

**Table 4.7:** Loss in infectious titers of PPRV isolates at 37°C over 30 hours

| Hours | PAK-VEH-12/NARC | PAK-ICT-12/NARC | PAK-KP-12/NARC | PAK-FGJ-14/NARC | PAK-ATT-13/NARC | PAK-MZD-13/NARC | PAK-LRS-13/NARC |
|---|---|---|---|---|---|---|---|
| **0** | 4.9 | 4.9 | 4.9 | 4.9 | 4.9 | 4.9 | 4.9 |
| **3** | 2.9 | 4.9 | 4.9 | 4.9 | 4.9 | 4.9 | 3.9 |
| **6** | 2.9 | 4.9 | 3.9 | 3.9 | 3.9 | 4.9 | 3.9 |
| **9** | 2.9 | 4.9 | 3.9 | 3.9 | 2.9 | 4.9 | 3.9 |
| **12** | 1.9 | 4.9 | 2.9 | 2.9 | 2.9 | 3.9 | 2.9 |
| **15** | 1.9 | 3.9 | 2.9 | 2.9 | 1.9 | 2.9 | 1.9 |
| **18** | 0 | 3.9 | 2.9 | 2.9 | 0 | 2.9 | 1.9 |
| **21** | 0 | 2.9 | 1.9 | 1.9 | 0 | 1.9 | 1.9 |
| **24** | 0 | 0 | 1.9 | 1.9 | 0 | 1.9 | 1.9 |
| **27** | 0 | 0 | 1.9 | 0 | 0 | 0 | 0 |
| **30** | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

The mean titer losses of all PPRV isolates at 37°C were calculated at defined time periods and are represented in the figure 4.3.

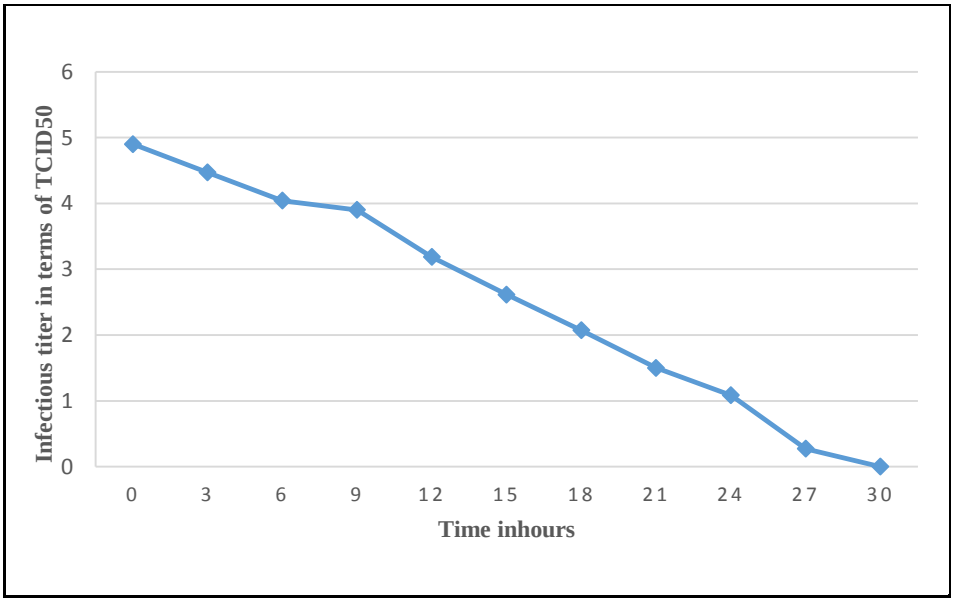



**Figure 4.3:** Mean titer losses of all PPRV isolates at 37°C over 30 hours

### 4.2.4 Effect of Heat Treatment on Infectivity of PPRV Isolates

In order to investigate the effect of heat treatment on infectivity of PPR virus, aliquots of seven isolates of PPRV were subjected to four progressive temperatures (56°C, 60°C, 62°C and 65°C) for 6 different time intervals (5min, 15min, 30min, 45min, 60min, 120min). At 56°C, PPR virus infectivity was lost within 20 to 25 minutes as shown in Table 4.8. PAK-LRS-07/NARC was the most stable isolate at this temperature as it showed titer of $10^{2.9}$ TCID50/ml after 15 minutes exposure which dropped to $10^{1.9}$ TCID50/ml after 30 minutes at 56°C.

PAK-ICT-12/NARC and PAK-MZD-13/NARC were moderately stable as they maintained their titer of $10^{2.9}$ TCID50/ml for 15 minutes while PAK-VEH-12/NARC and PAK-FGJ-14/NARC were least stable with $10^{1.9}$ TCID50/ml titer for 15 minutes. PAK-KP-12/NARC and PAK-ATT-13/NARC were highly labile isolates as they completely lost their infectivity at high temperature.

PPRV infectivity loss was more rapid at higher temperatures. PPRV in cell culture medium was impressible to heat and could be inactivated in 7 and 5 min when incubated at 60°C and 65°C respectively. PAK-ICT-12/NARC and PAK-MZD-13/NARC gave reasonable titer when exposed to high temperature of 60°C for 15 minutes. The most labile isolates were PAK-KP-12/NARC and PAK-ATT-13/NARC as they lost their complete titer after incubation at 60°C for 15 minutes.

**Table 4.8:** Loss in infectious titers of PPRV isolates at 56° C over 120 minutes

| Min | PAK-VEH-12/NARC | PAK-ICT-12/NARC | PAK-KP-12/NARC | PAK-FGJ-14/NARC | PAK-ATT-13/NARC | PAK-MZD-13/NARC | PAK-LRS-13/NARC |
|---|---|---|---|---|---|---|---|
| **0** | 4.9 | 4.9 | 4.9 | 4.9 | 4.9 | 4.9 | 4.9 |
| **15** | 1.9 | 2.9 | 0 | 1.9 | 0 | 2.9 | 2.9 |
| **30** | 0 | 0 | 0 | 0 | 0 | 0 | 1.9 |
| **45** | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **60** | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **120** | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

The mean titer losses of all PPRV isolates at 56°C were calculated at defined time periods and are represented in the figure 4.4.

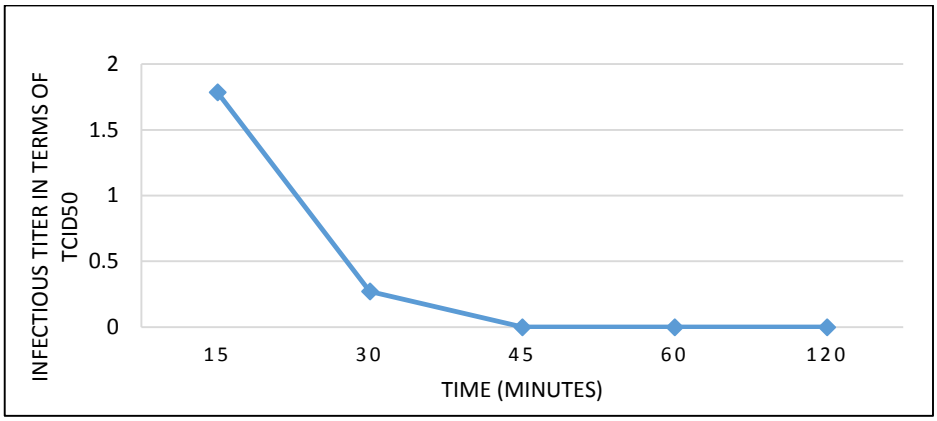



**Figure 4.4:** Mean titer losses of all PPRV isolates at 56°C over 120 minutes

On the basis of thermostability experiment, the seven isolates were categorized according to their thermostability potential as given in Table 4.9.

**Table 4.9:** Categorization of PPRV isolates in terms of their thermo-stability potential

| Most stable | Moderately stable | Least stable | Highly labile |
|---|---|---|---|
| PAK-LRS-13/NARC | PAK-ICT-12/NARC | PAK-VEH-12/NARC | PAK-KP-12/NARC |
| | PAK-MZD-13/NARC | PAK-FGJ-14/NARC | PAK-ATT-13/NARC |

### 4.2.5 Effect of UV Irradiation on PPRV Infectivity

To assess the effect of UV irradiation on PPR virus infectivity, aliquots containing 100µl of each PPRV isolate were placed under UV lamp for different time intervals and infectious titer was calculated immediately after exposure. All isolates were inactivated completely within 2 to 3 minutes under UV irradiation while aliquots of PPRV placed at RT as control for the same time intervals maintained the original titer for this time period.

## 4.3 Discussion

PPRV is an enveloped, negative stranded RNA virus in the family Paramyxoviridae and genus Morbillivirus. Like other members of this genus, it is a thermo-labile virus that can easily be inactivated at high temperatures, by UV irradiations, lipid based detergents and pH variations (inactivated at pH < 5.6 and >9.6) (Chazya et al., 2014).

Limited work has been done related to the stability of PPR virus and its inactivation at different environmental conditions. Although similar work has been performed on many other viruses like Hepatitis C virus (HCV) (Song et al., 2010), Hepatitis E viruses (HEV) (Emerson et al., 2005), and Newcastle disease virus (NDV) (Omony et al., 2016). In this study, thermostability of seven cell culture derived PPRV isolates was determined under different temperatures and inactivation of PPRV by heat and UV irradiation was evaluated.

The present study categorized PPR virus isolates recovered from field samples collected from different areas of the country into different classes based on thermal stability characteristics. PPRV isolates were comparatively stable at 4°C with no radical loss of infectivity for 3 weeks and were still detectable at 4$^{th}$ week. Though there was difference in reduction of infectivity between different isolates. At 25°C, the decline in infectious titer was slow and moderate. It was estimated that PPR virus could tolerate temperature of 25°C for about 10-15 days. The most stable isolate was PAK-ICT-12/NARC that did not lose its original titer up to the 4$^{th}$ day at room temperature. The study reveals that PPR virus in cell culture medium can tolerate 37°C for a maximum of 30 hours after which no residual infectivity was observed. The most stable isolates that showed low titer loss at 37°C were PAK-ICT-12/NARC and PAK-MZD-13/NARC as they maintained their original titer for first 12 and 9 hours post infection respectively. No titer reduction was observed until this time. The isolate PAK-ICT-12/NARC was originally isolated from Sind province of Pakistan, where in summer season temperature reaches up to 52-54°C. The most labile isolate at 37°C was PAK-ATT-13/NARC that was initially isolated from Punjab province where maximum temperature reaches almost 45-51°C. The isolate was completely inactivated after 18 hours of incubation at 37°C. The better heat tolerance in the circulating strains of these areas could be due to the higher temperatures in the region and circulating virus isolates might have adapted to survive these higher temperatures. In a similar study performed

on Hepatitis C virus (HCV), it was demonstrated that a genotype of HCV virus was comparatively stable at 4°C without profound loss of infectivity for 6 weeks and could tolerate 37°C and RT (room temperature) for a maximum of 2 and 16 days when grown in cell culture medium (Song et al., 2010).

Most of the viruses become inactivated when exposed to high temperatures. Heat generally denatures viral proteins and also results in formation of noninfectious viral protein subunits. In order to assess the effect of extreme heat on PPR virus infectivity, each virus aliquot was subjected to higher temperatures of 56°C, 60°C, 62°C and 65°C for various time intervals after which the loss of infectious titer was determined by calculating $TCID_{50}$ on Vero cells. PPRV was shown to be highly sensitive to extreme heat. At 56°C, PAK-LRS-13/NARC was most stable virus that remained active with reasonable titer for 30 minutes exposure. All other isolates were completely inactivated when exposed to 56°C for 30 minutes. PAK-ICT-12/NARC and PAK-MZD-13/NARC remained active for 15 minutes although the titers declined. PAK-VEH-12/NARC and PAK-FGJ-14/NARC lost their infectivity but were still detectable after 15 minutes. The most labile viruses at this high temperature were PAK-KP-12/NARC and PAK-ATT-13/NARC that were completely inactivated after 15 minutes at 56°C. In another study performed on Dongola strain of NDV, it was found out that by exposing the strain at extreme temperature of 56°C, titer was decreased by one logarithmic order as compared to the step-wise exposure for 15 minutes to increasing temperatures in which case the titer loss was almost double (Abdellatif and Mohammed, 2012). The results were also in agreement with another study performed on field isolates of NDV which concluded that NDV strains exhibit varying degree of thermostability at high temperatures (Omony et al., 2016). The difference of thermostability was also observed in HEV strains nevertheless all strains tested were effectively inactivated by heating at 60°C while in case of HAV, heating to 5-10°C higher temperature than HEV was required to inactivate HAV strains (Emerson et al., 2005).

None of the PPR virus isolates showed any titer when exposed to 60°C, 62°C and 65°C while studies on HCV demonstrated that the virus become inactivated at 60°C and 65°C in 10 and 4 minutes respectively while at 56°C, minimum time of 40 minutes was required for complete viral inactivation (Song et al., 2010).

To better understand the sequence variation among these isolates, genome sequencing of virulent genes is suggested.

PPR virus local isolates categorized, based on thermal stability characteristics helped in selection of suitable candidate strain for PPR vaccine. The strain was used later to develop a thermostable vaccine formulation that could improve the vaccine keeping required quality under field conditions and minimizing the dependence on cold chains and refrigeration. Such efforts would ultimately facilitate in progressive control of PPR in local areas within the country in an effective way.

# Chapter 5

## Attenuation and Pathogenicity in Goats of a Thermo-tolerant Strain of Peste des Petits Ruminants Virus

# Chapter 5: Attenuation and Pathogenicity in Goats of a thermo-Tolerant Strain of PPRV

## 5.1 Introduction

PPR is characterized by high fever, oculo-nasal discharges, oral erosions, bronchopneumonia, gastroenteritis, necrotic stomatitis leading to death (Hota et al., 2018). Sometimes the infection occurs without specific signs and symptoms of the disease (Couacy-Hymann et al., 2007). This is also true for rinderpest in which extensive variation exists between different strains due to difference in virulence (Taylor, 1986). The morbidity and mortality rates can reach up to 100% (Hailat et al., 2018) and 50-90% respectively in susceptible populations (Rojas et al., 2017).

PPRV exists as one serotype but is categorized into four lineages based on the partial sequences of F and N genes (Banyard et al., 2010; Clarke et al., 2017). Lineage I is mainly confined in Western Africa, Lineage II in central Africa, Lineage III in eastern Africa and Southern parts of Middle East and Lineage IV is predominant in Middle East and Asia (Kwiatek et al., 2011). Although there are recent reports of co-circulation of Lineage II, III and IV in Eastern Africa (Muniraju et al., 2014; Woma et al., 2015). Few live attenuated vaccines are available for controlling PPR. The first homologous vaccine belonging to lineage I was developed by successful attenuation of a virulent strain of PPRV (Nigeria 75/1) initially isolated from a sick Nigerian goat in 1975 (Liu et al., 2014). The vaccine provides a lifelong immunity in small ruminants (Zahur et al., 2014). Another live attenuated vaccine Sungri-96 was developed in India which belongs to Lineage IV. But the major drawback with these vaccines is their sensitivity to high temperatures especially in areas with hot climatic conditions. In many developing countries including Pakistan, the vaccine loses its potency under field conditions due to poor infrastructure and inadequate cold chain maintenance resulting in vaccine failure (Yaqub et al., 2016). After the successful eradication of rinderpest in cattle, PPR has been declared as next eradicating target by Food and Agriculture Organization (FAO) of United Nations and the World Organization for Animal Health (OIE) by 2030 (Baazizi et al., 2017). The disease control programs can be facilitated by the development of a thermostable vaccine to eliminate the cold chain associated problems in these areas.

In this chapter, results of PPR virus attenuation by successive passages using *in-vitro* Vero cell culture are described. The avirulent virus obtained possess the same characteristics as the Nigeria 75/1 vaccine developed by Diallo and his colleagues in 1989. The viral strain can serve as potential candidate for a homologous vaccine against PPR.

## 5.2 Results

### 5.2.1 Attenuation of PPRV Local Isolate

#### 5.2.1.1 Selection of Strain

As described in chapter 4, a total of seven PPR virus isolates were subjected to thermostability experiment in order to select a thermo-tolerant strain which could be used as a potential candidate for PPR vaccine development. One strain (Pak-LRS-13/NARC) which came out as most stable at high temperatures was selected for attenuation onto Vero cells.

#### 5.2.1.2 Determination of Infectious Titer

The titer of PPRV isolate (Pak-LRS-13/NARC) determined by Reed and Muench method was found to be $10^{4.9}$ $TCID_{50}$/ml. The calculation of infectious titer is given in the section 3.2.11.

#### 5.2.1.3 Attenuation onto Vero Cells

The virus was first isolated onto Vero cells after two blind passages from Vero to Vero cell line. The CPEs started to develop in 16-18 days post inoculation. The infection resulted in development of large rounded cells followed by cell shrinkage and clumping. Soon after, their number increased and they invaded the entire monolayer. Gradually, the cells died and detached from the bottom of flask. Initially, no viral effect was noticeable before 5 to 7 days because of adaptation of virus on new type of cells which acting as typical host (African green monkey kidney cells) made the virus to multiply slowly *in vitro*. The first 10 to 15 passages on Vero cells took 7 days for a single harvest and second viral harvest was collected after 9 to 12 days as shown in Table 5.1. However, after 30 to 40 passages, it was possible to take 2 harvests in 3 to 4 days' time period. And after 50 passages on Vero cells, all the virus was collected as a single harvest as early as 2 days. The growth medium was replaced with the

maintenance medium at alternative days and it was noticed that the progression of CPEs was rapid with the renewal of culture medium. The decline in infectious titer was due to the cells death and detachment from the bottom of culture flask. The release of large quantities of proteolytic enzymes in the culture medium resulted in the inactivation of virus by digesting the surface proteins necessary for multiplication of virus in the initial phase of its life cycle.

**Table 5.1:** Attenuation related changes in cytopathic effects (CPEs) with the virus at different levels of viral passage

| Passage levels | Vero cells | | | | |
|---|---|---|---|---|---|
| | Rounding | Clumping | Layer detachment | Syncytia formation | Days for 70% CPEs development |
| **Passage 1** | + | + | - | - | 16 |
| **Passage 2** | + | + | - | - | 8 |
| **Passage 5** | + | + | - | - | 7 |
| **Passage 10** | + | + | + | - | 7 |
| **Passage 15** | + | + | + | - | 7 |
| **Passage 20** | + | + | + | - | 5 |
| **Passage 25** | + | + | + | - | 4 |
| **Passage 30** | + | + | + | - | 3 |
| **Passage 35** | + | + | + | - | 3 |
| **Passage 40** | + | + | + | - | 3 |
| **Passage 45** | + | + | - | - | 3 |
| **Passage 50** | + | + | - | - | 2 |
| **Passage 55** | + | + | - | - | 2 |
| **Passage 60** | + | + | - | - | 2 |

### 5.2.1.4 Confirmation of Virus Recovery by RT-PCR

After every 5 passages, the viruses were re-tested by RT-PCR which confirmed the virus recovery at each passage (100%) (Table 5.2). The isolates were lyophilized and stored at -20°C till further analysis.

**Table 5.2:** Confirmation of virus recovery by RT-PCR and qRT-PCR for attenuated strain of PPR at different passage level

| **Passage levels** | **P-1** | **P-5** | **P-10** | **P-15** | **P-20** | **P-25** | **P-30** | **P-35** | **P-40** | **P-45** | **P-50** | **P-55** | **P-60** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **qRT-PCR ($C_t$ values)** | 35.25 | 28.63 | 28.22 | 28.23 | 28.30 | 28.29 | 29.09 | 29.65 | 28.99 | 28.80 | 30.87 | 26.43 | 27.31 |
| **RT-PCR** | + | + | + | + | + | + | + | + | + | + | + | + | + |

*P =Passage

*$C_t$ =Cycle threshold

### 5.2.2 Pathogenicity Trial

The experimental animals were kept in the same room for one week to observe their general behavior. After one week, animals were ear tagged (Figure 5.1) and placed in four separate groups (Table 5.3). Twelve goats were randomly allocated to four groups (Groups A, B, C and D) each containing 3 animals as described in Table 3.3. Two goats in groups A, group B and group C were inoculated subcutaneously through a syringe with 2ml of $10^{4.5}$ $TCID_{50}$/ml of attenuated viral suspension at passage 20, 40 and 60 respectively. One animal in each group (Groups A, B, C) was kept as transmission control. Group D was taken as placebo control and two goats in this control group were inoculated subcutaneously with 2ml of sterile GMEM without FBS (Figure 2.2). Each group was kept in separate room after virus inoculation.

**Table 5.3:** The experimental design and virus inoculation details

| **Group A** | | **Group B** | | **Group C** | | **Group D** |
|---|---|---|---|---|---|---|
| Infected animals | Transmission control | Infected animals | Transmission control | Infected animals | Transmission control | Sterile GMEM without PBS (3 goats) |
| Attenuated PPRV ($20^{th}$ passage) (2 goats) | (1 goat) | Attenuated PPRV ($40^{th}$ passage) (2 goats) | (1 goat) | Attenuated PPRV ($60^{th}$ passage) (2 goats) | (1 goat) | |


**Figure. 5.1 (a & b):** Experimental animals and their tagging

**Figure. 5.2:** Virus inoculation in goat inoculated subcutaneously through a syringe with 2ml of $10^{4.5}$ $TCID_{50}$/ml of attenuated viral suspension at passage 20, 40 and 60 respectively.

### 5.2.2.1 Clinical Observations

#### 5.2.2.1.1 Evaluation of Pathogenicity of the Virus at 20th Passage

The experimental animals were observed for signs of PPR using a Performa given in Appendix 1. After an incubation period of 6-7 days, there was slight hyperthermia in two animals of group A (105.8°F in animal 1 and 106.34°F in animal 2) although it lasted only for 48 hours. There was congestion of oral and nasal mucosa with mild discharges on day 10 post exposure. The dorsum of tongue and inner surface of lips got erosive lesions on day 12. One animal in this group had diarrhea on 13th day post exposure. The two animals became progressively weak and developed productive cough and difficult breathing on day 16. One of these animal died on day 20 while the other animal recovered from clinical disease (Figure 5.3)

A per-acute form of disease was observed in transmission control animal. The animal developed fever of 106.7°F on day 7th post exposure. There was mild oral and nasal discharges on day 11. There was no diarrhea and respiratory symptoms and animal also recovered from clinical disease.

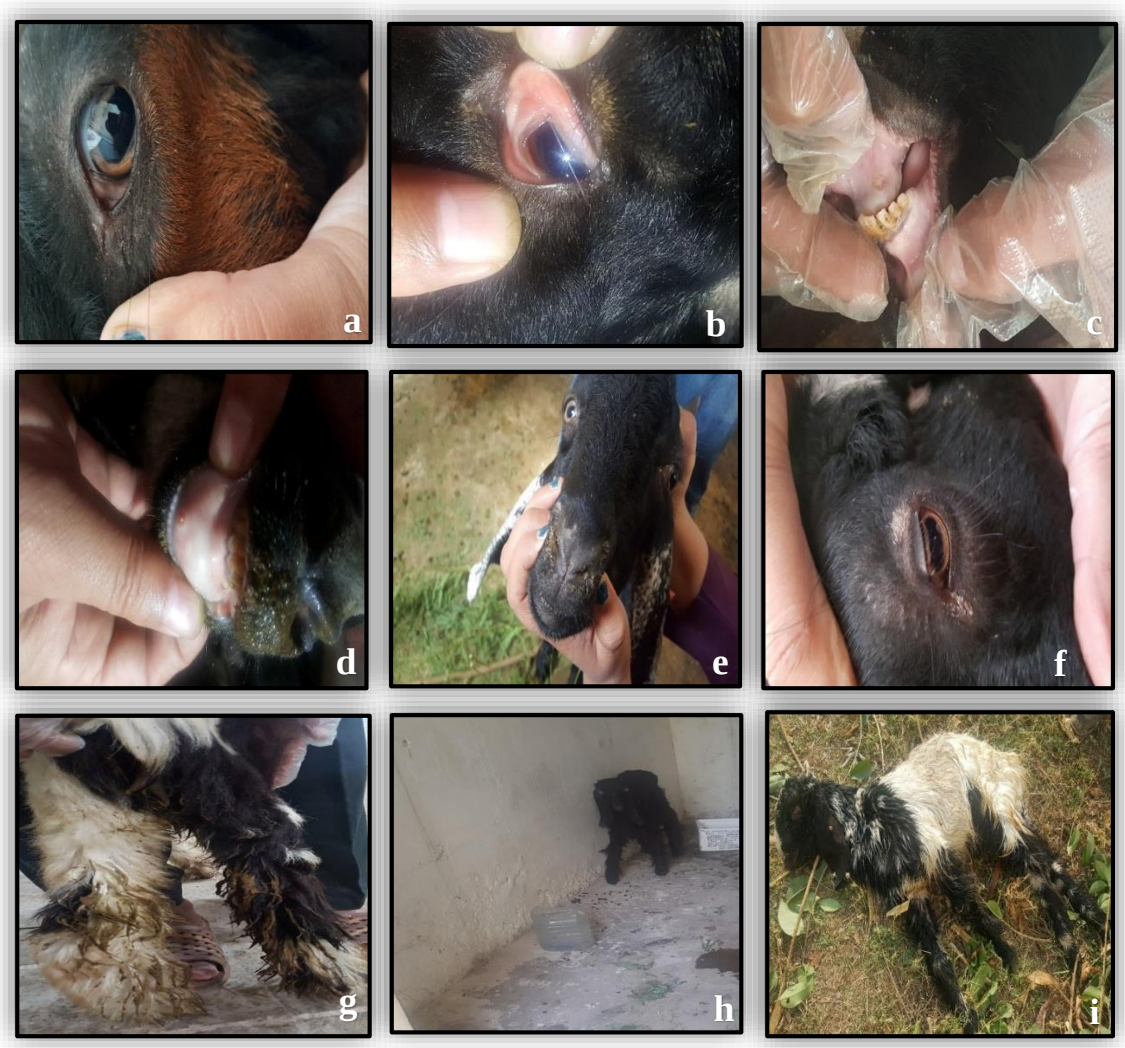



**Figure 5.3:** Experimental animals in group A showing clinical signs of PPR (a & b): Conjunctivitis. (c): Erosive lesion on upper gum (d): Erosive lesion on lower gum (e): Nasal discharge (f): Ocular discharge (g): Diarrhea (h): Animal in isolation (i): Dead goat

#### 5.2.2.1.2 Evaluation of Pathogenicity of the Virus at 40$^{th}$ and 60$^{th}$ Passage

No clinical signs related to the disease were observed in any animal within groups B and C and they remained healthy till 30 days or at the end of experiment and there was no mortality.

#### 5.2.2.2 Necropsy Observations

The signs of dehydration and evidence of diarrhea could be seen in carcasses of group A animals. The necrotic lesions were found on dental pad, gums and oral cavity. The oro-pharyngeal sections showed necrotic lesions on palatine tonsils and small fibrin deposits on the base of tongue, zebra striping in large intestine and consolidated lungs leading to pneumonia. The reactive mesenteric, retropharyngeal and mediastinal lymph nodes of animals were inflamed and reactive.

Animals in groups B, C and D did not show any gross pathological lesions at necropsy.

#### 5.2.2.3 Detection of PPRV from Swabs by RT-PCR

RT-PCR was performed from nasal, oral and ocular swabs of animals up to day 12 post exposure. The results of RT-PCR are shown in Table 5.4. All the swab samples were found negative for PPRV antigen on day 0. Swab samples were detected positive for PPRV genome on day 7 for group A. Two animals in this group were positive by RT-PCR on day 7 and virus shedding continued till last sampling day. While the transmission control animal in this group became positive on day 9 post exposure till day 11.

The animals in other 2 groups (Group B and group C) along with the control experimental group remained negative during the observation period for genome detection by RT-PCR (Figure 5.4)

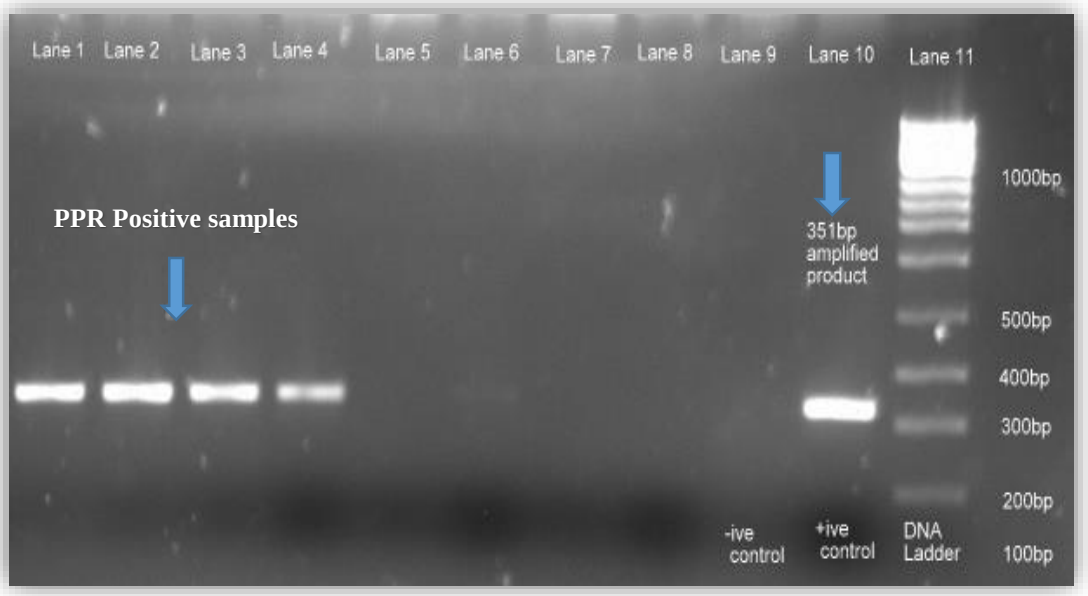



**Figure 5.4:** A representative gel image showing N gene based RT-PCR amplification with product size of 351bp (from left to right). Lanes 1-4: PPR positive swabs; Lanes 5-8: PPR negative swabs; Lane 9: Negative control; Lane 10: Positive control; Lane 11: 100bp DNA ladder

**Table 5.4:** Detection of PPRV genome by RT-PCR in swab samples (Nasal, ocular and oral) 12 days post exposure during pathogenicity trial experiment

| Treatment Groups | Observation period (in days) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| Group A | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 2/3 | 2/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| Group B | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| Group C | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| Group D | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |

The figures represent number of positive animals / Total number of animals in different groups

### 5.2.2.4 Isolation of PPR Virus on Cells

Although the animals in group A were tested positive for PPRV genome by RT-PCR but no virus was recovered on Vero cells from any tissue sample of experimental animals collected at necropsy. Same was the case with groups B, C and D.

### 5.2.2.5 Sero-conversion in Experimentally Infected Animals

The animals in groups B and C remained apparently healthy with no clinical signs of PPR disease on inoculation with attenuated strain of PPRV at passage level 40 and 60. No pyrexia was seen in animals of these two groups after inoculation. Sero-conversion was studied in experimentally infected (Group A, B and C) and control group (Group D) animals by cELISA for 30 days and antibody levels were expressed in PI (Percent inhibition) values. The presence of neutralizing antibody titers was observed after inoculation in groups B and C where diagnostically positive serum titers (PI values > 50) were found as early as 7 days post exposure and reached the plateau after 3 weeks. However, in the group A, the rise in antibody titers was observed on day 14 before death.

No sero-conversion (PI values < 50) was observed in the group D animals during the observation period.

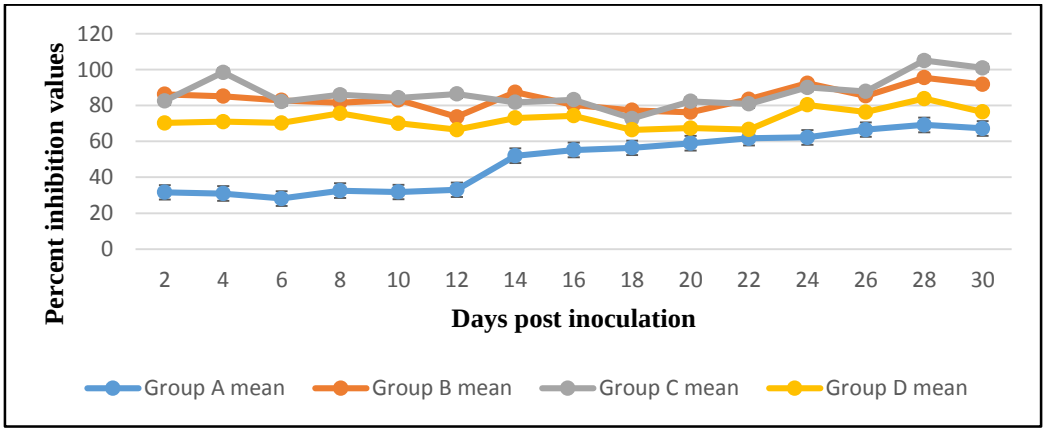



**Figure 5.5:** Serological results with c-ELISA (cut-off 50%) of different groups (A, B, C) inoculated with attenuated strain of PPRV at passage 20, 40 and 60 respectively and a placebo control group (D)

## 5.3 Discussion

Gilbert and Monnier were the first scientists who tried to adapt PPR virus for *in-vitro* culture using sheep kidney cells. The cytopathic effects of virus were manifested by the appearance of large syncytia (Gilbert and Monnier, 1962). After that, Laurent studied this adaptation on different cellular systems including calf goat and monkey kidney cells (Laurent, 1968). PPR virus along with other members of genus morbillivirus (rinderpest, measles and canine distemper) has the property to develop syncytia. However, in case of Nigeria 75/1 strain of PPRV, initial passages do not always reveal syncytia formation. The cells, after staining with hematoxylin-eosin were found with few nuclei and CPEs appeared as round, refractive cells which gradually detached from the flask surface. Plowright and Ferris while studying bovine viruses on calf kidney cells described the same type of polycaryons during the early stages of viral infection which subsequently were replaced by large syncytia (Plowright and Ferris, 1959).

All morbilliviruses have two external proteins (Hemagglutinin and Fusion proteins) which play an important part in initial phase of host cell interaction (Diallo, 1990). The H protein helps in binding of virus to host cell and cooperating with F protein to mediate the fusion of viral and cellular membranes resulting in penetration of virus inside the host cell at neutral pH (Das et al., 2000). In the current experiment, it was found that local strain of PPRV (Pak-LRS-13/NARC) within the same cellular system behaves in a different way in terms of CPEs as compared to other bovine viruses. In the beginning of serial passages, the CPEs were not visible until 4-6 days post infection and this time was reduced to almost 2 days subsequently with the adaptation of virus to the cells *in vitro*. Same was observed while attenuating the PPRV Nigeria 75/1 onto Vero cells. It might be possible that in both cases, the F protein was less expressed or its inactive form ($F_0$) was not fully cleaved which is otherwise executed by host cell proteases into two active forms ($F_1$-$F_2$), believed to be important for potential activity of F protein (Lamb and Kolakofsky, 2001).

Sabin and his colleagues (1954) while studying the variants of poliomyelitis virus revealed that using high doses of poliovirus during serial passages led to prompt development of mutants (Sabin et al., 1954). The same methodology was used by Plowright and Ferris during attenuation of rinderpest virus. They found that the virus during initial passages underwent exaltation in its pathogenicity followed by gradual

reduction which resulted in avirulent strain at 40th passage (Plowright and Ferris, 1959). PPRV local strain which adapted quickly to Vero cells after 20th passage when inoculated caused slight hyperthermia and mild oculo-nasal discharge in goats. The attenuated strain after 40th passage did not show any sign and symptoms of the disease in goats leading to good immunity. The same finding was observed in case of attenuated PPRV 75/1 strain (Diallo et al., 1989). Contrary to this observation, Gilbert and Monnier noticed during the attenuation of another strain of PPRV that the animals inoculated at 12th passage virus showed slight increase in their body temperature. But the strain was not completely avirulent until 65th passage on primary cultures of sheep kidney cells (Gilbert and Monnier, 1962). One of the reasons might be the low concentrations of inoculating virus that did not allow better chance to obtain mutants. The attenuated bovine virus being lymphotropic in nature ultimately lost its virulence to grow in mucous membranes which results in loss of transmissibility from one animal to other. PPRV strain attenuated onto Vero cells followed the same modification of tropism at 20th passage. Other viruses show similar changes involving surface proteins for attachment and penetration inside the host cell membrane which results in avirulent strains. Progressive loss of pathogenicity of attenuated PPRV strain could be due to mutations in the surface proteins of this virus.

The local strain attenuated and used in present study for inoculation in goats provided good immunity at 40th and 60th passage level and produced neutralizing antibodies as early as 7 days post inoculation. However, successive multiplication to animals was not performed hence no evidence for reversion to wild type could be demonstrated. Moreover, the strain did not produce abortion in 2 pregnant goats (Unpublished data of Zahur et al., 2014 group at NARC). These characteristics were also exhibited by attenuated Nigeria 75/1 strain and other bovine viruses which suggests that the attenuated strain of present study is a good candidate for PPR vaccine. Additionally, the strain has been selected on the basis of its genotyping, thermostability potential and immunogenicity, hence the development of homologous lineage vaccine would be a great effort towards the global eradication of PPR by 2030 as planned by international agencies like FAO and OIE.

# Chapter 6

## Comparative Efficacy of Different Chemical Stabilizers on the Thermostability of Newly Developed PPR Vaccine

# Chapter 6: Comparative Efficacy of Different Chemical Stabilizers on the Thermostability of Newly Developed PPR Vaccine

## 6.1 Introduction

Vaccination is an effective way to control Peste des Petits Ruminants (PPR). In the past, Plowright and Ferris rinderpest (RP) vaccine was widely used for a long time in African and other countries giving absolute protection and life-long immunity against both RP and PPR (Plowright and Ferris, 1962). But the major constraint with this vaccine was its dependence on cold chain under field conditions due to thermo-labile nature of both viruses. Later the development of a thermostable RP vaccine expedited its eradication around the world. However, due to interference in the sero-surveillance of RP after its successful eradication, the international organizations strictly restricted the use of this vaccine for PPR (Kumar et al., 2014) which paved the way to the prompt development of homologous vaccine against PPR. The vaccine was prepared using Nigerian strain (75/1) after its attenuation for 63 serial passages onto Vero cells (Liu et al., 2014). Clinical trials proved the safety of this vaccine with no detrimental effects on animals, providing immunity for at least three years (Zahur et al., 2014). This homologous vaccine is now being used effectively throughout Africa, Asia and Middle East for combating PPR. But the efficient use of this live attenuated vaccine in the endemic areas of hot climatic conditions is limited due to the thermo-labile nature of PPR virus (Silva et al., 2011). The vaccine must be refrigerated at the appropriate temperature throughout its transportation, storage, and delivery. After the successful Global Rinderpest Eradication Program (GREP), World Organization for Animal Health (OIE) and the Food and Agriculture Organization (FAO) have launched a worldwide campaign to control and eradicate PPR by the year 2030 (Jones et al., 2016). The most challenging task during the vaccination operations in tropical and subtropical regions is the dilemma of maintaining the cold chain especially in developing countries (Worrall et al., 2000). In order to accomplish the successful disease eradication programs, development of a thermostable live-attenuated vaccine will be a great step to avoid cold chain associated problems in these areas.

In this chapter, the development of a live attenuated thermostable PPR vaccine has been described. A comparative study was also conducted to evaluate the effects of stabilizers

on the thermostability of PPR vaccine by thermo-stabilization method used for RP vaccine. The results were compared by performing accelerated stability tests.

## 6.2 Results

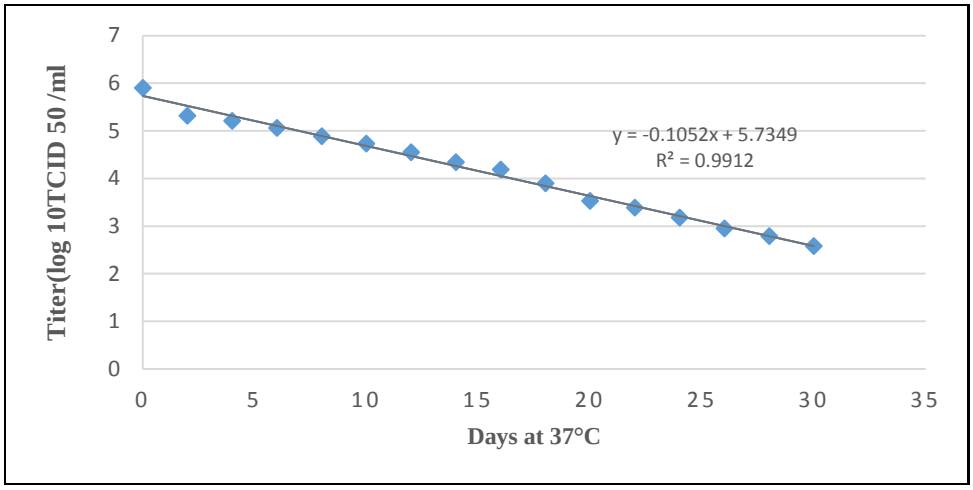
### 6.2.1 Comparative Efficacy of Different Chemical Stabilizers on the Thermostability of Newly Developed PPR Vaccine

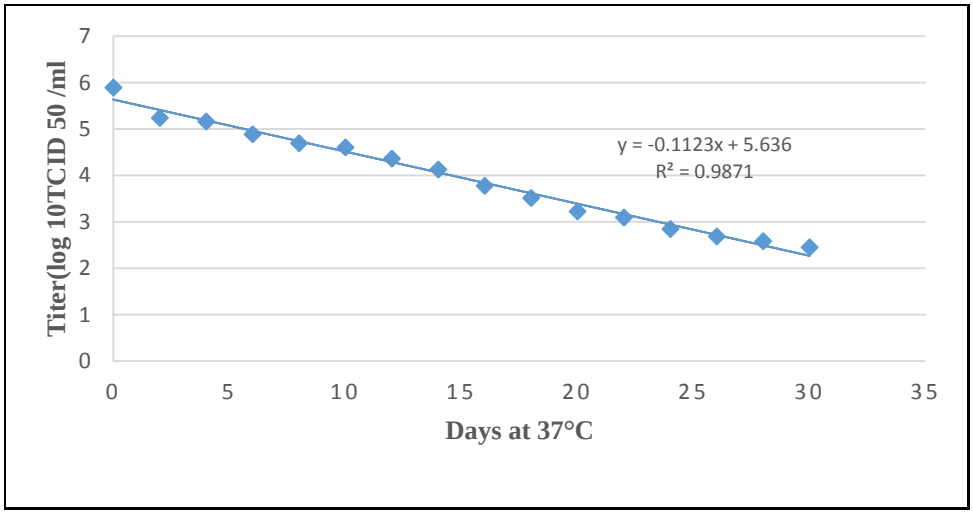
A live attenuated, thermostable, homologous lineage PPR vaccine was prepared using 3 different stabilizers following the method described by Mariner (Mariner et al., 2017) with some modifications as mentioned in section 2.3.3.4.1. The accelerated stability testing was performed at four different temperatures (25°C, 37°C, 45°C and 56°C) and the degradation curves at each temperature are shown in Figures 6.1-6.3. The degradation curve represented a biphasic degradation pattern comprising of a first phase of rapid loss followed by a second phase of gradual recession for newly developed vaccine lyophilized with three different stabilizers, Lactalbumin Hydrolysate-Sucrose (LAH), Weybridge medium (WBM), Trehalose dehydrate (TD).

At 37°C, the vaccine stabilized with LAH, TD and WBM as 25 dose presentation had the shelf life of 17, 15 and 10 days respectively (Table 6.1). The degradation constants (k) for WBM stabilized vaccine were found to be larger (0.1437 and 0.04424) than the vaccines stabilized with TD (0.11227 and 0.04364) and LAH (0.1052 and 0.04283) at 37°C and 45°C respectively. The shelf lives of vaccines as 25 dose vial at 45°C with stabilizers LAH, TD and WBM were found to be 46, 38 and 33 hours respectively (Table 6.2).

**Table 6.1:** Accelerated stability test at 37°C for newly developed PPR vaccine stabilized with three different stabilizers

| Stabilizer | Harvest titer | Product titer | N | Degradation constant | Intercept | Loss on lyophilization | Shelf life (Days) |
|---|---|---|---|---|---|---|---|
| **TD** | 5.9 | 5.53 | 16 | -0.11227 | 5.635956 | 0.37 | 15.47 |
| **WBM** | 5.9 | 5.45 | 16 | -0.1437 | 5.432353 | 0.45 | 10.70 |
| **LAH** | 5.9 | 5.67 | 16 | -0.1052 | 5.734853 | 0.23 | 17.44 |



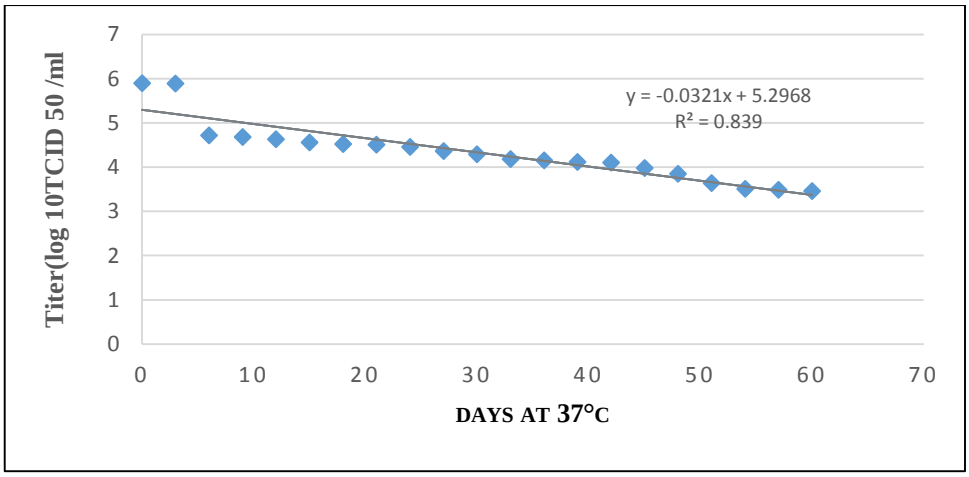
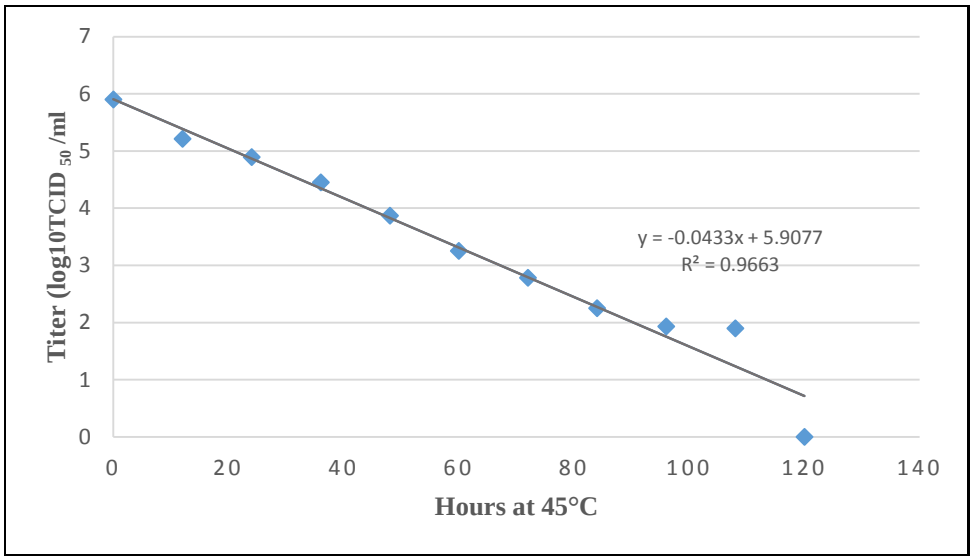
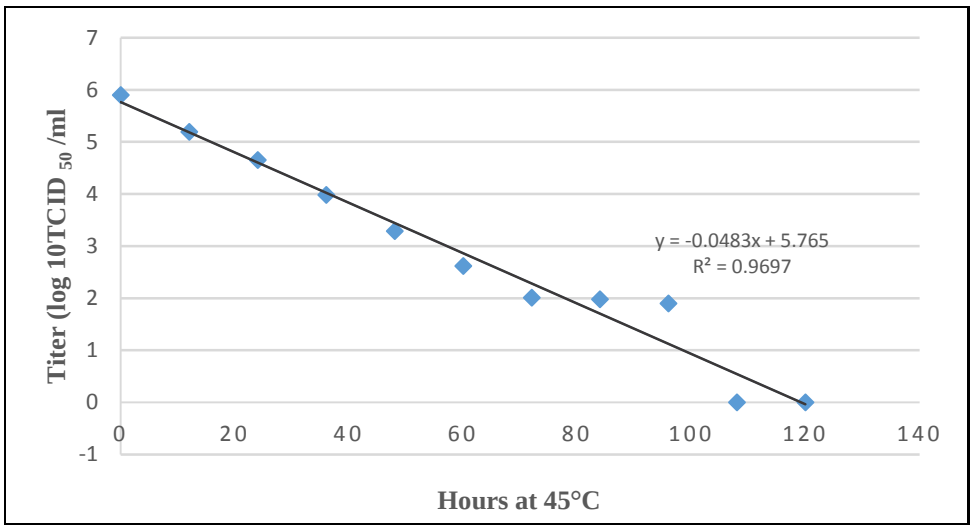




**Figure 6.1:** Degradation curve for newly developed PPR vaccine at 37°C stabilized with different stabilizers (a) LAH, (b) TD, (c) WBM

**Table 6.2:** Accelerated stability test at 45°C for newly developed vaccine PPR vaccine stabilized with different stabilizers

| Stabilizer | Harvest titer | Product titer | N | Degradation constant | Intercept | Loss on lyophilization | Shelf life (Hours) |
|---|---|---|---|---|---|---|---|
| **TD** | 5.9 | 5.53 | 13 | -0.04364 | 5.566703 | 0.37 | 38.20 |
| **WBM** | 5.9 | 5.45 | 13 | -0.04424 | 5.378022 | 0.45 | 33.41 |
| **LAH** | 5.9 | 5.67 | 13 | -0.04283 | 5.886044 | 0.23 | 46.40 |



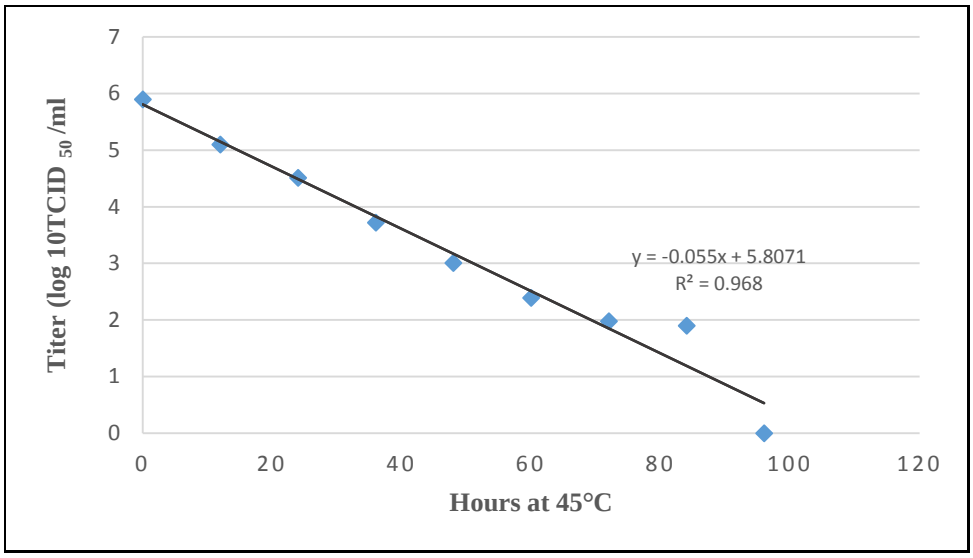
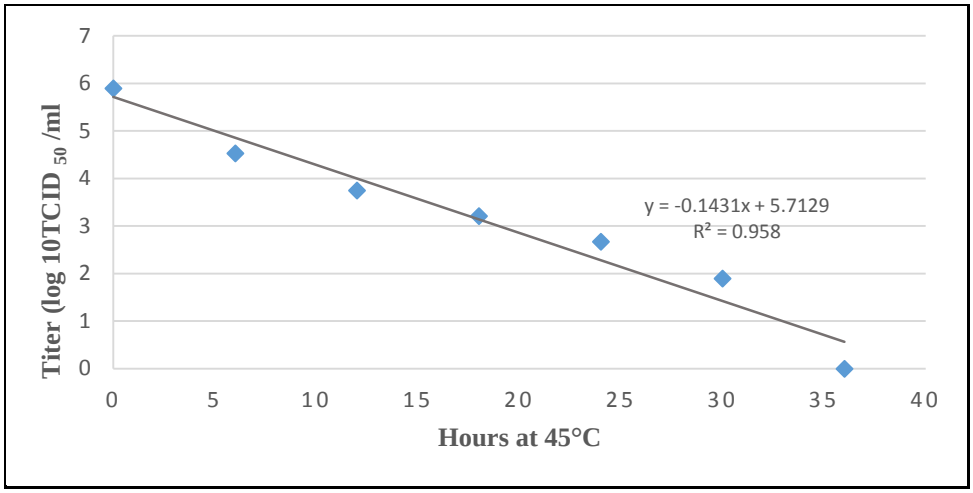


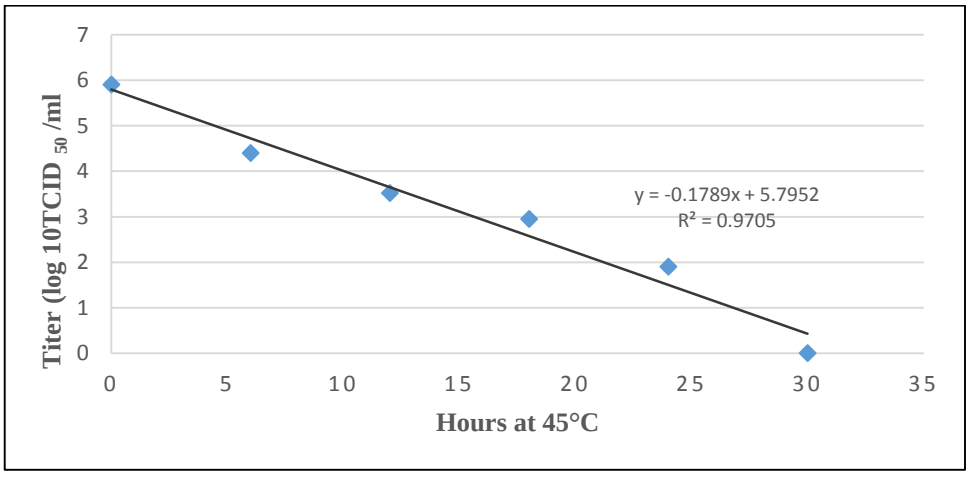

**Figure 6.2:** Degradation curve for newly developed PPR vaccine at 45°C stabilized with different stabilizers (a) LAH, (b) TD, (c) WBM

At 56°C, the degradation constant (k) for vaccines stabilized with WBM, LAH or TD were found to be -0.17565, -0.16738, -0.1431 and the shelf life as 25 dose preparation was 9, 11 and 13 hours respectively (Table 6.3).

**Table 6.3:** Accelerated stability test at 56°C for newly developed vaccine PPR vaccine stabilized with different stabilizers

| Stabilizer | Harvest titer | Product titer | N | Degradation constant | Intercept | Loss on lyophilization | Shelf life (Hours) |
|---|---|---|---|---|---|---|---|
| **TD** | 5.9 | 5.53 | 7 | -0.16738 | 5.68 | 0.37 | 10.7 |
| **WBM** | 5.9 | 5.45 | 7 | -0.17565 | 5.458929 | 0.45 | 8.9 |
| **LAH** | 5.9 | 5.67 | 7 | -0.1431 | 5.712857 | 0.23 | 12.7 |



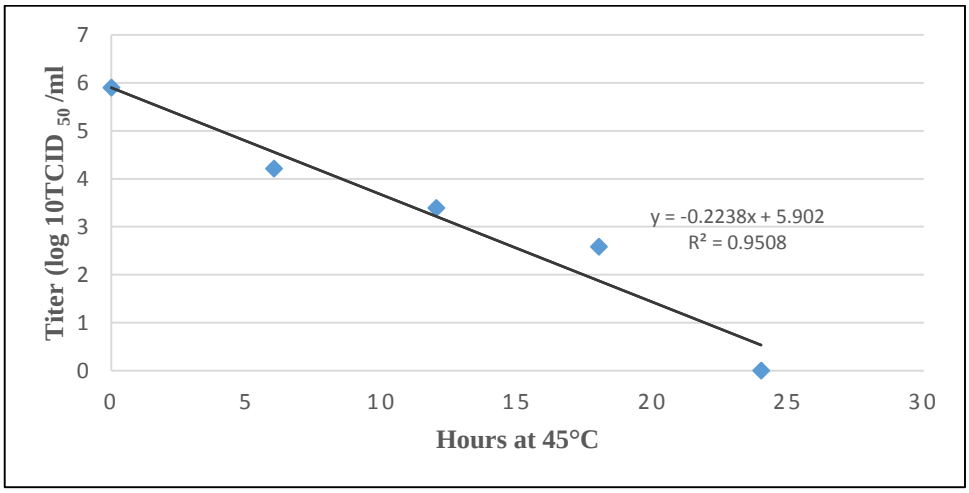




**Figure 6.3:** Degradation curve for newly developed PPR vaccine at 56°C stabilized with different stabilizers (a) LAH, (b) TD, (c) WBM

### 6.2.2 Experimental Immunization

The experimental animals were kept in the same room for one week to observe their general behavior. After one week, animals were ear tagged and placed in separate groups. Six goats were randomly allocated to three groups (Groups A, B and C) each containing 2 animals as described in Table 6.4. The goats in groups A and B were inoculated subcutaneously through a syringe with 2ml of $10^{4.5}$ $TCID_{50}$/ml of PPR vaccine (Nigeria 75/1) and 2ml of $10^{4.5}$ $TCID_{50}$/ml of PPR vaccine (Pak-LRS-13/NARC). Group C was taken as placebo control and two goats in this control group were inoculated subcutaneously with 2ml of sterile PBS. Each group was kept in separate room after vaccine inoculation.

**Table 6.4:** The experimental design and vaccine inoculation details

| **Group A** | **Group B** | **Group C** |
|---|---|---|
| PPR vaccine (Nigeria 75/1) | PPR vaccine (Pak-LRS-13/NARC) | Placebo control |
| (2 goats) | (2 goats) | (2 goats) |

#### 6.2.2.1 Clinical Observations

The experimental animals were observed for signs of PPR using a Performa given in Appendix 1. No clinical signs related to the disease were observed in any animal within groups A and B and they remained healthy till 30 days or at the end of experiment and there was no mortality.

### 6.2.2.2 Detection of PPRV from Swabs by RT-PCR

RT-PCR was performed from nasal, oral and ocular swabs of animals up to day 12 post exposure. The results of RT-PCR are shown in Table 6.5. All the swab samples were found negative for PPRV antigen on day 0 till day 12. The control group also remained negative during the observation period for genome detection by RT-PCR (Figure 6.4)

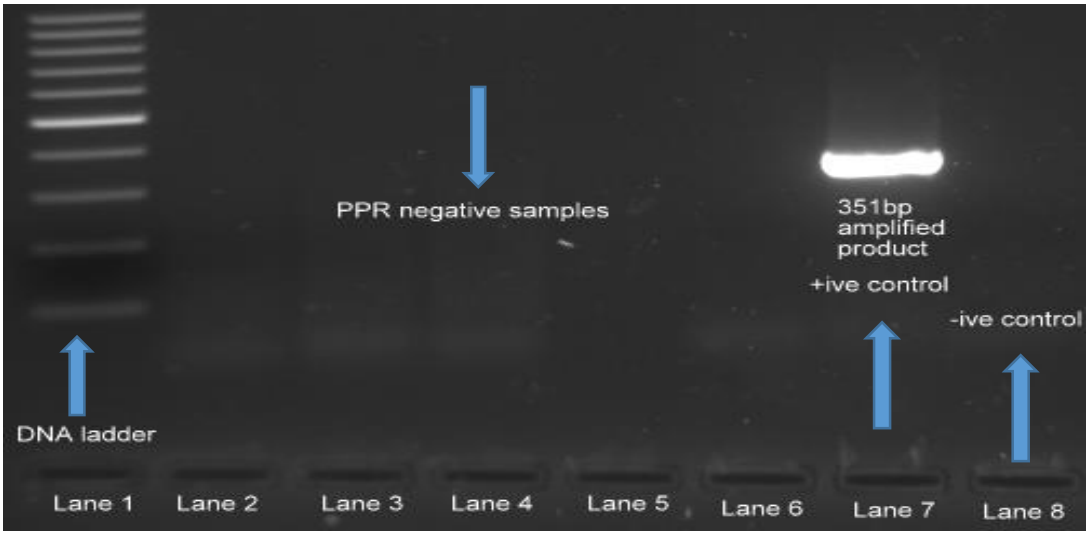



**Figure 6.4:** A representative gel image showing N gene based RT-PCR amplification with product size of 351bp (from left to right). Lanes 1: 100bp DNA ladder; Lanes 2-6: PPR negative swabs; Lane 7: Positive control; Lane 8: Negative control

**Table 6.5:** Detection of PPRV genome by RT-PCR in swab samples (Nasal, ocular and oral) 12 days post exposure during vaccine trial

| Treatment Groups | Observation period (in days) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| Group A | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 |
| Group B | 0/3 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 |
| Group C | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 |

The figures represent number of positive animals / Total number of animals in different groups

### 6.2.2.3 Humoral Immune Response in Animals

The animals in groups A and B remained apparently healthy with no clinical signs of PPR disease on inoculation with PPR vaccine (Nigeria 75/1) and PPR vaccine (Pak-LRS-13/NARC). No pyrexia was seen in animals of these two groups after vaccine inoculation. Humoral immune response was studied in experimental animals (Group A and B) control group (Group C) animals by cELISA for 22 days and antibody levels were expressed in PI (Percent inhibition) values. The presence of neutralizing antibody titers was observed after inoculation in groups A and B where diagnostically positive serum titers (PI values > 50) were found as early as 3 days post exposure and reached the plateau after 3 weeks. No sero-conversion (PI values < 50) was observed in the group C animals during the observation period.

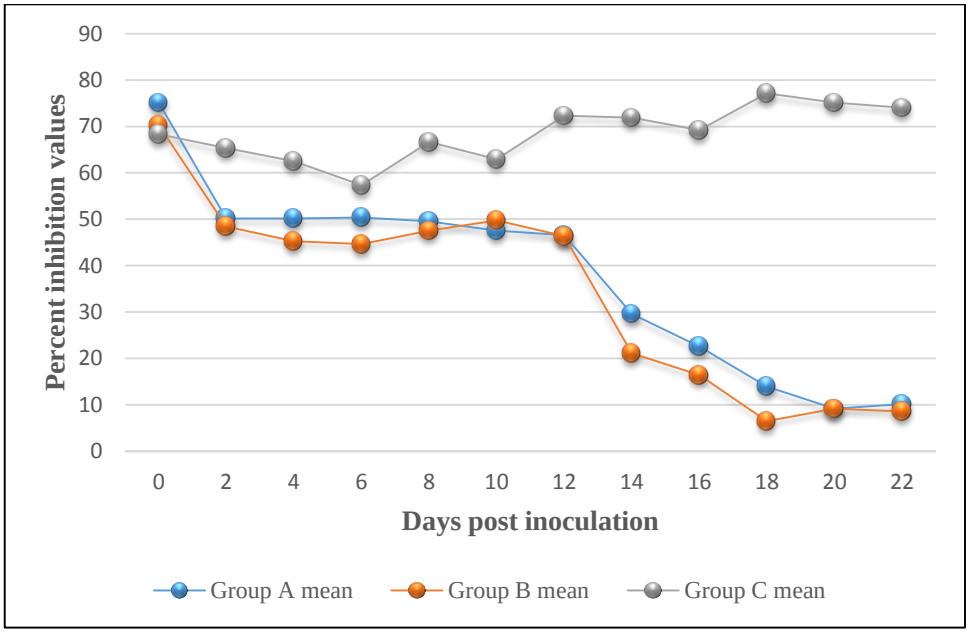



**Figure 6.5:** Serological results with c-ELISA (cut-off 50%) of different groups (A and B) inoculated with PPR vaccine (Nigeria 75/1) and PPR vaccine (Pak-LRS-13/NARC) and a placebo control group (C)

### 6.2.3 Challenge Protection Studies

The experimental animals in groups A and B and C previously used in the control trial and inoculated with 2ml of $10^{4.5}$ $TCID_{50}$/ml of PPR vaccine (Nigeria 75/1) (Group A) and 2ml of $10^{4.5}$ $TCID_{50}$/ml of PPR vaccine (Pak-LRS-13/NARC) (Group B) 2ml of PBS (Group C) respectively were challenged with a virulent strain (PAK-Fjg-14/NARC) of PPRV (Table 6.6).

**Table 6.6:** The experimental design and vaccine inoculation details

| **Group A** | **Group B** | **Group C** |
|---|---|---|
| PPR vaccine (Nigeria 75/1) | PPR vaccine (Pak-LRS-13/NARC) | Placebo control |
| 2ml of $10^{4.5}$ $TCID_{50}$/ml of virulent strain (PAK-Fjg-14/NARC) | | |
| (2 goats) | (2 goats) | (2 goats) |

Each group was kept in separate room after vaccine inoculation.

#### 6.2.3.1 Clinical Observations

The experimental animals were observed for signs of PPR using a Performa given in Appendix 1. No clinical signs related to the disease were observed in any animal within groups A and B and they remained healthy till 30 days or at the end of experiment and there was no mortality. While typical signs of disease were observed in animals within group C.

### 6.2.3.2 Detection of PPRV from Swabs by RT-PCR

RT-PCR was performed from nasal, oral and ocular swabs of animals up to day 12 post exposure. The results of RT-PCR are shown in Table 6.7. All the swab samples were found negative for PPRV antigen on day 0 till day 12 for groups A and B. While in control group (Group C) swab samples were detected positive for PPRV genome on day 6 and virus shedding continued till last sampling day (Figure 6.4)

**Table 6.7:** Detection of PPRV genome by RT-PCR in swab samples (Nasal, ocular and oral) 12 days post exposure during vaccine trial

| **Treatment Groups** | **Observation period (in days)** | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| Group A | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 |
| Group B | 0/3 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 |
| Group C | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 |

The figures represent number of positive animals / Total number of animals in different groups

### 6.2.3.3 Isolation of PPR Virus on Cells

The animals in group C were tested positive for PPRV genome by RT-PCR and PPR virus was recovered on Vero cells from tissue sample of experimental animals collected at necropsy.

### 6.2.3.4 Humoral Immune Response in Animals

The animals in groups A and B remained apparently healthy with no signs of PPR after challenge with a virulent virus. No pyrexia was seen in animals of these two groups after virulent virus inoculation. Humoral immune response was studied in experimental animals (Group A and B) and control group (Group C) animals by cELISA for 22 days and antibody levels were expressed in PI (Percent inhibition) values. The animals which showed percent inhibition values less than 50% were considered positive. The antibody titers did not diminish after challenge in group A and B and diagnostically positive serum titers (PI values < 50) were observed till the end of experimental period. The PI values ranged between 8 to 51. While in group C, the animals did not develop any increase antibody titer (PI values > 50) and remained weak till the end of observation period (Figure 6.6).

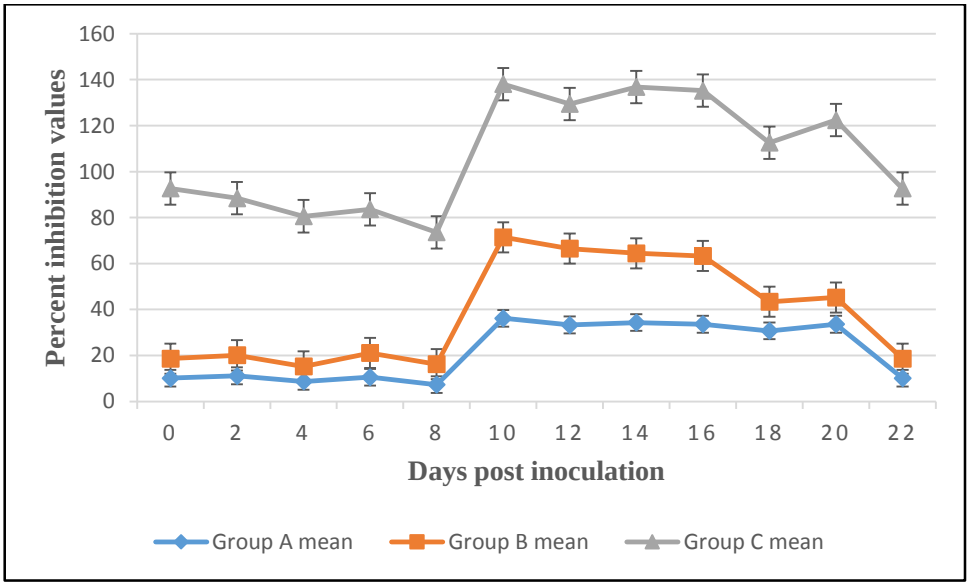



**Figure 6.6:** Serological results with c-ELISA (cut-off 50%) of different groups (A and B and C) challenged with virulent PPRV strain (PAK-Fjg-14/NARC).

## 6.3 Discussion

Thermostability refers to the quality of a substance to resist the degradation or change at high temperatures. The relative term refers to the rate of degradation which is generally augmented by increased temperatures (Mariner et al., 2017). Thermostability of vaccines however, is the capability to maintain the minimum required titer for certain time period at specified temperature. The stabilizers generally augment the stabilizing properties of vaccine that could ultimately help in eliminating the need for cold chain under field conditions (El-Bagoury et al., 2015). In this study, a homologous lineage, live attenuated, thermostable vaccine against PPR was developed using a local isolate of PPRV (Pak-LRS-13/NARC) after 60 serial passages onto Vero cells. The study was conducted to evaluate the effects of three different stabilizers on the thermostability of PPR vaccine by thermo-stabilization method already used for RP (Rinderpest) vaccine.

The results were compared by performing accelerated stability tests which revealed that the three stabilizers used in the present study remarkably extended the shelf life of newly developed PPR vaccine. However, LAH was found to be more effective over TD and WBM as it increased the shelf life of vaccine for 17 days at 37°C as compared to the two other stabilizers (15 days for LAH and 10 days for WBM).

As is evident for Zerovac method to dehydrate and preserve RP and PPR vaccines, the excipient consisting of 2.5% trehalose had very good stabilizing effect during freeze drying of PPR vaccine with loss of merely 0.15 $TCID_{50}$/ml (Worrall et al., 2000). The same methodology was used here for lyophilization of newly developed PPR vaccine. The results were in accordance with the study performed by Worrall and his co-workers and revealed that in case of LAH stabilizer, there was only 0.23 $TCID_{50}$/ml loss in viral titer during lyophilization as compared to the TD (0.37 $TCID_{50}$/ml) and WBM (0.45 $TCID_{50}$/ml).

The residual moisture plays an important role in thermostability of vaccines (Bora et al., 2015). Worrall and his co-workers found that at residual moisture of 5.36%, there was intolerable loss of vaccine titer in accelerated stability testing procedures. Secondary dehydration steps extended for 17 more hours reduced the residual moisture to less than 1% which further enhanced the thermostability of PPR vaccine. There was only 1.9 $\log_{10}$ $TCID_{50}$/ml loss in viral titer when the vaccine was subjected to 45°C for 14 days which showed that the vaccine maintained its protective titer (3.03 $\log_{10}$

$TCID_{50}$/ml) as compared to the vaccine batches which were lyophilized employing secondary drying for 2 hours (Worrall et al., 2000).

In another study carried out by Sarkar and his fellows, the high residual moisture (4.03 to 6.57%) in case of TD and LAH stabilizers due to the absence of secondary drying steps contributed to high deterioration in viral titer after exposure to high temperatures (Sarkar et al., 2003).

However, Mariner and his co-workers used the rinderpest method for thermostabilization of PPR vaccine which could resist the environmental stress for 30 days without maintaining the cold chain under field conditions. The vaccine stabilized to this extent would further help to lessen the vaccination costs which are otherwise implemented by use of vehicles for vaccine delivery. The thermostability of the vaccine batches prepared with lactalbumin hydrolysate (LAH) and sucrose (LS) in comparison with trehalose were measured at 37°C by executing accelerated stability tests. The two methods (Zerovac drying and Rinderpest lyophilization) for PPR vaccine stabilization were also compared using AST. It was found that vaccine produced using LS as stabilizer and lyophilized employing rinderpest method proved to be sufficiently thermostable. The shelf lives of vaccines also improved with rinderpest method as compared to the previously used Zerovac method. The results were in agreement with the present study in which newly developed vaccine prepared using LS and rinderpest method gave minimum required titer after exposure to high temperatures (Mariner et al., 2017). Yaqub and his colleagues while comparing the effects of different stabilizers on PPR vaccine using Nigeria 75/1 strain observed the same results proving LS as stabilizer of choice due to less titer loss during lyophilization with extended shelf life of 208 days at 4°C, 2.42 days at 25°C and 3.6 days at 37°C as compared to locally available vaccine having shelf life of 99 days at 4°C, 10.63 days at 25°C and 1.23 days at 37°C (Yaqub et al., 2016).

During the studies conducted by Riyesh and his co-workers, the two stabilizers namely LS and stabilizer E were compared and it was revealed that both stabilizers behaved equally good with marginal differences in shelf lives (44h and 40h) at higher temperatures (42°C and 45°C). Nevertheless, LS stabilizer was superior to the other (Stabilizer E) in terms of shelf life and infectious titer (Riyesh et al., 2011).

The efficacy of this newly developed PPR vaccine was evaluated by carrying out experimental immunization in goats. Dynamics of humoral immune response was monitored. Challenge protection studies were also carried out in animals previously inoculated with PPR vaccine made by Nigerian strain (75/1) and newly developed thermostable PPR vaccine formulation. A virulent field isolate catalogued as PAK-Fjg-14/NARC was used in challenge protection studies. It was found that antibodies were developed after 3 days post vaccination. The results were comparable to the Nigerian strain vaccine. The animals combated the challenge and did not produce any clinical signs of disease throughout the experimental period. PPRV was not detected in any of nasal ocular and oral secretions by RT-PCR. Results of humoral immune response revealed that immunization with vaccine prepared from local thermostable strain of PPRV with the new formulation provided equally good protection in goats. This proves that the newly developed thermostable vaccine would be beneficial in eliminating cold chain associated problems present in existing vaccine and also help in progressive control by PPR in the country.

# Chapter 7

General Discussion

# Chapter 7: General Discussion

Transboundary animal diseases (TADs) are highly contagious diseases and are of great global concern due to their serious socio-economic and public health consequences. They have potential for rapid spread across borders affecting the livelihood of poor farmers (FAO, 1999). Among other TADs, PPR (Peste des petits ruminant) is an enormusly contagious and transboundary viral animal disease affecting predominantly sheep and goats as well as wild small ruminants (Bwihangane et al., 2017). The disease has gained a significant economic importance because of its massive morbidity (100%) (Hailat et al., 2018) and mortality (50-90%) in susceptible populations (Rojas et al., 2017). PPR is a priority disease having a potential of rapid spread over vast areas. It is one of the major notifiable diseases of the World Organization of Animal Health (OIE).

The disease is capable of severely affecting whole of the susceptible host population by provoking epidemics, thus damaging economy, undermining food security and livelihood of poor farmers.

Pakistan has a mixed small ruminants population of 100 million (goats; 70.3 million and sheep; 29.8 millions); about sixty per cent (60%) of this is susceptible to PPRV infection. At present small ruminants producers in Pakistan are facing great economic losses due to PPR. The disease was first recorded in Pakistan during 1991 (Amjad et al., 1996) since then it has spread very rapidly across all provinces and regions of the country inflicting enormous economic losses to the poor livestock farmers and national economy. It has been estimated that losses due to PPR worth Rs. 20.53 billion (US$ 342 million) occur annually. The volume of the economic losses warrants measures to be taken for progressive control leading to eradication of PPR by 2030.

Few live attenuated vaccines are available to control this disease. These vaccines provide lifelong immunity in small ruminants with the preeminent drawback of their susceptibility to thermal degradation especially in areas of extreme hot weather. In developing countries including Pakistan where in summer seasons, the temperature goes up to 50-53°C, the vaccine loses its potency under field conditions due to poor infrastructure and breakage in cold chain which ultimately results in vaccine failure (Yaqub et al., 2016). PPR is now the next eradicating target after rinderpest eradication (Bataille et al., 2019). The disease control programs can be facilitated by the

development of a thermostable vaccine to eliminate the cold chain associated problems in these areas.

The present study was conducted to develop a live attenuated, homologous lineage thermostable vaccine against PPR. For this purpose, a total of 239 clinical samples including tissues (Lymphnode and spleen), swabs and blood were collected from 43 suspected outbreaks of PPRV reported during 2012-2014. The samples were identified by RT-PCR and Ic-ELISA and it was determined that RT-PCR was more effective test for PPRV identification. The study achieved an overall 65.58% of PPR positive samples. The results support a previous study which highlights that proper sampling and disease investigation contributes significantly towards high percentage of PPR positive samples (Zahur et al., 2008).

The highest number of outbreaks were reported from Khyber Pakhtunkhwa (KPK) province of Pakistan followed by Islamabad capital territory (ICT) and Punjab province because of more nomadic movements in these provinces than others (Zahur et al., 2011). These findings are in accordance with a previous study in which highest frequency of outbreaks was reported in Punjab province (Abubakar et al., 2018); (Abubakar et al., 2008); (Khan et al., 2007).

Different molecular epidemiological studies have been carried out to study the high sequence variability found in RNA viruses. The phylogenetic analysis of N gene undertaken in the current study showed that all present study isolates were of lineage IV of PPR virus. It was evident that Pakistani isolates showed more diversity and were found more closely associated with other isolates from neighboring countries like China, India and Iran. One reason for this sequence similarity might be due to the unrestricted animal movement among these countries. The results were in agreement with previous studies based on phylogenetic analysis of N and F gene which revealed that Pakistani isolates were fell in two groups. One was clustered together with Pakistani isolates while other had similarities with Indian, Iranian and Tajikistani isolates (Abubakar et al., 2018); (Zahur et al., 2014); (Anees et al., 2013). Another study based on F and N gene showed a different similarity pattern. The results for N gene were comparable to the present study while F gene had more similarities with isolates from Middle East (Munir et al., 2012).

The three PPRV strains (PAK-LRS-13/NARC, PAK-ICT-12/NARC and PAK-MZD-13/NARC) were sequenced completely using NGS technique. Amplification was made with two methods, SISPA or random sequencing method and specific sequencing method. The low rates obtained with SISPA can be explained by the fact that non-specific amplification was used with random primers. On the other hand, the amplification with specific primers resulted in high PPRV specific reads (96.7%). Although the time required for carrying out this method was less, but it is much expensive compared with other technique. Therefore, considering the cost of amplification and number of reads obtained with the method, sequencing with specific primers is highly suggested. The results of phylogenetic analysis revealed that phylogeographic distribution of different PPRV strains related to specific lineages were similar regardless of the type of sequencing used: complete, incomplete or partial sequence of specific protein (N) of PPRV. This suggested that phylogenetic analysis even from partial sequence of N gene is informative for a preliminary investigation of origin of a strain. Moreover, great diversity was observed in PPRV strains belonging to lineage IV which might be due to cross border PPRV transmission related to animal trade which takes place among these countries.

The complete genome sequencing generated 15,948 bp of sequence which was for all other PPRV genomes sequenced to date. The genome organization of these three Pakistani strains was also same and is in agreement with "rule of six" for Paramyxoviruses. There was a genome promotor region comprising of 107 nucleotides (nt) at the 3′ end and anti-genome promotor at 5′ end. In between these two regions, the transcriptional units for structural (N, P, M, F, H) and nonstructural proteins (P and V) were present. The results were in agreement with the previous studies in which sequencing of complete genome of PPRV resulted in the same genomic organization (Zhu et al., 2016; Dundon et al., 2018).

Comparative analysis of these strains with each other and with Nigerian strain 75/1 (HQ197753.1) revealed that N gene comprising of 1578 nucleotides was more conserved in comparison with Nigerian strain which showed highest identity (98.73%) with Pakistani isolates and lowest (89.57% and 92.7%) with the Lineage I isolate (ICV-89) and Lineage II isolate (Nigeria 75/1) respectively. However, single nucleotide polymorphisms (SNPs) were evidenced at different positions. The same mutations were

seen within N gene in another study reported by Bao and his co-authors (Bao et al., 2012).

The P gene of Nigerian strain showed differences in 102 to 103 bases/residues from three Pakistani isolates of PPRV however, among Pakistani isolates, the maximum difference was of 14 bases. The 16 amino acid motif (EQAYHVNKGLECIKSL) referred to as soyuz 1sequence was also shown at N termini of P protein of Pakistani strains as well as Nigerian strain of PPRV. The conserved motif was observed for the first time by Karlin and Belshaw while studying conserved motifs in N termini of Mononegavirales P proteins (Karlin and Belshaw, 2012). In another study, the same motif was found in P gene of Ghana/NK 1/2010 strain of PPRV (Dundon et al., 2014).

The M protein is considered highly conserved among different lineages of PPRV although the comparative analysis of Nigerian strain with three Pakistani strains revealed that an amino acid change was observed within Pakistani strains while the M protein of Nigerian strain showed 7 amino acid changes from Pakistani strains. The differences in different regions within M gene of a novel mutant of PPRV were also reported by Zhu and his coauthors (Zhu et al., 2016).

The amino acid motif GRRTRR found in F protein, described by Meyer and Diallo as cleavage site (Meyer and Diallo, 1995) was only found in PAK-ICT-12/NARC and PAK-MZD-13/NARC at position 127. The three glycosylation sites (NLS, NIT and NCT) shown by F2 protein which are considered as highly conserved in all morbilliviruses (Dundon et al., 2014) were also found only in PAK-ICT-12/NARC and PAK-MZD-13/NARC at positions 49, 81 and 87.

The H gene was found 1829 nucleotides long. It was considered the least conserved gene among morbilliviruses. Recent analysis of H protein of present study isolates revealed that glycine residue was present instead of histidine at position 481. The binding of MV to SLAM receptors on host cells was mediated by asparagine residues at this position. The study was in contrast with another study conducted by Dundon and his co-workers which revealed that histidine was found at this position in H proteins of most PPRV strains submitted to date (Dundon et al., 2014)

The largest among all the proteins of PPRV is L protein consisting of 6552 nucleotide and 2183 amino acids (Figure 3.14). The previously identified conserved motifs QGDNQ and GDDD known for RNA polymerase activity at positions 771 and 1464 in

lineage II PPRV strain (Ghana/ NK 1/2010) (Dundon et al., 2014) were missing in present study isolates (Lineage IV) as well as Nigerian strain (Lineage II). The L protein of PPRV was also studied by Zhu and his co-authors and it was found to be highly conserved at amino acid and nucleotide level (Zhu et al., 2016).

A comparative analysis of two cell lines (Vero-76 and BTS-34) was also made in this study which revealed that BTS cells showed more obvious pattern of CPEs with high virus titer in short time as compared to Vero cells. The SLAM protein present on BTS cell surface aids in the attachment of virus with the host cell facilitating efficient PPRV isolation. The study supports another similar work in which CHS-20 cells constitutively expressing SLAM receptors in monkey CV1 cells were used in comparison with most commonly used Vero cells and results revealed that CHS-20 cells induced syncytia in 1 to 2 days while Vero cells developed visible CPEs after 11 to 12 weeks post infection (Adombi et al., 2011). Another study performed on comparative analysis of Vero cells expressing canine SLAM and Nectin-4 revealing Vero Nectin-4 to be more efficient for PPRV isolation (Fakri et al., 2016) also supports the results of present study.

Like other morbilliviruses, PPRV is a thermolabile virus which may easily be inactivated at high temperatures, by extreme heat, lipid based detergents, UV irradiation and pH variations (inactivated at pH < 5.6 and >9.6) (Chazya et al., 2014). The present study evaluated the thermostability potential of 7 local isolates of PPRV and categorized them into different classes based on thermostability characteristics which helped in selection of suitable heat resistant PPRV isolate which would be used as candidate for PPR vaccine. PPRV isolates were comparatively stable at 4°C with no radical loss of infectivity for 3 weeks. Though there was difference in reduction of infectivity between different isolates. At 25°C, the decline in infectious titer was slow and moderate. It was estimated that PPR virus could tolerate temperature of 25°C for about 10-15 days and 37°C for a maximum of 30 hours after which no residual infectivity was observed. The most stable isolate at these temperatures was PAK-ICT-12/NARC that did not lose its original titer up to the $4^{th}$ day at 25°C and maintained their original titer for initial 12 hours at 37°C. The most labile isolate was PAK-ATT-13/NARC which was completely inactivated after 18 hours of incubation at 37°C. The better heat tolerance in some circulating strains could be due to the higher temperatures in the region and circulating virus isolates might have adapted to survive these higher temperatures. In a similar study performed on Hepatitis C virus (HCV), it was demonstrated that a genotype of HCV

virus was comparatively stable at 4°C without profound loss of infectivity for 6 weeks and could tolerate 37°C and RT (room temperature) for a maximum of 2 and 16 days when grown in cell culture medium (Song et al., 2010).

PPRV was shown to be highly sensitive to extreme heat. At 56°C, PAK-LRS-13/NARC was most stable virus that remained active with reasonable titer for 30 minutes exposure followed by PAK-ICT-12/NARC and PAK-MZD-13/NARC which remained active for 15 minutes although the titers declined. The most labile viruses at this high temperature were PAK-KP-12/NARC and PAK-ATT-13/NARC that were completely inactivated after 15 minutes at 56°C. Limited work has been done related to the stability of PPR virus and its inactivation at different environmental conditions. Although similar work has been performed on many other viruses like Hepatitis C virus (HCV) (Song et al., 2010), Hepatitis E viruses (HEV) (Emerson et al., 2005) and Newcastle disease virus (NDV) (Omony et al., 2016). The results were also in agreement with another study performed on field isolates of NDV which concluded that NDV strains exhibit varying degree of thermostability at high temperatures (Omony et al., 2016) The difference of thermostability was also observed in HEV strains nevertheless all strains tested were effectively inactivated by heating at 60°C while in case of HAV, heating to 5-10°C higher temperature than HEV was required to inactivate HAV strains (Emerson et al., 2005). None of the PPR virus isolates showed any titer when exposed to 60°C, 62°C and 65°C while studies on HCV demonstrated that the virus become inactivated at 60°C and 65°C in 10 and 4 minutes respectively while at 56°C, minimum time of 40 minutes was required for complete viral inactivation (Song et al., 2010).

One most thermo-tolerant PPR virus local isolate was selected based on thermal stability characteristics which was used later to develop a thermostable vaccine formulation.

PPR virus like other members of the genus morbillivirus has property to develop syncytia during late passages. Primary cultures of mammalian kidney and lungs show early formation of syncytia during infection process (Plowright and Ferris, 1959; Gilbert and Monnier, 1962). However, due to the difficulty in maintaining these cultures and batch to batch variation, PPRV was adapted to grow on goat, calf or monkey kidney cells (Laurent, 1968). The attenuated strain of PPRV (Pak-LRS-13/NARC) did not show syncytia formation during early serial passages onto Vero cells. This finding was in

accordance with a previous study which revealed that initial passages during attenuation of Nigerian strain did not exhibit syncytia (Laurent, 1968).

The current study revealed that during the attenuation of local strain of PPRV, initial passages did not show CPEs development until 4 to 6 days post infection but with the adaptation of PPRV to the atypical host (Vero cells), this time was reduced to 2 days later on. These results were supported by a previous study in which Nigerian strain 75/1 was attenuated by serial passages. The possible reason might be the less expression of F protein or non-cleavage of its inactive form which is important for potential activity of F protein (Lamb and Kolakofsky, 2001).

The local strain of PPRV attenuated using Vero cells, when inoculated in goats caused slight hyperthermia and oculo-nasal discharge. The same strain after $40^{th}$ passage failed to develop any clinical signs of PPR. Same was true for Nigerian strain (Diallo et al., 1989). However, this observation was contrary to the Gilbert and Monnier who noticed that another strain of same virus did not lose its pathogenicity until $65^{th}$ passage on primary cultures (Gilbert and Monnier, 1962). The reason might be the low concentrations of virus used that did not develop mutants. The present study also revealed that local strain of PPRV provided good immunity after inoculation in goats at $40^{th}$ and $60^{th}$ passages and produced humoral immune response as early as 3 days post inoculation which suggests attenuated strain a good candidate for PPR vaccine.

Vaccine stability is generally enhanced to a certain extent by the addition of suitable stabilizer (El-Bagoury et al., 2015). After successful attenuation of PPRV onto Vero cells by 60 serial passages, PPR vaccine was prepared. A comparative study was also conducted to evaluate the effects of stabilizers on the thermostability of PPR vaccine by thermo-stabilization method used for RP vaccine. The results were compared by accelerated stability tests. Among the three chemical stabilizers used in this study namely Lactalbumin Hydrolysate-Sucrose (LAH), Weybridge medium (WBM), Trehalose dehydrate (TD), LAH extended the shelf life of PPR vaccine for 17 days at 37°C as compared to the two other stabilizers (15 days for LAH and 10 days for WBM). The results were in accordance with a previous study which revealed that trehalose provided better stabilizing effect with minimal titer loss (Worrall et al., 2000). The residual moisture plays an important role in thermostability of vaccines (Bora et al., 2015). Therefore secondary drying steps were employed in the present study and it was found that titer loss in case of LAH stabilizer was only 0.23 $\log_{10}$ $TCID_{50}$/ml. The

results were in agreement with the findings of Worrall and his co-workers who found secondary drying steps for 17 more hours reduced the residual moisture to less than 1% which further enhanced the thermostability of PPR vaccine (Worrall et al., 2000). One previous study also revealed that high deterioration was evident at high temperature due to absence of secondary drying during lyophilization (Sarkar et al., 2003).

In the present study, rinderpest method was used for PPR vaccine stabilization and it was found that LAH stabilizer and rinderpest method protected the vaccine sufficiently as is evident by minimal titer loss in case of LAH (0.23 $\log_{10}$ $TCID_{50}$/ml). The results were in agreement with another study in which Mariner and his co-workers found that vaccine produced using LS as stabilizer and lyophilized employing rinderpest method proved to be sufficiently thermostable (Mariner et al., 2017). Yaqub and his colleagues while comparing the effects of different stabilizers on PPR vaccine using Nigeria 75/1 strain observed the same results proving LS as stabilizer of choice due to less titer loss during lyophilization with extended shelf life (Yaqub et al., 2016).

During the studies conducted by Riyesh and his co-workers, the two stabilizers namely LS and stabilizer E were compared and it was revealed that LS stabilizer was superior to the other (Stabilizer E) in terms of shelf life and infectious titer (Riyesh et al., 2011).

# Chapter 8

Concluding Remarks

# Chapter 8: Concluding Remarks

## 8.1 Summary of Work Presented in This Thesis

The present study was conducted to develop a live attenuated, homologous lineage thermostable vaccine to control PPR in Pakistan. To achieve this objective, a total of 43 outbreaks were attended and a repository for PPRV isolates was developed.

It is evident from the results of the current study that RT-PCR was more sensitive and specific assay for confirmation of PPR from suspected field outbreaks. The highest number of outbreaks were reported from Khyber Pakhtunkhwa (KPK) province of Pakistan followed by Islamabad Capital Territory (ICT) and Punjab province respectively. The prevalent production systems for small ruminants in Pakistan (Sedentary, Transhumant, nomadic, and household) involve a lot of movement of animals. Furthermore, KPK and ICT serve as a gateway for seasonal migration of small ruminants to high altitude alpine pastures in north of Pakistan.

The phylogenetic studies undertaken revealed that Pakistani isolates showed more diversity and were found more closely associated with other isolates from neighboring countries like China, India and Iran. The sequence similarity might be due to the frequent movement of the stock across the border of China and Iran. Likewise during early nineties India and Pakistan were affected by a massive outbreak of PPR prior to the fencing of border. These sequence similarities could be the result of that outbreak.

The comparative analysis of two cell lines (Vero-76 and BTS-34) revealed that BTS cells showed more pronounced CPEs associated with PPRV along with high virus titer in short time than on Vero cells. The SLAM protein expressed on BTS cell surface aids in the attachment of virus with the host cell facilitating efficient PPRV isolation.

The thermostability potential of 7 local isolates of PPRV was evaluated to select a more suitable candidate for the development of PPR vaccine. PPRV was shown to be highly sensitive to extreme heat. One local isolate (PAK-LRS-13/NARC) was found thermo-tolerant at higher temperature. The isolate remained active with reasonable titer for 30 minutes exposure followed by PAK-ICT-12/NARC and PAK-MZD-13/NARC which remained active for 15 minutes although the titers declined. The most labile viruses at this high temperature were PAK-KP-12/NARC and PAK-ATT-13/NARC that were

completely inactivated after 15 minutes at 56°C. However, PAK-LRS-13/NARC appeared more resistant maintaining reasonable titer after heat treatments.

Thus, the attenuation of a local thermo-tolerant isolate of PPRV was conducted by serially passaging up to 60 passages onto Vero cells. The pathogenicity trials were also conducted to monitor the degree of attenuation and progressive loss of pathogenicity after $20^{th}$, $40^{th}$ and $60^{th}$ passage. It is evident from the results that after $20^{th}$ passage, slight hyperthermia and oculo-nasal discharge were observed in goats. However, after $40^{th}$ and $60^{th}$ passage, no clinical manifestations were noticed. It was concluded that $60^{th}$ passage virus could be used for development of PPR vaccine. PPR vaccine was prepared following OIE guidelines.

The effects of various stabilizers on the thermostability of PPR vaccine was also evaluated by using thermo-stabilization method for RP vaccine. The results were analysed by accelerated stability tests. Among the three chemical stabilizers, the loss in viral titer during lyophilization in case of Lactalbumin Hydrolysate-Sucrose (LAH) was only 0.23 $TCID_{50}$/ml as compared to the Trehalose dehydrate (TD) (0.37 $TCID_{50}$/ml) and Weybridge medium (WBM) (0.45 $TCID_{50}$/ml).

The efficacy of this newly developed PPR vaccine was evaluated by carrying out experimental immunization in goats. Dynamics of humoral immune response was monitored. It was found that antibodies were developed after 3 days post vaccination. The results were comparable to the Nigerian strain vaccine.

Challenge protection studies were carried out in animals previously inoculated with PPR vaccine made by Nigerian strain (75/1) and newly developed thermostable vaccine formulation. A virulent field isolate catalogued as PAK-Fjg-14/NARC (Un-attenuated) was used in challenge protection studies. The results revealed that the animals did not produce clinical disease in experimental goats.

## 8.2 Advances Made in This Work

- A repository of local PPRV isolates was developed. A total of 18 PPRV isolates were recovered during this study. Complete biological and molecular characterization was carried out. The isolates were stored in Animal Health repository for further analysis.
- Three local strains of PPRV were sequenced completely using Next Generation Sequencing (NGS).
- The current study provides an update of data regarding phylogeny of PPRV.
- A comparative analysis of Vero-76 and BTS-34 cell line was conducted for the first time in the country regarding the isolation of PPR virus.
- A comprehensive evaluation was conducted to assess the thermostability potential of local isolates of PPRV for the first time.
- The development of live attenuated, homologous lineage, thermostable PPR vaccine was successfully attempted for the first time in the country along with the most suitable stabilizer for use in PPR control programs.
- The cost of locally produced PPR vaccine was greatly reduced due to development of a thermostable vaccine that would help in minimizing the dependence on cold chain under field conditions.

## 8.3 Recommendations and Future Prospects

The current study has made way for future investigations in following areas

1. RT-PCR assay should be used to understand the epidemiology and dynamics of PPRV in the country.
2. Prior to the use of this newly developed PPR vaccine, a field trial should be conducted in different ecological zones and/or all production systems for small ruminants in the country.
3. A cost effective sero-diagnostic assay such as Haemagglutination inhibition (HI) which is quite simple to conduct should be developed for post vaccination monitoring.
4. The lyophilization method used for thermostabilization of PPR vaccine is expedient to reproduce and commercialize and can aid further for progressive control of PPR in the country.
5. A repository of local PPRV isolates was developed during this study. The isolates can be used for further studies to understand the dynamics of PPRV transmission and its pathogenicity under controlled conditions.
6. The technology developed during this study can be transferred to PPR vaccine production units in the country.

# Chapter 9

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# Chapter 9: References

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# APPENDIX

## Appendix -1

### Performa for clinical observations of experimental animals

**IDENTIFICATION OF ANIMAL**

| Group | | | |
|---|---|---|---|
| Animal Tag | | | |
| Temperature $^0$F **(am)** | | | |
| Temperature $^0$F**(pm)** | | | |

**Clinical Signs**

| Absence of Clinical Signs | ☐ | ☐ | ☐ |
|---|---|---|---|

| Respiratory Troubles | Scoring | | | |
|---|---|---|---|---|
| Nasal Discharge | 1 | ☐ | ☐ | ☐ |
| Important Nasal Stuffing | 2 | ☐ | ☐ | ☐ |
| Ocular Disorders | | | | |
| Lacrimation | 1 | ☐ | ☐ | ☐ |
| Lacrimation important | 2 | ☐ | ☐ | ☐ |
| Cough | | | | |
| Cough | 1 | ☐ | ☐ | ☐ |
| Coughing Fit | 2 | ☐ | ☐ | ☐ |
| Digestive Disorders | | | | |
| Mild/ Light Lesions | 1 | ☐ | ☐ | ☐ |
| Stomititic Lesions | 2 | ☐ | ☐ | ☐ |
| Diarrhea | 3 | ☐ | ☐ | ☐ |
| General Behavior | | | | |
| Isolation | 1 | ☐ | ☐ | ☐ |
| Hair pic | 2 | ☐ | ☐ | ☐ |
| Decubitus | 3 | ☐ | ☐ | ☐ |
| Other Clinical Signs | | ☐ | ☐ | ☐ |

## Appendix-2

**1x TBE buffer**

| | |
|---|---|
| Tris base | 10.8g |
| Boric acid | 5.5g |
| $Na_2EDTA$ | 0.5 M |

Dissolve 10.8 g Tris and 5.5 g Boric acid in 900 ml distilled water. Add 4 ml 0.5 M Na2EDTA (pH 8.0). Adjust volume to 1 Liter. Store at room temperature.

## Appendix-3

**Growth media**

| | |
|---|---|
| GMEM | 1.25g |
| $NaHCO_3$ | 0.27g |
| Water | 80ml |
| TPB | 10ml |
| FBS | 10ml |

## Appendix-4

**5% LAH (Lactalbumin Hydrolysate-Sucrose)**

| | |
|---|---|
| 5% LAH | 5g |
| 10% Sucrose | 10g |
| HBSS | 100ml |

Dissolve 5% Lactalbumin hydrolysate (LAH) and 10% sucrose in Hank's balanced salt solution (HBSS) at pH 7.2. Incubated at 37°C for 48 hours and then store at 4°C.

## Appendix-5

**WBM (Weybridge medium)**

| | |
|---|---|
| 2.5% LAH | 2.5g |
| 5% Sucrose | 5g |
| 1% Sodium Glutamate | 1g |
| HBSS | 100ml |

Dissolve 5% sucrose, 1% sodium glutamate and 2.5% LAH in HBSS at pH 7.2. Incubated at 37°C for 48 hours and then store at 4°C.

## Appendix-6

**TD (Trehalose Dehydrate)**

| | |
|---|---|
| 5% Trehalose | 5g |
| Distilled Water | 100ml |

Incubated at 37°C for 48 hours and then store at 4°C.

## Appendix-7

**Hanks's Balanced Salt Solution (HBSS)**

| **Chemicals** | **mg/L** |
|---|---|
| KCl | 400 |
| $KH_2PO_4$ | 60 |
| NaCl | 8000 |
| $NaHCO_3$ | 350 |
| $Na_2HPO_4$ | 48 |
| D-Glucose | 1000 |
| $CaCl_2$ | 140 |
| $MgCl_2.6H_2O$ | 100 |
| $MgSO_4.7H_2O$ | 100 |

## Appendix-8

**Maintenance Media**

| | |
|---|---|
| GMEM | 1.25g |
| $NaHCO_3$ | 0.27g |
| Water | 96ml |
| TPB | 2ml |
| FBS | 2ml |

## Appendix-9

**Tryptose Phosphate Broth (TPB)**

| | |
|---|---|
| TPB (Sigma) | 2.95g |
| Deionized distilled water | 100ml |

Sterilize by autoclaving at 121°C at 15Ib/ sq inch for 15 minutes

# PUBLICATIONS & PRESENTATIONS

***Part of the research work presented in this thesis has been published in the following papers*** *(Copy attached)*

1. **Latif A**, Zahur AB, Libeau G, Zahra R, Ullah A, Ahmed A, Afzal M and Rahman SU, 2018. Comparative analysis of BTS-34 and Vero-76 cell lines for isolation of Peste des Petits Ruminants (PPR) virus. Pak Vet J, 38(3): 237-242.
2. **Latif A**, Akhtar Z, Ullah RW, Zahur AB, Ullah A, Irshad H, Malik AR, Farooq U, Hussain M, Mahboob K, Afzal S., 2014. Evaluation of haemagglutination assay (HA) for the detection of peste des petits ruminants virus (PPRV) in faecal samples of recovered goats. Res. J. Vet. Pract. 2 (1S): 11 – 13.

***And presented as poster presentations in the following conferences:***

1. **A. Latif**, A. Bin Zahur, R. Zahra, Ullah A, Afzal M. Selection and successful attenuation of a thermo-tolerant strain of Peste de petits ruminant (PPR) virus to be used as thermostable vaccine formulation. Regional Conference to Promote Safe and Secure Science in the Middle East, South and Southeast Asia. Held Kuala Lumpur, Malaysia on February 5-9, 2018.
2. **A. Latif**, A. Bin Zahur, R. Zahra, A. Ahmed, R. Wasee Ullah, U. Farooq. 2014. Evaluation of thermal stability and inactivation of PPR virus cultivated in cell culture" IMED (International Meeting on Emerging Diseases) conference held in Vienna, Austria on 31-10-2014 to 3-11-2014.
3. **A. Latif**, A. Bin Zahur, R. Zahra, R. Wasee Ullah, U. Farooq, K. Naeem, 2014. Isolation of PPR virus from the blood samples collected during acute phase of infection. 16$^{th}$ International Congress on Infectious Diseases, Cape Town, South Africa, 2-4-2014 to 5-4-2014.
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Other contributions in the field of Microbiology:

1. Ali A, Zahur AB, Farooq U, **Latif A**, Naeem K, Afzal M, Ullah RW, Shabana and Muhammad ZY, 2019. Comparative Sensitivity of LFBK and LFBK αVβ6 Cell Lines for Isolation of Foot and Mouth Disease Virus from Riverine Buffaloes by Using Oro-Pharyngeal Fluids. J Virol Antivir Res, 8:1.
2. Navid, M. T., Farooq, U., **Latif, A**., Awais, M. M., Anwar, M. I., Akhtar, M., Zahur, A. B. 2018. Prevalence of foot and mouth disease virus in apparently healthy buffaloes brought to Islamabad slaughterhouse in Pakistan. Tropical Biomedicine. 35(1), 161-167.
3. Afshan Ahmed, **Asma Latif**, Rabaab Zahra, Amir Bin Zahur, Umer Farooq, Aman Ullah, Muhammad Abubakar, Aatka Jamil and Muhammad Afzal, 2017. Current appraisal of the suitability of Foot and Mouth Disease strains in two commonly used commercial vaccines for control of FMD in Pakistan. Journal of Animal and Plant Sciences. 27 (2), 446-450.
4. Sidra Maryam, Tahir Rasheed, **Asma Latif**, Rabaab Zahra, Aamer Bin Zahur, Aitezaz Ahsan and Muhammad Afzal, Umer Farooq, 2017. One-step real time loop mediated isothermal amplification process (RT-LAMP) validation and its application for the detection of foot and mouth disease virus and its serotypes. Turkish Journal of Veterinary and Animal Sciences. 41: 435-443.
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6. Ullah, R, A. B. Zahur, **A, Latif**, J. I. Dasti, H. Irshad, M. Afzal, T. Rasheed, A. R. Malik and Z. U. qureshi, 2016. Detection ofr Peste des Petits Ruminants viral RNA in fecal samples of goats after an outbreak in Punjab province of Pakistan: A longitudinal study. Biomed Research International, http:// dx.doi.org/10.1155/2016/1486824
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**10.** Zahur, A.B., H. Irshad, A. Ullah, M. Afzal, **A. Latif**, R.W. Ullah, U. Farooq, M.H. Samo, M. Jahangir, G. Ferrari, M. Hussain and M.M. Ahmad. 2015. Short communication: Peste des Petits Ruminants vaccine (Nigerian strain 75/1) confers protection for at least three years in sheep and goats. EMPRES-Animal Health 360 (45): 23.

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**14.** Zahur, A.B., Irshad, H., Ullah, A., Afzal, M., **Latif, A**., Ullah, R.W., Farooq, U., Samo, M.H. and Jahangir, M. 2014. Peste des Petits Ruminants Vaccine (Nigerian Strain 75/1) Confers Protection for at Least 3 Years in Sheep and Goats. Journal of Biosciences and Medicines. 2, 27-33.

**15.** Umer Farooq, Hamid Irshad, Riasat Wasee Ullah, Aman Ullah, Muhammad Afzal, **Asma Latif**, Aamer Bin Zahur, 2014. Snake Bite in Jersey Cattle; a Case Report. Research Journal for Veterinary Practitioners. 2 (5): 82 – 83.

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