Standardization of qRT-PCR for detection and quantification of Peste Des Petitis Ruminant virus (PPRV) from 2013-2015 outbreaks in Pakistan

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By

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DECLARATION

The material contained in this thesis is my original work and I have not presented any part of this work elsewhere for any other degree.

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DEDICATED TO MARTYRS

OF

APS & CHARSADDA

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This thesis by Tahir Rasheed is accepted in its present form by the Department of Microbiology, Quaid-I-Azam University, Islamabad, for the requirement of the degree of Master of Philosophy in Microbiology.

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ACRONYM/ABBREVIATIONS

PPR	Peste des Petits Ruminants
PPRV	Peste des Petits Ruminants Virus
PCR	Polymerase Chain Reaction
RT-PCR	Reverse transcriptase Polymerase Chain Reaction
qRT-PCR	Quantitative Reverse transcriptase Polymerase Chain Reaction
HA	Hemagglutination Assay
RT-qPCR	Reverse transcriptase Quantitative Polymerase Chain Reaction
GDP	Gross Domestic Product
CCPP	Contagious Caprine Pleuropneumonia
CPD	Contagious Pustular Dermatitis
FMD	Foot and Mouth Disease
OIE	Office des International Epizooties
TAD	Transboundary Animal Diseases
ELISA	Enzyme Linked Immuno Sorbent Assay
CIEP	Counter Immuno-Electrophoresis
AGID	Agar Gel Immuno-Diffusion
RP	Rinderpest
RPV	Rinderpest Virus
FAO	Food and Agriculture Organization
GIT	Gastrointestinal Tract
"N" Protein	Nucleoprotein
"M" Protein	Matrix Protein
"F" Protein	Fusion Protein
"H" Protein	Hemagglutinin Protein
"L" Protein	Large Polymerase Protein
DIVA	Differentiation Between Infected And Vaccinated Animals
AD	Anno Dominie
CDV	Canine Distemper Virus
UTR	Untranslated Region
GP	Genome Promoter
UTR	Untranslated region
ORF	Open reading frame
SLAM	Signalling lymphocytic activation molecule
RdRp	RNA-dependent RNA polymerase
OIE	Office international des epizooties
CTL	Cytotoxic T cell
CCPP	Contagious caprine pleuropneumonia
CPD	Contagious pustular dermatitis
VNT	Virus neutralization test
MAbs	Monoclonal antibodies

DTT	Dithiothreitol
UDG	Uracil DNA Glycosylase
Ct	Cyclic Threshold
RNA	Ribonucleic Acid
DNA	Deoxyribonucleic Acid
cDNA	Complementary Deoxyribonucleic Acid
R Value	Regression value
RBCs	Red Blood Cells
TBE	Tris/Borate/EDTA
RT-LAMP	Reverse transcription loop-mediated isothermal amplification assay
ELISA	Enzyme Linked Immunosorbent Assay
RP	Rinderpest
RPV	Rinderpest Virus
VI	Virus Isolation
OD	Optical density
AGID	Agar gel immunodiffusion test
CIE	Counter immunoelectrophoresis
ic-ELISA	Immunocapture ELISA
c-ELISA	Competitive ELISA
HI	Hemagglutination inhibition test
VNT	Virus Neutralization Test
PBS	Phosphate Buffer Saline
PI	Percent Inhibition

Acknowledgement

*All Praises to Almighty **Allah**, The Light of Heavens and Earth, the omnipotent, the most compassionate and His **Prophet Muhammad** (ﷺ) The Most perfect among all human beings ever born on the surface of this earth, who is forever a source of guidance and knowledge for humanity as a whole.*

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Tahir Rasheed

Abstract

Abstract:

Peste des petits ruminant (PPR) is the most important disease of small ruminants (sheep and goats), having huge economic impact and with high morbidity and mortality rates. Peste des petits ruminant virus (PPRV) is the causative agent of it, this virus belongs to the morbillivirus family and its four lineages prevail in the world but in Pakistan linage IV is prevalent. PPR is a viral disease, so no specific treatment is required, just symptomatic and supportive treatment is recommended. For the prevention PPR vaccine is available that provides four year (which is the average age of small ruminants) protection. Hence for the eradication and control, its precise diagnosis is very important.

PPR can be diagnosed clinically and in the laboratory. In the laboratory there are many assays developed and standardized for PPRV detection. Most assays give qualitative results while few studies have reported the tests for quantification. In the current study, optimization of single step qRT-PCR (TaqMan) was done along with the development of SYBR Green based qRT-PCR utilizing newly designed primers for the diagnosis of PPR.

A total of 46 field samples (20 swabs and 26 tissue samples) were collected during 2013-2015 from 15 suspected outbreaks that were investigated for the screening of PPR disease, based on clinical signs. For the primary screening of collected samples, hemagglutination assay (HA) was performed while for the confirmation of PPRV, gold standard RT-PCR assay was used. Conventional RT-PCR is a laborious multistage process. Thus, real-time one-step reverse transcriptase-PCR was performed along with the development of a quantitative PCR (SYBR Green). The validity of this analysis was compared with HA; one step quantitative RT-PCR with primers (PPRNF & PPRNR) and TaqMan probe; and classical gold standard RT-PCR with primers (NP3 and NP4). In the tested 46 field samples, the percentage of positive results from HA, RT-PCR, RT-qPCR (probe based) and RT-qPCR (SYBR Green) were 93, 47%, 58.69%, 81.48% and 86.95% respectively , which shows that the newly developed test is more sensitive in diagnosis of PPRV.

CHAPTER: 01
INTRODUCTION

Introduction

Livestock division plays an important part because its contribution exceeds 56% to the agriculture division and 12% to its GDP which implies significance of livestock in the country (Economic Survey, 2014-15). In Pakistan, around 30-35 million people of rural society rear their domestic animals, sheep and goats as an additional livelihood (Sarwar et al., 2002). Pakistan comprises of 34 goat and 28 sheep breeds respectively (Isani and Baloch, 1996). Pakistan comprises 97.8 million of overall population of sheep and goats which contribute to livestock sector (Economic Survey, 2014-15). The goat population (68.4 million) exceeds in number as compared to other livestock species which reveals its importance. Its huge number attributes to its adjustments to diverse environments in Pakistan and browsing nature. Goats show high reproductive rates with short gestation length. Sheep and goats comprise of total of 54% of ruminant population and contributing 671 thousand tons of red meat per year (34%) (Economic Survey of Pakistan, 2015). The conventional farming system still exists in Pakistan. Generally, goats are reared for mutton production (Khan, 2004) although due to high cost and non-availability of cow and buffalo milk in some regions of Pakistan, the alternate is goat milk. In Pakistan, goat is said to be "poor man's cow". While in some areas of Pakistan like Balochistan, goat milk has preference over bovine milk. The obstructions for the animal productivity are parallel extension, shortage of fodder, rough yield remnants, conventional feeding systems, dietary deficiencies and malnutrition, lack of communication, reduced farmers' economy, under-developed markets, deprived vaccination and drug practices.

Peste des petits ruminants (PPR) holds a dominant position among the significant diseases of animals (small ruminants). Mainly sheep and goats are affected by this viral disease. It is of viral origin and is extremely contagious disease of sheep and goat with high morbidity and mortality rates. PPR virus is responsible for high mortality in camels with no signs in bovines and wild animals. Initially in 1942, PPR disease was detected in West Africa (Gargadennec and Lalanne, 1942). Initially PPRV was thought to be prevalent in Africa only, but later on PPR outbreaks were confirmed in different countries of the world like in the Middle East, and South Asia.

For the first time recognition of PPRV was done from cell culture of sheep (Gilbert and Monnier, 1962) and further in 1967 it was confirmed by electron microscope

(Bourdin and Vautier, 1967). The Peste des petits ruminants virus (PPRV) effected animal display sudden pyrexia, purulent ocular and nasal discharge with congested conjunctiva, erosion, respiratory distress, ulceration of mucous membrane and gastroenteritis as a specific clinical signs. Following the 4-6 days of pyrexia the animals might die and expecting animals might terminate. The animals kept in close vicinity are responsible for virus transmission. Goats are more prone to this disease as compared to sheep although sheep show fast recovery rate while as cattle display no clinical signs and work as reservoir host (Abubakar et al., 2008). The occurrence of PPR depends on the season, although PPR prevalence is recorded throughout the year. The disease signs of PPR are frequently mixed with secondary bacterial infections and based on the clinical picture the Peste des petits ruminants (PPR) is often confounded with contagious caprine pleuropneumonia (CCPP) or pasteurelosis, bluetongue, CPD and FMD (Singh et al., 2009). For PPRV confirmation lab testing is required.

PPR is also mentioned in Office International des Epizooties (OIE) list, made for the important diseases of animals. (OIE-Listed diseases, 2014). It has been identified by Institute for Livestock Research of Kenya, the prevention of PPR in different regions will help in poverty alleviation demonstrating the significance of disease to be the center of attention (Diallo, 2006; Perry, 2002). Due to high mortality rates and morbidity rates of 90% and 100% respectively PPR had imposed the threat to sheep and goat production with 20% dangerous blow on flock yield (Elzein et al., 2004). PPR is declared as an acute widespread disease in Pakistan with disease rate of 65.37%, death rate of 26.51% and case fatality rate of 40.40% (Zahur et al., 2014).

Clinical signs, symptoms, necropsy lesions and epidemiology are the basis for PPR diagnosis and in different techniques like immuno-capture ELISA, counter immuno-electrophoresis (CIEP) or agar gel immuno-diffusion (AGID) can be used for the recognition of PPRV antigens in laboratory (Obi and Patrick, 1984) and for the tentative diagnosis, indirect ELISA, can be used but shortcoming of these tests are that they cannot distinguish among PPRV and RP (Palaniswami et al., 2005). For the diagnosis of PPR, the cell culture and molecular methods are very speedy and extremely definite. All the above mentioned tests have some pros and cons over one another as they have unlike specificity and sensitivity standards.

Numerous RT-PCR procedures are being used for the detection of PPRV. Although conventional RT-PCR for the detection of virus follows supplementary steps, which increases the possibility of contamination (Bao et al., 2008). As compared to RT-PCR, the RT-qPCR is extra beneficial as it has high sensitivity, specificity, less time consumption, less contamination possibility and results accuracy. With the help of N-gene and M-gene, numerous qRT-PCR based on TaqMan were produced (Balamurugan et al., 2010; Bao et al., 2008) but quantification and recognition on the basis of TaqMan probe is costly and requires more proficiency to build up the primers and probe (Schmittgen et al., 2000). On the other hand SYBR green is more specific, cost effective, easy implemented and executed thus proved better than for the quantification and recognition than over TaqMan probe.
Up-till now a few studies have been conducted on SYBR Green qPCR assay for PPR diagnosis. The current study was carried out to compare this assay with other commonly used tests for PPR diagnosis. For this study a newly developed set of primers (N gene) was used for SYBR green assay. For the primers designing N gene was selected because N gene has many conserved regions (Abera et al., 2014). That's why it seems to be a good target for identification. There have not been many studies about the comparison of sensitivity and specificity of PPR quantitative assays i.e. SYBR green based qPCR and probe based qRT-PCR.

Optimization of sophisticated molecular methods should be done for PPRV recognition which will enable us to study its surveillance up to great extent and also for analysis of carrier state of infected animals. Not much work has been done on this till now so, there is immense need to work on the project concerning quantification of PPRV through PCR. The objectives of current study were as following:

1. To optimize a panel of diagnostic assays for quantification of PPR virus load in moribund field samples.
2. To optimize 1-step quantitative RT-PCR for PPR diagnosis
3. To develop a quantitative RT-PCR assay (SYBR Green) for PPR diagnosis
4. To evaluate newly developed assay with the 1-step quantitative RT-PCR for PPR diagnosis.

CHAPTER: 02

LITERATURE REVIEW

2. REVIEW OF LITERATURE

"The Transboundary Animal Diseases (TADs), are those diseases that are not possible to restrict in borders or limitations, and most important of these diseases is that they can cause high mortality, and are transmissible to surrounding animals. So in this respect they not only affecting economy of country but also a major concern about public health regarding food values (Fao, 2014). In an agriculture country livestock impart its major role in development and growth. While in livestock small ruminants are main source of income for the poor farmers and nomads. PPR is also a TADs listed disease which is caused by morbillivirus, sheep and goats are its typical hosts, but also cause disease in wild animals, camelid family and some reports also reported in felines. PPR is also included in the list of OIE and notifiable disease (Murphy and Parks, 1999).

PPR is extremely infectious, contagious disease of sheep and goats present in the South Asia, major part of Africa, Middle East. It causes major economic losses, due to high mortality, case fatality and morbidity rate. Vaccination against PPRV is available and its efficacy is good, but main drawback is that it is not easy to differentiate between diseased and vaccinated flock. PPR control and eradication program is in process and OIE and Food and Agricultural Organization (FAO) mark it for eradication by year 2030. Although vaccination is only solution to control and eradicate but with improved marker vaccination is required (Parida et al., 2015). Nigeria/75/1 strain is used for vaccine production which is mono-valent live attenuated vaccine, used for the immunization of sheep and goat against PPR and it provide protection for at least three years (Life time) (Zahur AB et al., 2013).

PPR is known by many names depending upon the area and language of peoples like Kata, rinderpest of small ruminants, plaque of sheep and goat, etc. But globally French abbreviation "PPR" is used. PPR is initially considered as rinderpest disease of small ruminants that cannot cause any clinical signs in bovines but later on it was identified that it was caused by other virus (Morbillivirus). Although it is a close relative of RPV (causing disease in cattle and buffalos) (Kumar et al., 2014). It is an emerging disease of the world so a great threat to the developed/ not effected countries. That's why it has major focus for eradication of the world. But this would be possible with advanced research and dedication, reminding DIVA (Differentiation between infected and vaccinated animals) concept in mind (Parida et al., 2015).

2.1. Historical background

As early as fourth-century AD PPR was mixed with its closely-linked rinderpest virus of morbillivirus (Curasson, 1932). But in late 1942 PPR was described during the 2nd world war in Ivory Coast, West Africa (Gargadennec and Lalanne, 1942). Early remarks proposed that the disease was not transmitted from sheep and goats to other animals (Gargadennec and Lalanne, 1942). After 1st identification it was subsequently reported in other African countries like Ghana, Senegal and Nigeria. That's why earlier it was thought PPR was restricted only in African continent. But in other parts of the world a Rinderpest like disease of goats identified such as in Sudan was reported in 1972 and it was confirmed as PPR after investigation (Diallo et al., 1988). On early stages of the disease, many cases were wrongly diagnosed as Rinderpest which could actually be the virus of PPR. PPR became important due to its diversification, economic significance (Lefevre and Diallo, 1990b) and some hinders which it created in universal eradication of Rinderpest (Couacy-Hymann et al., 2002)

2.2. Causative agent

Peste des petits ruminants (PPR) is caused by PPRV that scientifically is placed in genus Morbillivirus, sub-family Paramyxovirinae, family Paramyxoviridae, and order Mononegavirales. This genus has great importance in medical and veterinary field because it comprises of Measles virus (Disease in Human), CDV (in dog), RPV (in bovines), Phocine distemper virus (in dolphins) and the morbilliviruses of marine mammals, the cetacean morbilliviruses. Recently new morbilliviruses were described including Feline morbilliviruses (in cat) (Woo et al., 2012) and rodents/ bats morbilliviruses (Drexler et al., 2012). Commonly it was thought that morbilliviruses causing disease in its specific host like measles only in human, RP in Artiodactyla. But in contrast PPRV can cause disease not only in small ruminants, but also in Camelids (El-Hakim, 2006; Roger et al., 2001), and also in felids. Molecular knowledge of PPRV mainly based on other morbilliviruses viruses like measles, RPV (prototype virus) and CDV. That's why PPRV was uncharacterized regarding its replication, pathogenesis, immunity, transcription etc. But this virus was considered conserved regarding its characters with its group viruses. Hence literature review of PPR is generalized for morbilliviruses, along with PPRV specific researcher achievements.

2.3. Viral genome

PPR virus is pleomorphic in shape, size of variable size ranges from 150nm to 700nm. (Bourdin and Vautier, 1967). This virus has –ve sense single-stranded RNA genome which is in covered by ribo-nucleoprotein complex. Genome is comprised of 15948 nucleotides, 6 genes that have coding of 8 proteins as shown in figure 2.2. This ribo-nucleoproteins complex is placed inside the viral enveloped. Inside envelop is a bead like lining of M protein while F and H proteins are embedded in the outer lining of envelop just like studs (Fig. 2.1) (Rager et al., 2002). PPR viral genome comprises of six transcriptional components in the sequence of N, P, M, F, H and L that translate into 8 protein i.e. N, P, C, V, M, F, H and L (Bailey et al., 2005). C and V are the non-structural proteins while rest are structural (Fig. 2.2) (Mahapatra et al., 2003). Conserved regions are present between each gene. PPR genome contains 30 and 50 un-transcribed sequence that play central role in the replication as controlling elements (Banyard et al., 2005). The virus leader region, including 30 untranslated region (UTR) of the N gene, constitutes the genome promoter (GP) and, similarly, the 50 UTR of L gene with a short trailer sequence form the anti-genome promoter (AGP). The UTR between the M and F gene ORF is unusually long (1080 nucleotides) compared to other UTRs along the virus genome and is highly rich in G and C nucleotides (68–72% GC across the region). Overall, the virus genome is relatively conserved with a maximum divergence of 12% at nucleotide and 8% at amino acid sequence level (Muniraju et al., 2014).

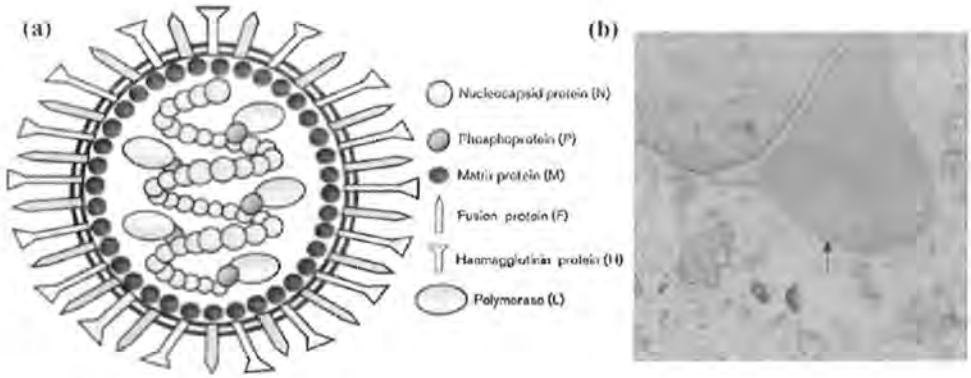


Figure 2.1: Schematic diagram of Peste des petits ruminants virion structure (adapted from Banyard et al., 2010). The PPRV glycoproteins (F and H) are embedded within the viral envelope. The M protein lines the inner surface of virus envelope. The ribonucleoprotein complex is composed of N, P and L proteins in association with the RNA genome. (b) Electron micrograph of peste des petits ruminants nuclocapsid in the cytoplasm of an infected cell. The viral RNA, completely encapsidated in the viral N protein has a herring-bone like appearance (arrow).

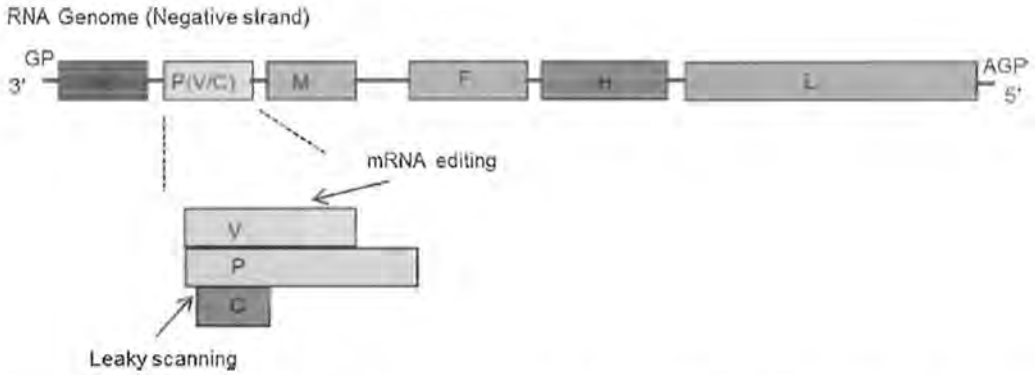


Figure 2.2: Schematic representation of Peste des petits ruminants virus genome organization. The PPRV genome is a non-segmented, single-stranded negative sense RNA molecule. The genome consists of six transcriptional units (encoding the nucleoprotein [N], phosphoprotein [P], matrix protein [M], fusion protein [F], hemagglutinin protein [H] and the large/polymerase [L] protein) that are flanked by a 30 genome promoter (GP) and a 50 anti-genome promoter (AGP) on the negative sense genome RNA. The P gene encodes for two additional non-structural proteins, namely C and V. The V protein is produced due to co-transcriptional P mRNA editing by insertion of non-template G residues at an editing site. The C protein is produced from an alternative reading frame downstream of the P initiation codon. Expression of C occurs following leaky scanning by the polymerase that reads through the first ATG and initiates at the second ATG.

2.4. PPRV Replication and Life Cycle

For the virus replication 1st step is attachment between infectious particle and host. PPR virus attaches via its hemagglutinin and neuraminidase protein (present on the outer envelope) interacts with receptors (sialic acid) on the susceptible host cell membrane. Most probably this is the reason behind which cause hemagglutination of the chicken, human, pig and goat RBCs (Renukaradhya et al., 2002). Now it was confirmed that SLAM are theco -receptors of PPRV (Pawar et al., 2008). After attachment of virus fusion occurred with the help of F-protein of virus that resulted in the discharge of nucleo-capsid from the virus. After this L-protein comes into action and act as RNA dependent RNA-polymerase (RdRp) and start the production of mRNA inside the host cell. RdRp attaches with the genome-promotor after which open reading frame starts transcription in stop & start manner. Sequence present at the gene-junction determine the quantity of protein required to produce. As N protein is required in abundant quantity so its mRNA production is more as compared to others and L-protein uses is least so its mRNA production is very less.

The process of transcriptase activity of RdRp shift to replicase is a complex one (Kolakofsky et al., 2004). RdRp requires other viral-proteins for proper functioning. M-protein of the virus controls RdRp activity, however during virus assembly and budding this regulatory mechanism of M-protein doesn't require (Banyard and Parida, 2015). Neuraminidase is also involved in the viral-budding process by cleaving sialic-acid from the host cell membrane (Scheid and Choppin, 1974). H-protein of PPR virus have neuraminidase and hemagglutinin functions, so it is referred to as HN rather than just H (Fig. 2.3) (Seth and Shaila, 2001).

Based on the knowledge of other morbilliviruses, it was assumed that in PPR virus, editing activity controls the production of P&V proteins. It is also assumed that depending upon the stage of the disease/infection proteins expression varies that dampen the host-immunity. But this information regarding PPR virus requires to be explored and validated.

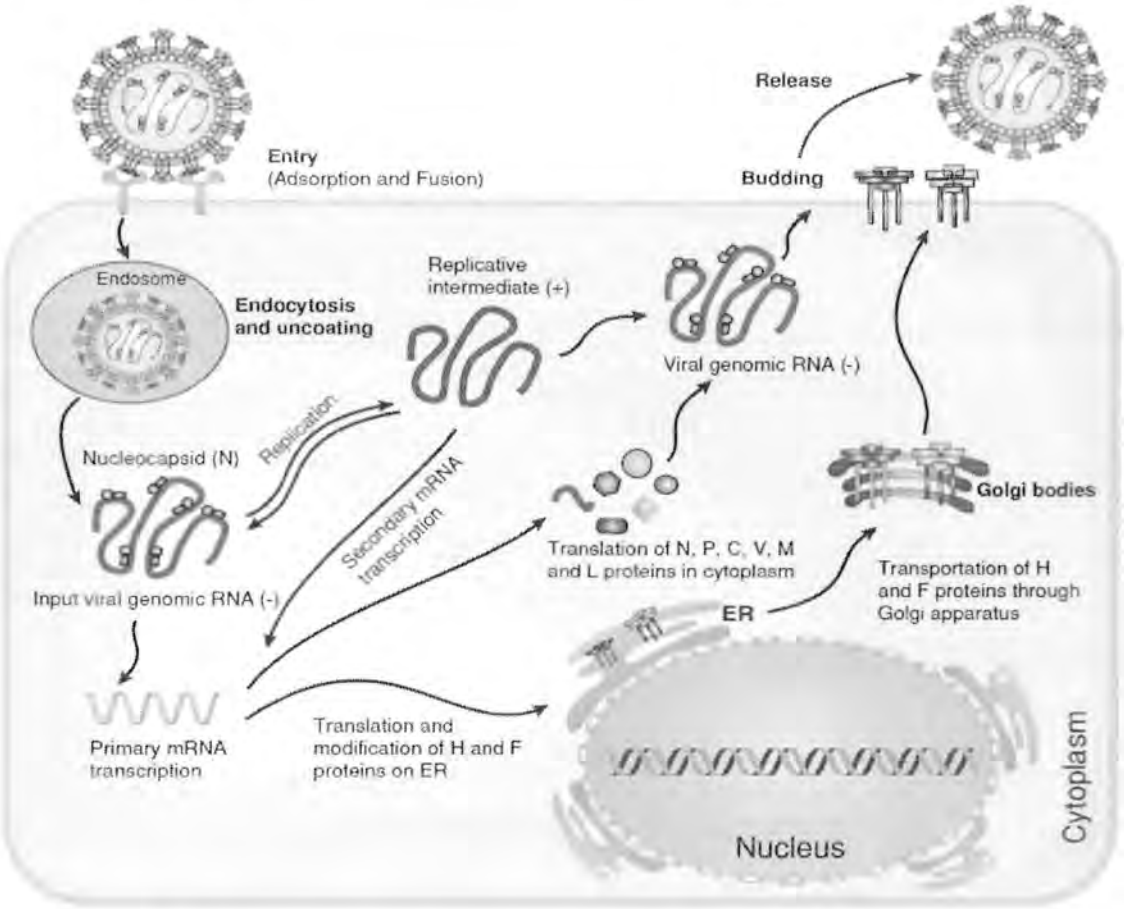


Figure 2.3: Replication cycle. Interaction of the viral HN and F proteins with the host plasma membrane leads to viral entry by binding of the HN protein to receptors (SLAM and other unidentified) for PPRV. The rest of the proteins are involved in replication of virus. Briefly, the P protein regulates transcription and replication and assembly of the N protein to nuclocapsid, the M proteins mediate viral assembly, and the HN protein facilitates the budding process when it acts as a neuraminidase in PPRV. Viral genome copies are formed from the genome through replicative intermediates. The role of the C and V proteins in PPRV is still not clear. It is believed that these proteins have abilities to abrogate the cellular interferon (IFN-a/b) responses and hence contribute in the virulence of PPRV.

2.5. Geographical distribution

First case of PPRV infection was reported in West African country, Nigeria in 1942. At present, it is endemic in vast spread area of West Africa. But, virus epidemics are under reported and very less characterized because of poor reporting system and lack of performing molecular analysis. West Africa is classified in 16 different regions of 5 million square km each. PPRV epidemic is seen in lot of countries of this continent. In past years, tests performed in Regional Reference

Laboratories have proved the existence of antibodies to the PPRV and identified viral nucleic acid in specimens from various countries of the region. Studies of cases of PPRV in Nigeria used hemagglutinin experiments with fecal matter to recognize PPRV excretion and recommended that healthy animals can act as carriers for PPRV (El-Yuguda et al., 2009; Ibu et al., 2008).

PPRV is found endemic in a number of countries of East Africa. This was found with genetic typing of the virus in 1996 which detected a virus prevalent in Ethiopia. Virus was related to lineage III. Previous isolations were performed from wild life in Oman related to the same lineage as from East African counties. Molecular tests also confirmed the relation of viruses with lineage III in Sudan, Uganda and Tanzania. Lineage IV Viruses were also detected from some regions of the continent (Khalafalla et al., 2010).

PPRV was diagnosed in a district of Kenya in 2006. This disease then disseminated to other districts of the country and caused disaster for food security and affected livelihood of local population. PPRV also attacked Somalia in 2006 with central areas most severely affected. But spread of disease was prevented here by adoption of necessary measures in time. In addition, ring vaccination was also adopted across the country to prevent the dissemination of the virus.

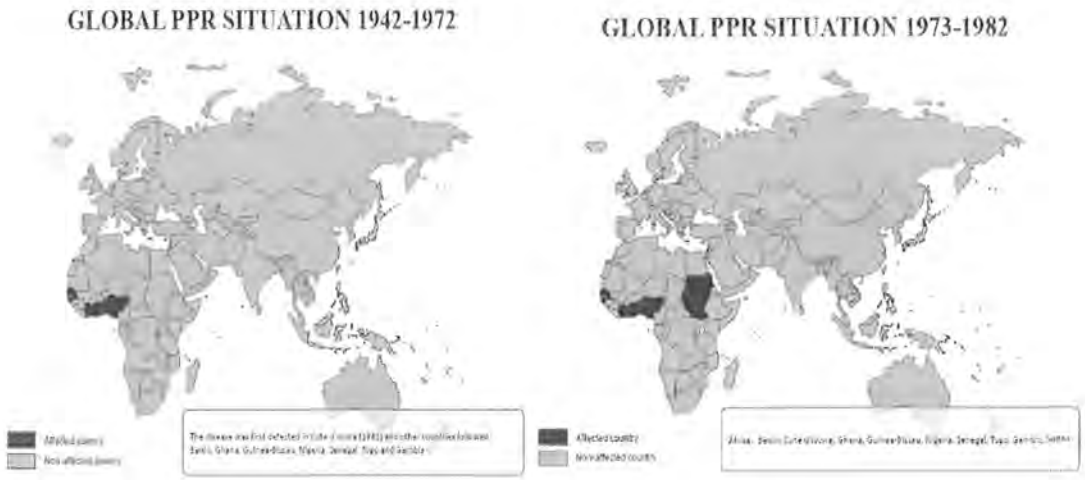
PPRV infection was also detected in the countries of North Africa. Especially in Egypt the cases of this disease was seen in 1987 and in 1990. Recently, a surge of the PPRV cases happened in 2006 in Aswan province of the country. This outbreak showed the potential of diseased goats to remain asymptomatic for a handsome period. After this period severe clinical disease was developed in these animals. Except Egypt whole countries of North Africa were considered PPR free but outstanding outbreak of PPRV infection in Morocco in 2008 negated this concept (El-Hakim, 2006).

The status of PPRV was analyzed in Saudi Arabia in 2000. This analysis suggested that the disease was not endemic in the country (Al-Naeem et al., 2000). But in April 2002 an outbreak of infection in goats and sheep with 100% mortality rate was taken place in the country. Camelsin was considered responsible for the dissemination of virus in goats. Seroprevalence of both goats and sheep was also reported in Jordan and in Lebanon. PPRV related to lineage IV has been identified in

a game reserve in United Arab Emirates. But in Qatar viruses related to both lineages III and IV were isolated from goats in 2010 (Al-Dubaib, 2008, 2009; Elzein et al., 2004).

The cases of PPRV in Pakistan have been reported since 1991. Early occurrence of disease was seen in Punjab region. This virus was characterized by applying PCR in 1994. Later on, continual increase is observed in virus dissemination as reported in different cases of the disease. Serum samples from normal animals in a goat flock were seropositive in different studies in 2005, 2008 and in 2009. Current virus present in Pakistan is related to lineage IV. (Abubakar et al., 2008; Mehmood et al., 2009).

The latest studies about PPRV in India have shown that virus is belonged to lineage IV. The virus is reported to exist across India with recent identification of PPRV in Rajasthan was reported (Mehmood et al., 2009). The existence of this virus is poorly characterized in Iraq, Iran and Afghanistan. The disease was first time reported in Iraq in 2000 where high morbidity with low mortality was observed (Barhoom et al., 2000).



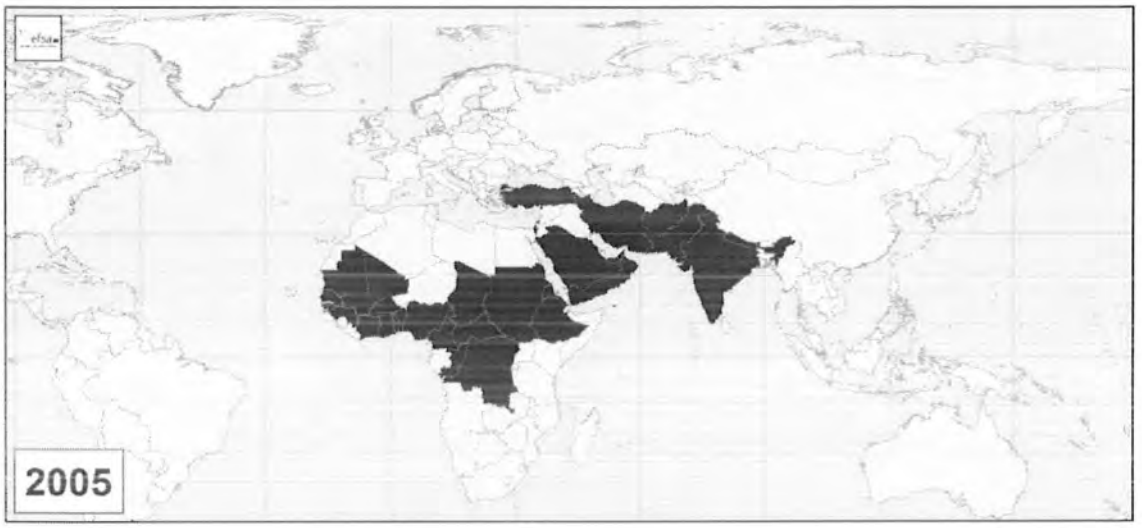
A B

GLOBAL PPR SITUATION 1983-1987

C

GLOBAL PPR SITUATION 1988-1995

D



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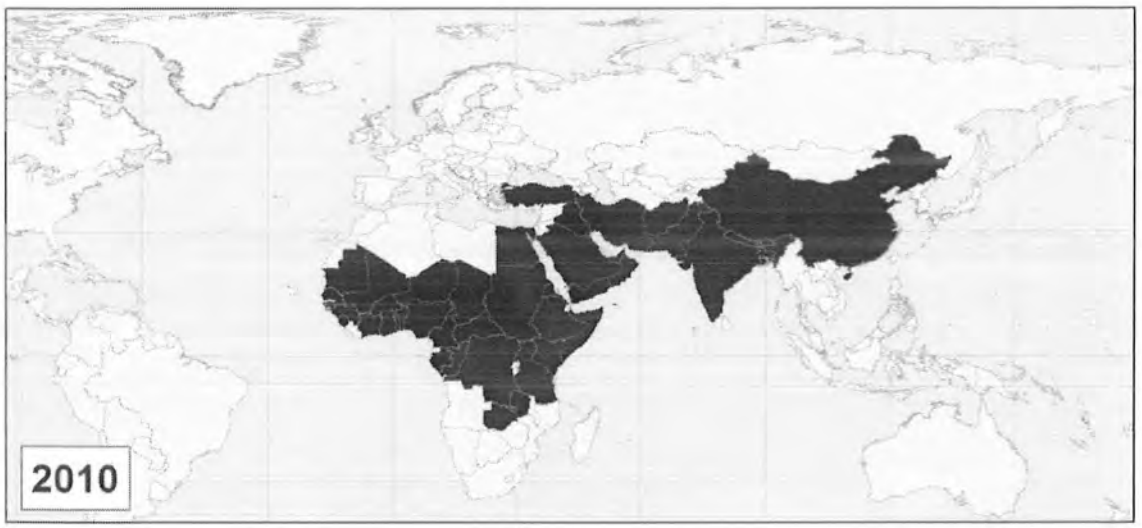

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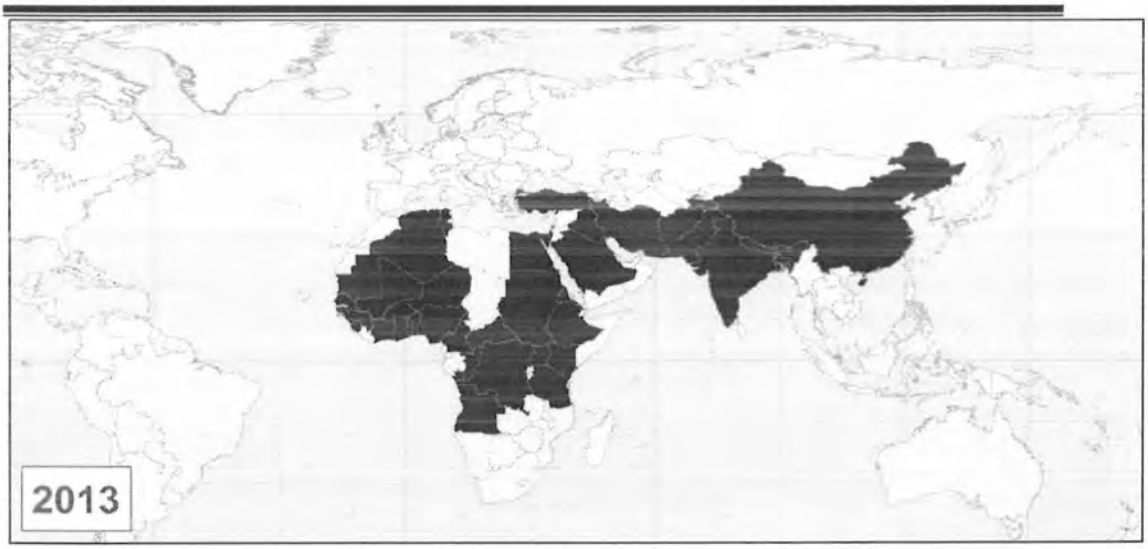

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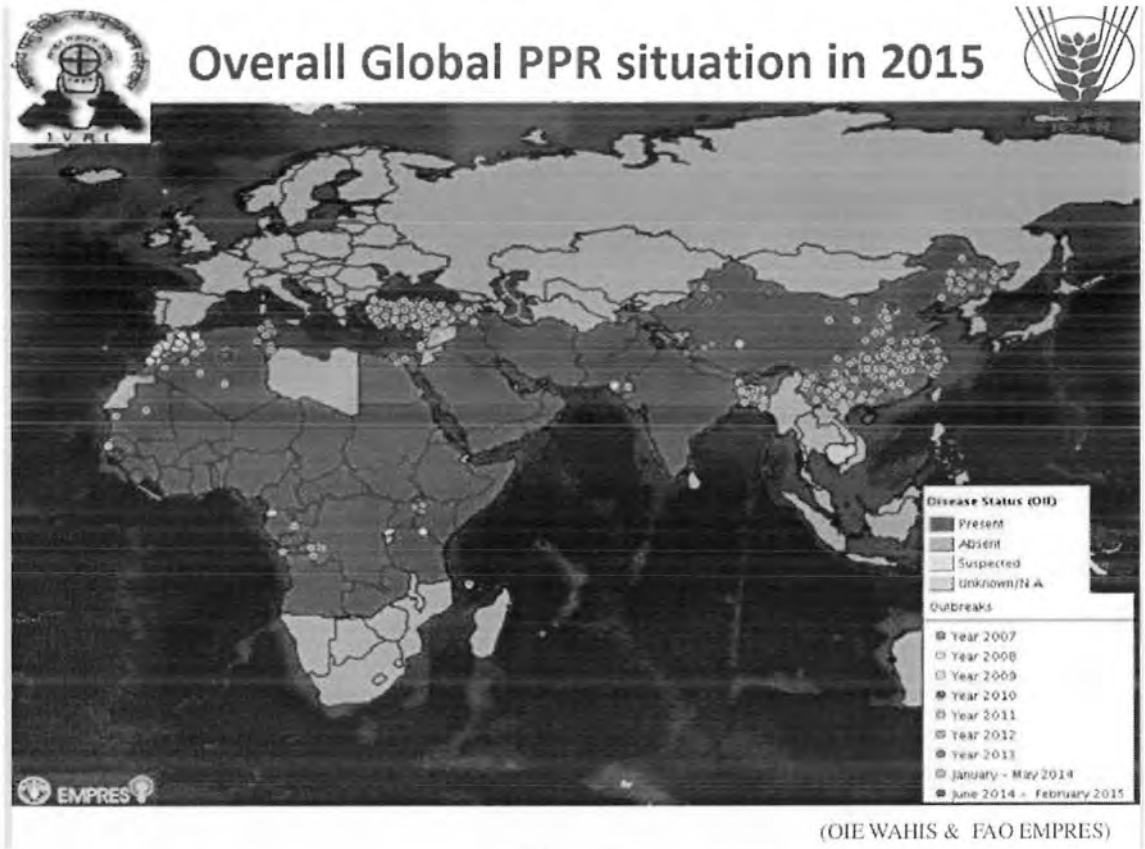


H

Figure: 2.4: Steps on PPR geographical distribution

A: Global PPRV distribution 1942-1972, **B:** Global PPRV distribution 1973-1982

C: Global PPRV distribution 1983-1987, **D:** Global PPRV distribution 1988-1995

E: Global PPRV distribution 2005, **F:** Global PPRV distribution 2010

G: Global PPRV distribution 2013, **H:** Global PPRV distribution 2015

The PPRV can be classified geographically into four different lineages. The West Africa have Lineage I and II, while the lineage III is present in East Africa. The lineage IV is found in Middle East and South East Asia including Pakistan (Albina et al., 2013). Following figure represent the distribution lineages of PPRV.

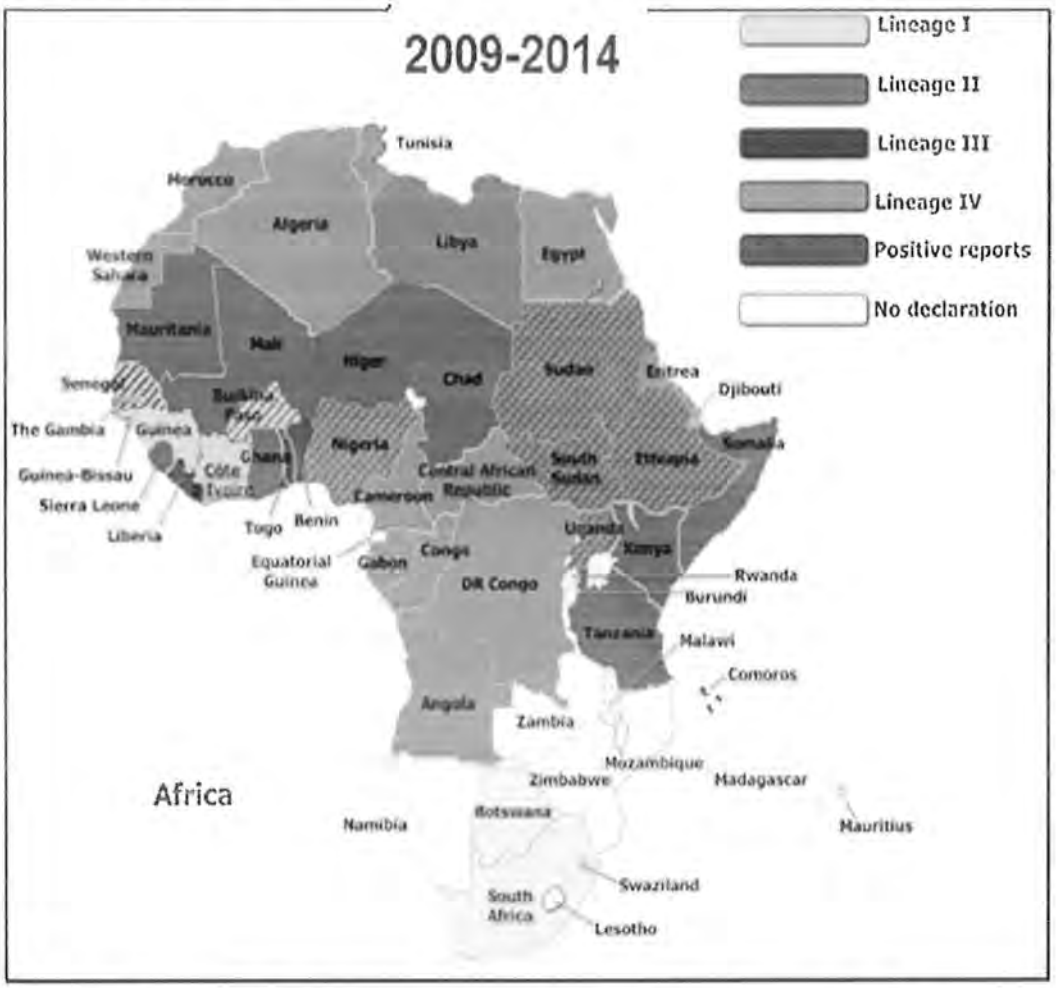


Figure 2.5: Distribution of different lineages of PPRV. Lineage IV is the most abundant all over the country, majorly cover area in the Asia. Lineage III is less prevalent in Asia, lineage I & II are prevalent just Africa.

2.6. Epidemiology

2.6.1. Transmission

As it is known PPR is a contagious disease, so mostly outbreaks of it caused by close contact from the diseased to healthy animals. Major route of transmissions are nasal discharge from air, through grazing on contaminated soil. Diseased animals spread virus in air, in soil through oral and nasal secretions, and excretions (faeces, urine and semen). PPR virus can't for a longer time in the environment outside its

host, almost 10 days is the maximum survival time. PPR can transmit from mother to young ones through milk feeding although exact survival period of PPRV is not known. Although survived animals from PPR virus infection acquire strong immunity but they become reservoir and shed virus for a long time (almost 11 weeks) (Ezeibe et al., 2008). Transmission through inanimate objects have less possibility because PPRV is a fragile entity in the hot and dry environment (Taylor et al., 2007). Virus excretion was reporetd 2 days after the onset of infection. Mostly outbreaks of PPR are occure due to introduction of new animals, animal movements, in the livestock markets, communal grazing and in stress condition.

2.6.2. Host Range

Sheep and goat are the principal host of PPR. Virus, but with some reports of disease in camelids (Khalafalla et al., 2010; Kwiatek et al., 2011; Roger et al., 2001; Saeed et al., 2010). PPRV can also cause disease in wild ruminants. While large ruminants such as cow and buffalo doesn't show any disease picture and also can't be able to shed virus particles so they are not playing any epidemiological role (Anderson and McKay, 1994; Lembo et al., 2013; Sen et al., 2014). PPRV cause more lethal disease in goats than sheep. Sheep comparatively resist this virus any clinical picture is less critical (Lefevre, 1980; Singh et al., 2004a; Tripathi et al., 1996; Wosu, 1994). It is also reported that lineage present in Asia have more susceptibility to goat than sheep (Balamurugan et al., 2012a). PPRV infection was reported in 3 ungulate-families i.e. Gazellinae, Caprinae, and Hippotraginae (Fentahun and Woldie, 2012; Munir et al., 2013). In UAE PPRV infection was investigated in *Capra ibex*, *Gazelladorcas*, *Ovisorientalislaristenica*, *Oryxgazella* and *Bosephalustragocamelus*(Furley et al., 1987). This disease also present in *Sylvicarpagrimmia* (Ogunsanmi et al., 2003). Disease was also reported in *Gazellathomsoni* and Dorcas Gazelle In Saudi Arab (Elzein et al., 2004). *Ovis orinetalis*and (*Capra aegagrusblythi*) also susceptible to PPRV infection repoted in Pakistan (OIE, 2000; (Abubakar et al., 2011)

PPRV be the reason of huge mortality in camel (El-Hakim, 2006; Roger et al., 2001) and it is also strange to find disease in felids in a report (Balamurugan et al., 2012a), but its further validation and confirmation is required.

Following table show PPRV infection in the wild animal reported by different researchers

Table 2.1: Reported infection of wildlife species by Peste des petits ruminants' virus.

Common name	Zoological Name	Diagnostic method	Country	Reference
Afghan Markhor goat	*Capra falconeri*	Clinical, serological and molecular examination	United Arab Emirates	Kinne *et al.* (2010)
African grey duiker	*Sylvicapra grimma*	Serological examination	Nigeria	Ogunsanmi *et al.* (2003)
Arabian gazelle	*Gazella gazella*	Clinical, serological and molecular examination	United Arab Emirates	Kinne *et al.* (2010)
Arabian mountain gazelle	*Gazella gazella cora*	Clinical, serological and molecular examination	United Arab Emirates	Kinne *et al.* (2010)
Arabian oryx	*Oryx leukoryx*	Serological examination	Saudi Arabia United Arab Emirates	Frolich *et al.* (2005)
Barbary sheep	*Ammotragus lervia*	Clinical, serological and molecular examination	United Arab Emirates	Kinne *et al.* (2010)
Bharal	*Pseudois nayaur*	Clinical, serological and molecular examination	China	Bao *et al.* (2011)
Bubal hartebeests	*Alcelaphus buselaphus*	Serological examination	Ivory Coast	Couacy-Hymann *et al.* (2005)
Buffalo	*Syncerus caffer*	Serological examination	Ivory Coast	Couacy-Hymann *et al.* (2005)
Bushbuck	*Tragelaphus scriptus Kinne*	Clinical, serological and molecular examination	United Arab Emirates	Kinne *et al.* (2010)
Defassa waterbuck	*Kobus defassa*	Serological examination	Ivory Coast	Couacy-Hymann *et al.* (2005)
Dorcas gazelle	*Gazella dorcas*	Clinical examination	United Arab Emirates	Furley *et al.* (1987)
Gemsbok	*Oryx gazella*	Clinical examination	United Arab Emirates	Furley *et al.* (1987)
Impala	*Aepyceros melampus*	Clinical, serological and molecular examination	United Arab Emirates	Kinne *et al.* (2010)
Kob	*Kobus kob*	Serological examination	Ivory Coast	Couacy-Hymann *et al.* (2005)
Laristan sheep	*Ovis gmelini*	Clinical examination	United Arab Emirates	Furley *et al.* (1987)
Nubian ibex	*Capra nubiana*	Clinical examination	United Arab Emirates	Furley *et al.* (1987)
Persian gazelle	*Gazella subgutturosa*	Serological examination	Turkey	Gur and Albayrak (2010)
Rheem gazelle	*Gazella subguttorosa marica*	Clinical, serological and molecular examination	United Arab Emirates	Kinne *et al.* (2010)
Sindh ibex	*Capra aegagrus blythi*	Clinical and serological examination	Pakistan	Abubakar *et al.* (2011)
Springbuck	*Antidorcas marsupialis*	Clinical, serological and molecular examination	United Arab Emirates	Kinne *et al.* (2010)
Thompson's gazelle	*Eudorcas thomsonii*	Clinical examination	Saudi Arabia	Abu Elzein *et al.* (2004)
White-tailed deer	*Odocoileus virginianus*	Clinical examination	United States of America	Hamdy and Dardiri (1976)
Wild goat	*Capra aegagrus*	Clinical and serological examination	Kurdistan	Hoffmann *et al.* (2012)

2.6.3. Pattern of Disease

Along with species, breed of animal has also influence on the sequel of the PPRV infection and disease occurrence. This influence is also variable among breeds. (Lefevre and Diallo, 1990a). The sheep and goats resident in coastal area of West Africa have high inborn immunity and suffered from subclinical infections. In contrast, sheep and goats resident in south of coast and nomadic animals in the Middle East have less innate immunity (Munir et al., 2013). No data is available regarding comparison of breeds for PPRV vulnerability in India except a study at small level conducted by (Pawar et al., 2008). Case Fatality of 65% was seen in Barbari-Black Bengal goats. In recent past a devastating outbreak of PPR among Tellichry breed of goats was reported with 100% case fatality rate in kids and above 85% mortality among adults. Some breeds like Salem and Kanni did not show disease at all. (Majumder, 1997; Narayanan et al., 2008).

Relationship between age of animal and severity of disease has also been reported by (Obi and Patrick, 1984)and (Taylor, 1984). According to these investigators, young animals of less than 1 year age are more prone to disease than mature animals. However, the frequency of occurrence of disease is elevated in older goats. (Gulyaz and Ozkul, 2005; Toplu, 2004). In Africa especially in Nigeria the disease was observed to occur in sporadic intervals of 3-5 years (Bourdin, 1973).

2.6.4. Environmental Influence

PPR disease is endemic in most of the areas, but its cases rapidly increased at the end of autumn and start of the winter-season (Dec and Jan) perhaps it's the season of kidding and lambing, feed shortage and so stressed animals more prone to disease(Zahur et al., 2008). But season mostly play role due to imposing stress in extreme conditions. That's why in areas of Sindh Pakistan PPR is common during summer. Although animals movements has also greater influence on disease occurrence. Similarly in the extreme hot weather PPR is common in some Indian areas. (Singh et al., 2004a). In the winter and rainy season PPR cases increased in Nigeria (Opasina and Putt, 1985). All in conclusion it shows that season not only the risk to increase disease occurrence but mainly stress due to any reason like nutritional-deficiencies, housing capacity, animal movements, ignorance of quarantine system (Taylor and Barrett, 2008).

2.7. Pathology

2.7.1. Pathogenesis

Pathogenesis of PPRV is mainly concluded from the rest of the morbilliviruses mechanism, So PPRV pathogenesis is not clear. Most of the morbilliviruses after entry are taken up by antigen presenting cells (APCs) in the respiratory epithelial mucosa. After this virus move to the initial replication site that is nearly located lymphoid tissue of the respiratory system. Probably this is the reason of immunosuppression caused by PPRV and other morbilliviruses. (Kumar et al., 2014). After replication vires moves to circulatory (Blood & lymphatic) system via infected lymphocytes. (Osunkoya et al., 1989; Pope et al., 2013). PPRV replicate in lymph nodes and epithelial cells that's why it causes conjunctivitis, stomatitis, rhinotracheitis, gastroenteritis and pneumonia. Through circulatory system virus get access to other body organs. Respiratory and gastrointestinal system is mostly effected, so when viral load is high pneumonic signs are visible. In the GIT PPRV causes substantial necrosis of payer patches. PPRV also causes splenomegaly. This in turn cause leucopenia, hence animal immunity depressed and prone to secondary infections. ((Kul et al., 2007; Kumar et al., 2004)Incubation periods of PPRV is about 3-5 days but it mainly depends upon the animal immune status, infectious dose, virulence of virus, route of entry. Pyrexia usually develops 3-7 days after infection. After pyrexia other clinical signs like erosive stomatitis, occulo-nasal discharge, diarrhea etc. appear. In the case of more virulent strain of PPRV and stressed animal death may occur due to pulmonary congestion. Hence Prognosis of PPR depends upon animal and virus strain (Olaleye et al., 1989). Histopathology of PPRV is also similar to other morbilliviruses. Characteristic syncytia is formed in lymph nodes, in respiratory and GIT epithelium. After syncytia apoptosis of these cell occur (Pope et al., 2013). Necrosis of GIT lymph nodes causes diarrhea. No nervous signs is reported in PPR natural infection but other morbilliviruses show CNS complications (Galbraith et al., 2002).

2.7.2. Clinical Picture

Clinical picture of the PPR mainly depends on the animal condition, and virulency of PPRV. That's why PPR can be in per-acute, acute, sub-acute or sub-clinical form. Normally in the adult sheep and goat, PPR is in acute form but in lambs, kids (less

than 4 Months) or above 4 month of age, having very less immunity suffer per-acute form of it.

In per-acute stage of PPR rapid onset of fever (104°F-108°F, anorexia, depression, hemorrhages on mucous membranes, occulo-nasal secretions, difficult breathing, severe diarrhea leading to dehydration and ultimately death of the animal occur within four to five days. In per-acute stage incubation period is only two days. (Munir et al., 2013)

During acute form of PPR incubation period is bitter longer ranges from three to four days which leads to fever, and other PPR specific clinical signs like occulo-nasal discharge, congestion of mucous lining of upper GIT, conjunctivitis, and vulvovaginitis. This precedes to the watery diarrhea, and later on bloody diarrhea (Abubakar et al., 2008). Dehydration and at the end death in untreated animals. In the acute form of disease animal show following general signs sequentially

- Incubation period ranges from two to seven days, fever appear in three to ten days from 103°F-106°F.
- After two to three days of pyrexia congestion of conjunctiva and oral mucosa occur.
- Occulo-nasal secretions start after four to seven days of infection and remain for two to four days, with time these serious-secretion turned mucopurulent, and later these congestions appear as lesions in oral cavity.
- Sometimes in severe conditions these lesions are visible as ulcer in five to nine days after infection. Caseous-material also appear around tongue and soft palate.
- Diarrhoea starts after four to ten days of infection, emaciated, weight-loss, dehydration and death.
- But in mild cases animal recover itself after ten to fifteen days of infection.

Sub-acute is mild form of disease, no prominent clinical-signs, and very low mortality observed. In this animal may suffer from fever (102-104) but no other clinical signs develop and mostly animal recover within ten to fourteen days. Sometimes sub-clinical disease also develop in cattle and buffaloes but no clinical signs and virus

clear completely just strong immune response in the form of antibodies against PPRV. (Couacy-Hymann et al., 2007)

2.7.3. Post Mortem Findings

Two systems (GIT & Respiratory) are affected by PPRV, so pathological lesions observed in these organs (Taylor and Barrett, 2008). On external examination PPRV infected carcass is apparently dehydrated, emaciated, watery diarrhea around perineal region is visible. Mucopurulent discharges may also present around eyes, nasal and oral cavity area. Inside the buccal cavity cheesy material, along with severe hemorrhages on mucosal lining is observed. Widespread congestion in the large intestine (caecum, colon and rectum) that is visible as Zebra striping. On the ileocecal valve hemorrhages are seen and in severe conditions edematous and ulcerative mucosa in throughout the GIT (Munir et al., 2013).

In the PPR infected carcass hyperemic upper-respiratory tract is seen along with corrosions. In the lower part of the respiratory tract (caudal-trachea and bronchi) foamy material may also aggregate (Emikpe et al., 2013). In the lungs pneumonic condition mostly observed, congestion and edematous with various degree of consolidations. Lungs coloration change from red to purple, and hardness can be felt by touch (Brown et al., 1991).

2.7.4. Immunity

2.7.4.1. Innate Immune Response

In case of PPRV infection classical inflammatory response activate i.e. increased release of cytokines including variety of interferons and interleukins. Although generally morbilliviruses stop the production of interferon. (Atmaca and Kul, 2012; Baron et al., 2014), while in some researches it is observed that V protein of morbillivirus interact with interferons signaling (Caignard et al., 2009; Takeuchi et al., 2003), and block interferon I and II in a different way.

On the other hand a few information regarding PPRV, how it trigger innate-immune response. For this it is required to characterize PRRs after PPRV-infection or vaccination. These PRRs are considering play important role in the activation of cell-mediated immunity that is involved in the PPRV specific antibodies production.

2.7.4.2. Adaptive Immune Response

Generally morbillivirus infection produce immense immune response that leads to immuno-suppression. Neuraminidase, hemagglutinin and fusion proteins of morbilliviruses are responsible for the production of humoral and cell mediated immune response (Naik et al., 1997; Sinnathamby et al., 2001a). CD4 & CD8 T cell response and virus-specific antibodies are observed after exposure to Morbilliviruses which play an important role in the clearance of virus and defense from re-infection (Griffin, 1995). F and H of PPR virus and RP virus causes an abrupt defensive, neutralizing-antibody response (Munir et al., 2013; Sinnathamby et al., 2001b). Furthermore, cell mediated immunity also plays an important role in protection. Similarly, MV and RPV infection generate N- specific T cells which are a main part of virus specific memory cells (Griffin, 1995; Hickman et al., 1997; Ohishi et al., 1999). PPRV induces a lethal infection in goats because a great reduction of CD4 T cells is noticed in below than 4 months old kids rather than immuned (Herbert et al., 2014). Furthermore, after 7-days, a slight percentage increase of CD8 T-cells in both vaccinated and naïve animals suggest induction of CTL response by PPR virus affected animals (Herbert et al., 2014).

Cell-mediated immunity is also induced by attenuated MV which is suggested to be important for natural defense (Lund et al., 2000). However, it is not evident which immune effectors are responsible for protection following PPRV vaccination: cytotoxic T cell, neutralizing antibodies or mucosal immunity. Conversely, passive transfer of immunity by colostrum are most likely to be involved in protection (Ata et al., 1989; Gans and Maldonado, 2013). Although further testing is required, however a link of life-long immunological memory following PPRV infection exists.

Newborn goats have detectable levels of passively acquired maternal antibodies before six month age. These antibodies level decreases from the 3rd month, that's why newborn kids and lambs should be vaccinated after 3 month of age (Ata et al., 1989; Balamurugan et al., 2012a).

2.8. Diagnosis

2.8.1. In field diagnosis

PPR normally diagnosed on the bases of its specific clinical signs and symptoms. But these clinical signs confuses with other diseases like RP, sheep and goat pox, CCPP, CPD, and many others. Although these clinical signs narrow down its diagnosis but for the confirmation and characterization of virus laboratory diagnosis is compulsory.

2.8.2. PPR Laboratory Diagnosis

PPR diagnosis on the basis of clinical sign and symptoms, necropsy and epidemiology is not definite. Different serological techniques like indirect ELISA, CIE and AGID have been used for diagnosis of this disease but differentiation of PPR from other related diseases is not possible (Balamurugan et al., 2012a; Liermann et al., 1998). Molecular based and Cell culture techniques (virus isolation) can be used for sensitive and specific detection of PPRV. Although cell culture techniques are laborious and time consuming while molecular techniques like RT-PCR, real time PCR, LAMP (Cartee et al., 2003) are not only sensitive and specific for PPR diagnosis but are also very rapid techniques. Along with virus isolation and antibody detection, nucleic acid sequencing is also used for PPRV detection (Diallo et al., 1995). However, molecular based techniques are more reliable and help in definitive diagnosis of PPRV.

2.8.2.1. Virus Isolation Methods (Cell culture Technique)

It is considered as gold standard for PPR diagnosis. Virus isolation can be achieved by using primary sheep and bovine cells (Scheid and Choppin, 1974). Additionally, cell lines like Vero and B95a are now mostly used for PPRV isolation (Diallo et al., 2007). Cytopathic effect (CPE) produces during the PPRV infection i.e structural change of infected cells (round in shape), vacuolation, cell cytoplasm granulation and then monolayer cells fusion and syncytia formation. Due to failure of virus separation at first passage, many blind passages are required. To overcome this problem, cell line derivatives with receptors for morbillivirus have been introduced which are capable of virus isolation within a week without need of blind passages e.g. CV1 with goat SLAM (Adombi et al., 2011) and Vero cell line with dog SLAM. Virus isolation technique cannot be used routinely for diagnosis because it is time consuming but still

it is very effective for isolation of virus from different field samples which are then further studied by molecular based methods.

2.8.2.2. Antigen detection methods

2.8.2.2.1. Virus neutralization (VNT)

VNT is the serological test used for detection of antibodies specific for PPR. Primary cells (Lamb kidney cells) and cell line (Vero), both can be used to perform this test. This test can be used to differentiate PPR virus from Rinderpest (RP) (Horvath and Lamb, 1992; Malur et al., 2002) with the help of either simply tubes or microtitre plate (96 well) i.e. micro-VNT (Malur et al., 2002). With the help of this test, PPRV is differentiable from RP, CDV and MV (Rapaport and Shai, 1994). Although this test is beneficial due to its sensitivity and specificity but it is also cumbersome and slow, thus time consuming as well (Taylor and Abegunde, 1979).

2.8.2.2.2. Hemagglutination Assay (HA)

HA is an effective, non- expensive, easy and rapid test for PPRV diagnosis and does not require sophisticated laboratory equipment (Elzein et al., 2004). First time Wosu in 1985 demonstrated about the hemagglutination activity of PPRV. PPRV has the ability of causing agglutination of RBCs of chicken, pig, Goat and even Human (Group O) (Elzein et al., 2004). HA test is especially used for PPR diagnosis from live animals. In this test, agglutination time is observed that is time required by RBCs for settling down in wells while there is no agglutination in wells with positive samples. However, in some cases elution starts after agglutination, thus reading of HA test should be taken within certain period of time i.e. in case of PPRV, reading must be done within one hr. However, this test is not very sensitive and some studies also reported that false positive results have been obtained by using HA test.

2.8.2.2.3. Agar gel immunodiffusion (AGID)

AGID is the cheap and simple test capable of being performed under both laboratory and field conditions (Abraham et al., 2005; Obi and Patrick, 1984). This test can be used not only for the detection of antibodies but also antigens as well (Obi and Patrick, 1984). This test has many limitations like, this test is unable to differentiate PPRV from RP although its specificity is about 92% and it is not helpful in detection of mild PPR due to its low sensitivity.

2.8.2.2.4. Counter immunoelectrophoresis (CIEP)

CIEP is rapid as well as reliable test for PPR diagnosis by detecting both antibodies and antigen. When results obtained by AGID were compared with CIE, it was clear that CIE (80.3 %) was more effective and sensitive than AGID (42.6 %) for PPRV detection (Obi and Patrick, 1984). It is operated simply by using electrophoresis bath, attached with the source of high voltage.

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

This test was first time developed in CIRAD-EMVT (World Reference Laboratory in France) and then achieved great acceptance globally for PPRV (antigen) detection. In this test biotinylated monoclonal antibody (N) for PPRV epitope is used for capturing of PPRV antigen.

MAPs (multiple antigenic peptides) were also developed for identification of antibodies against PPRV. MAPs that are highly specific for polypeptides of PPRV are used in this test which give high titer in ELISA. icELISA with the use of two MAbs against N protein facilitate fast recognition of PPRV (Libeau et al., 1995).

2.8.2.3. Antibodies detection methods

2.8.2.3.1. Hemagglutination Inhibition (HI)

Antibodies against PPRV present in serum samples can be quantitatively measured with the help of this test. Serum samples are diluted twofold in microtitre plate. Serum sample titre can be identified by the antibodies dilution requires for inhibition of agglutination. Thus, with the help of HAI and HA tests, titration of PPRV antigen can also be achieved (Wosu and Ezeibe, 1991).

2.8.2.3.2. Competitive ELISA (c-ELISA)

Because of inability of indirect ELISA in differentiation of PPRV from RPV, new alternatives were searched (Anderson et al., 1982). Then competitive ELISA was developed, in which MAbs for H protein of PPRV were used to produce differentiation between PPRV and RPV (Anderson et al., 1991).This test is based on the competition between monoclonal antibodies and antibodies of test serum. c-ELISA based on specific MAbs against H protein (Anderson et al., 1991; Saliki et al., 1993; Singh et al., 2004b) and N protein (Libeau et al., 1995) was done for PPRV

identification. This test proved to be more sensitive and specific when compared with VNT (Singh et al., 2004b)

2.8.2.4. Molecular diagnostic techniques

2.8.2.4.1. RT-PCR

It is considered as an effective and sensitive method due to its ability of detection of minute amount of DNA or RNA even in samples with low virus titer. This test has high sensitivity than ELISA technique (Forsyth and Barrett, 1995). Due to its rapidity and specificity, it is highly acceptable for PPRV detection (Nanda et al., 1996). For identification and differentiation of PPTV from RPV, RT-PCR based on two proteins of PPRV was mostly performed i.e Phospho (P) and Fusion (F). Now N (Nucleoprotein) protein based RT-PCR has also been developed for PPR diagnosis .Sensitivity of RT-PCR is 1000 times more than virus isolation technique (Couacy-Hymann et al., 2002).With the help of RT-PCR, PPRV can be diagnosed by using ocular samples before the appearance of its symptoms. Products obtained from PCR can further be utilized in sequencing of nucleic acid so that relationship can be established between isolates and geographical locations (Shaila et al., 1996). Different types of PCR like multiplex (George et al., 2005) and qRT-PCR (e.g. (Bao et al., 2008; Batten et al., 2011; Kwiatek et al., 2011) were further proved to be more effective for PPRV detection due to their higher specificity and sensitivity than conventional PCR. LAMP also appeared as a useful method for PPRV diagnosis with similar sensitivity when compared with real time PCR (Li et al., 2010).

2.8.2.4.2. Real Time Quantitative RT-PCR (qRT-PCR)

RT-PCR is considered as a gold standard test for PPRV detection but it is tedious, time consuming and labour extensive assay. That's why many researchers develop quantitative RT-PCR, although it is comparatively expensive but gives quick and quantitative results.

Quantitative RT-PCR/ Real time RT-PCR assay is more sensitive, specific than RT-PCR (conventional). It is more advantageous assay than others like quick result, contamination chances decrease, less time consuming and is more specific (Agüero et al., 2007; Shaw et al., 2007). In real time RT-PCR number of steps decreases to reach

final conclusion, results as amplification occur are visible on screen, no need to load on agarose gel and later steps as in conventional RT-PCR. (Bao et al., 2008)

1st time Bao et al in 2008 develop, a rapid and specific TaqMan-based, one-step real-time quantitative reverse transcription PCR (qRT-PCR) assay for the detection of PPRV. He used N-gene sequence based primers and probe for detection. This assay was able to detect almost all type of lineages of PPRV. This assay was has high specificity and along with it much more sensitive than conventional RT-PCR. (Bao et al. 2008). Similarly one other study was carried out for qRT-PCR by Batten et al in 2011 for rapid detection of PPRV (Batten et al., 2011).

Balamurugan optamize SYBR green qRT-PCR assay using M-Gene sequence for PPR detection in 2011. This assay is rapid, sensitive and less expensive as compared to the probe based assay develop by Bao and Batten. He also compared it with conventional RT-PCR and TaqMan Rt-PCR. He claimed that this assay is more sensitive than TaqMan RT-PCR and conventional RT-PCR. This is a good alternative to TaqMan qRT-PCR and conventional RT-PCR for early detection of PPRV (Balamurugan et al., 2012b). Later on Abera et al in 2014 develop SYBR green based RT-PCR using N-gene sequence for the detection of PPRV. In this assay N-gene was used because it is most expressed gene in PPRV. This was two step assay but it is more sensitive, specific and rapid for the finding the presence of PPRV (Abera et al., 2014).

2.8.2.4.3. Nucleic acid hybridization

This test also proved to be suitable for PPRV diagnosing from field materials as well. cDNA probes (radiolabeled) were developed by using N gene of PPRV (Diallo et al., 1995; Muthuchelvan et al., 2006). This test has the advantage of having ability to differentiate between PPRV and RP without requirement of virus isolation technique. cDNA probes that target F,M and P gene are not very specific (Muthuchelvan et al., 2006). Furthermore, probes cannot be used widely due to their small half-life and sensitivity of this test is less as compared to PCR.

2.8.2.5. Penside tests

A dot-ELISA test by utilizing MAbs for N (Singh et al., 2009) and M (Obi and Ojeh, 1989) can be used as a Penside test for PPRV identification. It facilitates in screening of greater number of samples suspected for PPRV and capable of performing both in field as well as in laboratory. Its specificity and sensitivity is greater than s-ELISA (Singh et al., 2009). Lateral flow test can also be used for PPR diagnosis but not in routine use up till now. But it is advantageous of being fast, simple and easy thus can be used as penside test for diagnosis. Dipsticks by utilizing MAb for M protein have also been developed. Furthermore, c-ELISA for rapid diagnosis of PPRV was developed (Choi et al., 2005). Immunofiltration ELISA also has high specificity and sensitivity, thus can be used as screening test for PPRV (Raj et al., 2008).

Now a days, polyclonal antibodies have been produced against M protein (recombinant) which showed high specificity in immunofluorescence and western blot. Thus are much better and safe, so, can be utilized for surveillance and diagnosis of PPRV in both non-enzootic and enzootic regions throughout the world (Liu et al., 2013).

CHAPTER 03

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Sample Collection

Samples were collected from fifteen suspected field outbreaks of PPR in different regions of the country reported by a focal person in each region during 2013 to 2015. The samples form outbreaks occurring in 2013 and 2014 were obtained from NARC Animal Health repository. However, PPR suspected outbreaks occurring during Nov, 2014 to 2015 were inspected and clinical examination of effected animals and post mortem examination of dead animals was conducted. Appropriate samples including ocular, oral, and nasal swabs and tissue samples from organs like spleen, lungs and lymph nodes (mediastinal, retropharyngeal and mesenteric) were taken during post mortem examination.

3.1.1. Swab Samples

In case of live animals, swabs were collected from ocular, nasal and oral cavity. The cotton swabs (Sure Care Corporation) were inserted and rubbed in circular fashion deep into the mouth and nostrils. Ocular swabs were collected by gentle rubbing of sterile swab just beneath conjunctiva. Marking of all swab samples were done with specific numbers, date and area. These swabs were transported to lab in ice packs by dipping in 0.5 ml PBS solution. A total of 20 swab samples were collected from different suspected outbreaks.

3.1.2. Tissue Samples

In case of dead animals, postmortem was done and tissue samples were collected from lungs, spleen, mesenteric and bronchial lymph nodes according to pathognomonic lesions. These tissue samples were also tagged with date, number and place of outbreak. Tissue samples were placed in container with ice packs to avoid post-necropsy changes during transportation. In the lab these samples were stored at -20 °C until processing. Total of 26 different tissue samples were collected from suspected outbreaks.

3.2. Sample Processing

After collection samples were processed for further laboratory analysis.

3.2.1. Swabs Samples

Cotton bud from swab stick was removed with the help of sterilized scissor and forceps and was placed in PBS with pH 7.4 in Eppendorf tube. Approximately 10% (w/v) suspension was prepared by squeezing out all material from cotton bud.

Suspension was mixed thoroughly with the help of vortex for 1 minute and then it was centrifuged at 12,000 rpm for 5 minutes. Debris and cotton bud settled down, supernatant was collected and stored at -70 °C for further experimentation.

3.2.2. Tissue Samples

Tissue samples were also processed in same way. One gram of representative part of tissue sample was dissected, crushed into small pieces and minced with the help of pestle and mortar. Approximately 15% (w/v) homogenized mixture of tissue sample was prepared in PBS. It was centrifuged at 12,000 rpm for 5 min. Debris settled down and supernatant was collected and stored at temperature of -70 °C until its further use.

3.3. Peste des Petits Ruminants (PPR) Outbreak Confirmation

3.3.1. Hemagglutination Assay

Although it is not a specific test for PPRV diagnosis, it is performed for earlier detection of PPRV.

3.3.1.1. Preparation of washed chicken red blood cells

Washed chicken RBCs solution was prepared by following four steps.

1. Blood collection
2. Washing of RBCs
3. Calculating packed cell volume (PCV)
4. Dilution of RBCs as per required working solution.

3.3.1.1.1. Collection of the blood:

Blood was collected from the wing vein of chicken, transferred in heparinized vacutainer, and was mixed gently

3.3.1.1.2. Washing of red blood cells:

Heparinized blood was transferred to the falcon tube and centrifuged at 2000 rpm for 10 min. After discarding serum with the help of pipette, PBS was added in equal volume as remaining blood cells in the tube for washing. Then again it was centrifuged at 2000 rpm for 10 minutes. Washing step was repeated twice. (Grimes, 2002)

3.3.1.1.3. Measuring the packed cell volume

After washing thrice RBCs pellet was obtained. Volume of this pellet was measured for dilution.

3.3.1.1.4. RBCs Dilution

- To make 25 % solution of RBCs, 3 parts of PBS/ N.S was added to 1 part of RBCs.
- Then 1% dilution was made by adding 24 parts PBS/ N.S in just 1 part of 25% RBCs solution.
- Then 0.5 % dilution was prepared by adding 50 parts of PBS/ N.S in 1 part of 25% RBCs solution.

3.3.1.2. Assay protocol

A 96 wells round bottom plate was taken. 25ul of PBS was added in all wells. 6 samples were loaded in 1st column from A-F. 25ul of each sample was added in 1st well and then it was diluted from 1st to 12th well and after 12th well, 25ul was discarded. In the plate 6 samples were loaded in 1st 6 rows and 7, 8 rows were controls. After that, 25ul of 1% RBCs were added in each well. Plate was covered and incubated at 4^0C temperature for 45 min. After this results were noted (Wosu, 1985).

3.3.2. RT-PCR

Field outbreaks samples were analyzed with single-step RT-PCR using already published primers (NP3 & NP4) (Couacy-Hymann et al., 2002).

3.3.2.1. RNA Extraction Procedure

For RNA extraction, 200 µl quantity of already processed swab or tissue sample was used. Extraction was done with the help of Invitrogen RNeasy kit. RNA extraction was done as described by the product use protocol. First of all, mixture of carrier RNA and lysis buffer was prepared according to the following mentioned formula,(Carrier RNA solution was prepared by adding 310 µl of Nuclease free water

in vial containing 310 µg of Carrier RNA, and then mixed it thoroughly by vortexing).

$$N \times 0.21ml = A\ ml$$

$$A\ ml \times 28\ \mu l/ml = B\ \mu l$$

N=number of samples to be processed

A=lysis buffer volume used

B= volume of Carrier RNA solution used

Thawing of processed samples was done at room temperature and vortex for 30 seconds. For preparation of Lysate, 25µl of proteinase K was added in each Eppendorf tubes located according to the number of samples. Then, 200 µl of sample was added was added in each tube. After this, 200 µl of prepared lysis buffer was added in each tube. Then tubes were vortex for 15 sec and incubation was done at 56^0C in heat block for 15 min. After being centrifuged for short time, 250 µl of ethanol (96-100% pure) was added to each tube containing lysate. Incubation was done at room temperature for 5min. After brief centrifugation, lysate was transferred onto the spin column (placed on collection tubes). Columns were then centrifuged at 6800×g for at least one min. Collection tubes with flow through in them were discarded and spin columns were placed on new wash tubes. Then 500 µl of wash buffer available in kit was added to each column and centrifugation was again done at 6800×g for 1 min. Wash tubes with flow through was discarded and and after placing columns on new wash tubes, again 500 µl of wash buffer was added and columns were centrifuged at 6800 ×g for 1 min. Flow through with wash tubes were discarded. Spin columns were placed on new wash tubes and then centrifugation was done with full speed for 1 min so that membrane could be completely dried. Then wash tubes were discarded and columns were placed on recovery tubes. For elution, 40 µl of RNase free water was added to each column and incubated at room temperature for 1 min. Again columns were centrifuged at full speed far 1 min. Columns were now discarded and recovery tubes were tightly closed and labeled properly according to the samples. They were then stored at -70^0C.

2.2. RT-PCR

For PPRV detection, primers specific to Nucleoprotein (N) gene were used for amplification (Couacy-Hymann et al., 2002). Optimization of reaction was done by using different annealing temperatures from 52-58°C and primer concentrations ranging from 100nm to 500nm were used in the final concentration. Master Mix was prepared by reagents described in Table 3.1

Table 3.1: Master Mix for RT-PCR

Sr. No	Reagents	Volume
1	Verso Enzyme Mix	0.5ul
2	2X PCR thermo-Start Master Mix	12.5 (1X)
3	RT Enhancer	1.0ul
4	Forward primer (10 µM)	0.25, 0.5, 0.75 & 1.0
5	Reverse primer (10 µM)	0.25, 0.5, 0.75 & 1.0
6	Template (RNA)	2.5
7	Water, nuclease-free	To 25ul
8	Total	25ul

Table 3.2: Sequence of forward (NP3) and reverse (NP4) primers

Name of primer	Target	Sequence (5'-3')	Positions	Reference
NP3(Forward)	Nucleoprotein gene	5'TCTCGGAAATCGCCTCACAGACTG3'	1232–1255	(Couacy-Hymann et al., 2002)
NP4 (Reverse)	Nucleoprotein gene	5'CCTCCTCCTGGTCCTCCAGAATCT 3'	1583–1560	

Gradient RT-PCR was performed initially for the optimization of annealing temperature of primers by using temperature range from 52-58°C. Master Mix was prepared with 1X 1-Step PCR Hot-Start Master Mix containing primers of 300nm concentration, enzyme 0.5ul as manufacturer instruction, RT Enhancer 1ul and nuclease free water. Total volume with RNA template was up to 25ul. After the

optimization of annealing temperature primer concentration was optimized by running reaction from 100nm-500nm. For optimization, extracted RNA of PPRV vaccine strain was used.

Table 3.3: Final Thermal profile for Conventional RT-PCR of PPRV

Sr. No	Stage	Temperature	Time	No. of cycles
1	c-DNA synthesis	50°C	20 minutes	1 cycle
2	Inactivation	95°C	15 minutes	1 cycle
3	Denaturation	95°C	20 seconds	40 cycles
4	Annealing	57°C	30 seconds	
5	Extension	72°C	30 seconds	
6	Final extension	72°C	5 minutes	1 cycle

Table 3.4: Master Mix for RT-PCR

Sr. No	Reagents	Volume (ul)
1	Verso Enzyme Mix	0.5
2	2X PCR thermo-Start Master Mix	12.5 (1X)
3	RT Enhancer	1.0
4	Forward primer (10 µM)	0.5
5	Reverse primer (10 µM)	0.5
6	Template (RNA)	2.5
7	Water, nuclease-free	7.5
8	Total	25ul

3.3.2.2.Gel Electrophoresis

Results of PCR products were evaluated on 1 % agarose gel with the help of gel Electrophoresis technique. For preparation of 1% agarose gel, 1g agarose was weighed and poured into an Erlenmeyer flask containing 100ml of 1X TBE buffer and then it was mixed by swirling and heated in microwave oven for almost 30 seconds or more until complete desolution of agarose . Then clear solution of agarose obtained was placed at room temperature for 15-20 minutes. 9ul/ (50ug/100ml) working solution of ethidium bromide was added into electrophoresis tray for staining. This mixture was kept at room temperature, solidified in a few minutes. Comb was removed and gel was placed on the gel tank and it was filled with 1X TBE buffer. 5ul of amplified sample was mixed with 2ul of loading dye (6X orange dye solution), and mixture was loaded in all well except 1st one. 5ul of 100bp DNA ladder was loaded in 1st well. After loading gel tank lid was closed and, electrophoresis was run at 100 V for 30-40 minutes.

3.3.2.4 Gel Documentation/ Visualization of amplified product:

Agarose gel was placed inside the gel documentation system to visualize amplified samples (GenoSens 1520, BIO-EQUIP) with the help of Ultra violet light. Gel picture was saved for record purpose.

3.3.3. Standardization of qRT-PCR with Taqman probe for Detection of PPRV

3.3.3.1. Standard RNA

Reference strain (vaccinal strain) of PPRV i.e Nigeria 75 was used for preparation of standard curve. Its RNA was extracted by following same protocol as described in section 3.3.2.1.

3.3.3.2. Standardization of qRT-PCR (Taqman Probe)

Standardization of qRT-PCR technique was done by using Thermo Scientific Verso 1-step qRT-PCR kit, sequence specific primers and probes described in Table 3.7 The template RNA solution was immediately kept on ice after taking from -70^{0}C. After thawing all reagents were kept on ice. Reagents and their volumes used in preparation of master mix are mentioned in Table 3.5 Total 22.5µl volume of master mixture was

used. Then 2.5µl of extracted RNA (PPRV) was added and total volume of mixture used for standardization became 25µl.

Initially, conventional RT-PCR was done in thermocycler "Applied Biosystems Veriti" by using different temperatures ranging from 53-58°C to check the proper annealing of primers.

Table 3.5: Master Mix of RT-PCR for optimization of annealing temperature for qRT-PCR (TaqMan probe)

Sr. No	Reagents	Volume µl
1	Nuclease free water	6.5
2	2X 1step qPCR master Mix	12.5 (1X)
3	RT Enhancer	1.0
4	Verso enzyme mix	0.5
5	Forward primer (10 µM)	1
6	Reverse primer (10 µM)	1
7	Template (RNA)	2.5
8	Total	25ul

Table 3.6: Thermal Profile for Optimization of qRT-PCR

Sr. No	Stage	Temperature	Time	No. of cycles
1	c-DNA synthesis	50°C	20 minutes	1 cycle
2	Inactivation	95°C	15 minutes	1 cycle
3	Denaturation	95°C	20 seconds	45 cycles
4	Annealing (Gradient)	**52-58°C**	20 seconds	
5	Extension	72°C	10 seconds	
6	Final extension	72°C	2 minutes	1 cycle

Table 3.7: Sequence of Primer and Probe of qRT-PCR (TaqMan)

Purpose	Target	Sequence (5'-3')	Position	Reference
Forward	N gene	5'CACAGCAGAGGAAGCCAAACT 3'	1213-1233	(Bao et al., 2008)
Reverse	N gene	5'TGTTTTGTGCTGGAGGAAGGA 3'	1327-1307	
Probe	N gene	5'CTCGGAAATCGCCTCGCAGGCT3'	1237-1258	

For the optimization of primers concentration in the final mix a range of primer concentrations (100 nmoles-500 nmoles) was used. After optimization of primers annealing temperature and concentration in conventional RT-PCR, qRT-PCR was performed using following master Mix and thermal profile.

Table 3.8: Master Mix for qRT-PCR by TaqMan probe.

Sr. No	Reagents	Volume(µl)
1	Nuclease free water	5.75
2	2X 1step qPCR master Mix	12.5 (1X)
3	RT Enhancer	1.25
4	Verso enzyme mix	0.25
5	Forward primer (10 µM)	1
6	Reverse primer (10 µM)	1
7	Probe (5 µM)	0.75
8	Template (RNA)	2.5
9	Total	25ul

Table 3.9: Thermal profile for qRT-PCR (TaqMan Probe)

Sr. No	Stage	Temperature	Time	No. of cycles
1	c-DNA synthesis	50°C	30 minutes	1 cycle
2	Inactivation	95°C	5 minutes	1 cycle
3	Denaturation	95°C	20 seconds	40 cycles
4	Annealing and extension	60°C	60 seconds	

For rRT-PCR assay ABI7500 real time PCR system (Applied Biosystem) was used. Ct value for each sample was determined at annealing step of each cycle which is the description of threshold (when fluorescence overreach its threshold limit).

3.3.3. Standardization of Reaction qRT-PCR with SYBR Green for PPRV Detection

Standardization of qRT-PCR with Syber Green was done with the extracted RNA of same vaccinal strain of PPRV i.e. Nigeria 75/1 .

3.3.3.2. Standardization of qRT-PCR with SYBR Green

Thermo Scientific Maxima SYBR Green/ROX qPCR kit was used to standardize qRT-PCR with SYBR Green. For Nucleoprotein (N) gene q-PCR primer designing initial genomic data was taken from Gene Bank (accession no. KJ466104, KJ867544, AJ849636, FJ905304, EU267273, KM463083, KM212177, KM091959, KF727981, X74443, KP789375), aligned using Clustal W and designed with geneious 9.0.4. Primers sequence was given in Table 3.10 Gradient RT-PCR was performed in Veriti 96 well Thermocycler of applied Biosystems for the optimization of annealing temperature of primers. For this purpose, Verso 1-step Hot start RT-PCR kit was used. Master Mix was prepared according to manufacturer instructions. Briefly, nuclease free water (7.5 µl), PCR mix (12.5 µl), Verso enzyme mix (0.5 µl), RT enhancer (01 µl) and 0.5 µl of both forward primer (200nmoles in final volume) and reverse primer (200nmoles in final volume) were mixed. 2.5 µl of extracted RNA was added as template. Total volume of the solution was 25 µl. In order to determine optimized annealing temperature for primers, gradient temperature range from 50-56°C was checked.

Table 3.10: Sequence of Primer for qRT-PCR (SYBR Green)

Name of primer	Target	Sequence (5'-3')	Positions	Designed with
Forward	Nucleoprotein gene	5'–CCTCGTGAGGCTCAAAGATC– '3	1593–1612	geneious 9.0.4
Reverse	Nucleoprotein gene	5'– GTTGTCTTCTCCCTCCTCCT– 3'	1665–1685	

Table 3.11: Thermal Profile for Optimization of Primers annealing temperature for qRT-PCR (SYBR Green)

Sr. No	Stage	Temperature	Time	No. of cycles
1	c-DNA synthesis	50°C	20 minutes	1 cycle
2	Inactivation	95°C	15 minutes	1 cycle
3	Denaturation	95°C	20 seconds	45 cycles
4	Annealing (Gradient)	**50-56°C**	20 seconds	
5	Extension	72°C	10 seconds	
6	Final extension	72°C	2 minutes	1 cycle

3.3.3.3. Reverse Transcription (cDNA Synthesis)

Reagents used for cDNA Synthesis were random hexamer primers, Ribonuclease inhibitor, 5X Reaction Buffer, Oligo dT, DTT stock solution, dNTP Mix, and Nuclease free water. Synthesis of cDNA was performed in two steps, in 1st step sample denaturation and primer annealing was done. In a nuclease free microtube (1st tube) 5ul of nuclease free water, 1ul of Random Hexamer primers and 4ul of template RNA solution was taken. After giving incubation at 70°C for 5 minutes, tube was then placed on ice. In the 2nd step a reaction of mixture was prepared by adding 2.5ul nuclease free water, 4.0ul RT buffer, and 1.0ul of 10mM dNTPs, 1.0ul DTT stock solution, 1.0 ul RNase inhibitor and 0.5ul Reverse Transcriptase in another nuclease free microtube (2nd tube). After sample denaturation and primer annealing 1st tube contents were mixed with 2nd tube. Then after gentle mixing, incubation was given to tube first at 42°C for 10 min, then at 50°C for one hour, and in last 70°C for 10 min in thermocycler. The solution was chilled on ice for 15 minutes and store at -20°C till use.

Table 3.12: 1st tube mixture contents for cDNA synthesis

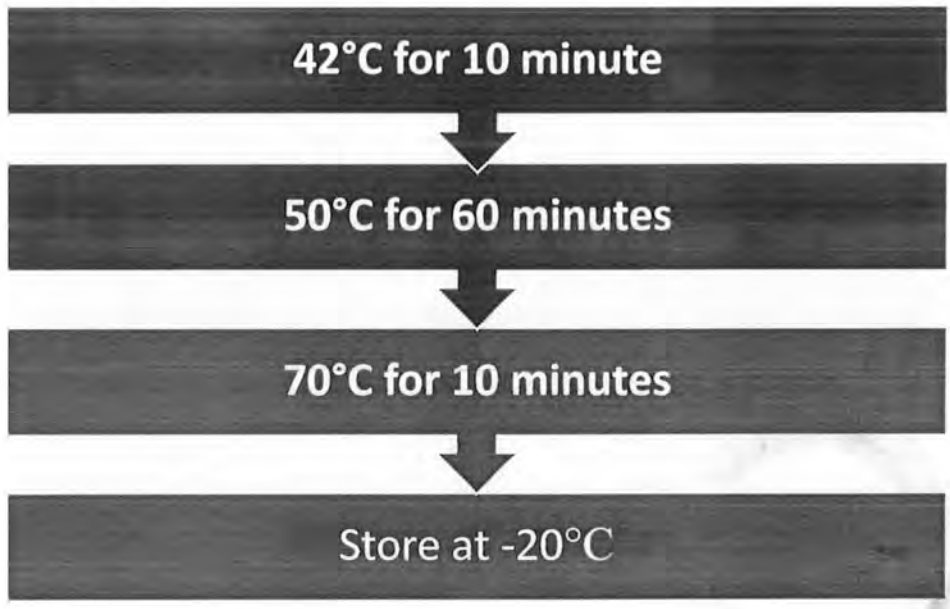
Sr. No.	Reagents	Volume (ul)
1.	Nuclease free wáter	5.0
2.	Random Hexamer Primers	1.0
3.	RNA template	4.0
4.	Total	10ul

These reagents were mixed in a nuclease free microtube, incubated for 5 min at 70°C and then kept in ice.

Table 3.13: 2nd tube mixture contents for cDNA synthesis

Sr. No.	Reagents	Volume (ul)
1.	Nuclease free wáter	2.5
2.	RT Buffer	4.0
3.	10mM dNTPs	1.0
4.	DTT stock solution	1.0
5	RNase inhibitor	1.0
6	Reverse transcriptase	0.5
7	Total	10ul

1st tube content were mixed with 2nd tube contents gently. And incubated as follows



3.3.3.4. Quantitative Polymerase Chain Reaction (SYBR Green)

qRT-PCR with SYBR Green dye was developed and standardized. PCR was done with final reaction volume of 25µl using Thermo Scientific Maxima SYBR Green/ROX qPCR kit. Primer sequences of Nucleoprotein genes are given in Table 3.10 Reagents mentioned belowin Table 3.14 were used in preparation of master mix.

Table 3.14: Contents for qPCR by SYBR Green

Sr. No.	Reagents	Volume (ul)
1.	Nuclease free wáter	9.0
2.	Maxima SYBR green Master Mix	12.5
3.	Forward primer (10 µM)	0.75
4.	Reverse primer (10 µM)	0.75
5	cDNA template	2.0
6	Total	25

Thermal Profile for the reaction was set according to primer annealing Temperature optimized by RT-PCR in section 3.3.3.2

Table 3.15: Thermal profile for qPCR (SYBR Green)

Sr. No	Stage	Temperature	Time	No. of Cycles
1	UDG Pretreatment	50°C	2 minutes	1 Cycle
2	Initial Denaturation	95°C	10 minutes	1 Cycle
3	Denaturation	95°C	15 seconds	40 cycles
4	Annealing	52°C	20 seconds	
5	Extension	72°C	20 seconds	

For q-PCR,AB17500 real time thermocycler (Applied Biosystems) was used.During annealing step of each cycle Ct values for each sample was calculated.

CHAPTER: 04
RESULTS

CHAPTER 4
RESULTS

4. Results

4.1. Standardization of RT-qPCR (Probe)

RT-qPCR (TaqMan) was standardized using PPRV vaccine (Nigeria 75/1). Tenfold serial dilutions of the RNA extracted from PPRV vaccine, was prepared up to six level. These dilutions were used for the standardization of assay. For estimation of nucleic acid concentration, NanoDrop was used. Table 4.1 shows the cyclic threshold values of the amplification of template.

Table 4.1: Serial dilutions results of RT-qPCR (Probe)

Serial Dilutions	RNA Concentration (µg/µl)	Copy Number	Cyclic threshold (Ct)values (Probe)
Log 10	9.0	$5x10^7$	19.87
Log 10^{-1}	0.9	$5x10^6$	23.33
Log 10^{-2}	0.09	$5x10^5$	25.9
Log 10^{-3}	0.009	$5x10^4$	30.43
Log 10^{-4}	0.0009	$5x10^3$	35.67
Log 10^{-5}	0.00009	$5x10^2$	37.54
Log 10^{-6}	0.000009	$5x10^1$	undetected

Ct values are increasing as the copy number decreases. However as dilution increases, copy number of RNA decreases up to a certain limit after which no florescent signal was detected by PCR machine. These results show that this assay can detect $5x10^2$ copy numbers at minimum. The R value for this assay was 0.9884.

This assay can detect 5.0×10^2 RNA copies in a reaction mixture with its Ct-value 37.54. The assay dynamic range was over 10-log-unit span of viral RNA conc, stretching from 5.0×10^1 to 5.0×10^7 RNA copy number per reaction mixture. Additionally, tenfold further cDNA transcripts serial dilutions in parallel with copy

numbers of 5.0 x 10^2 to 5.0 x 10^6 per reaction mixture were used to construct a standard curve (Figure 4.1)

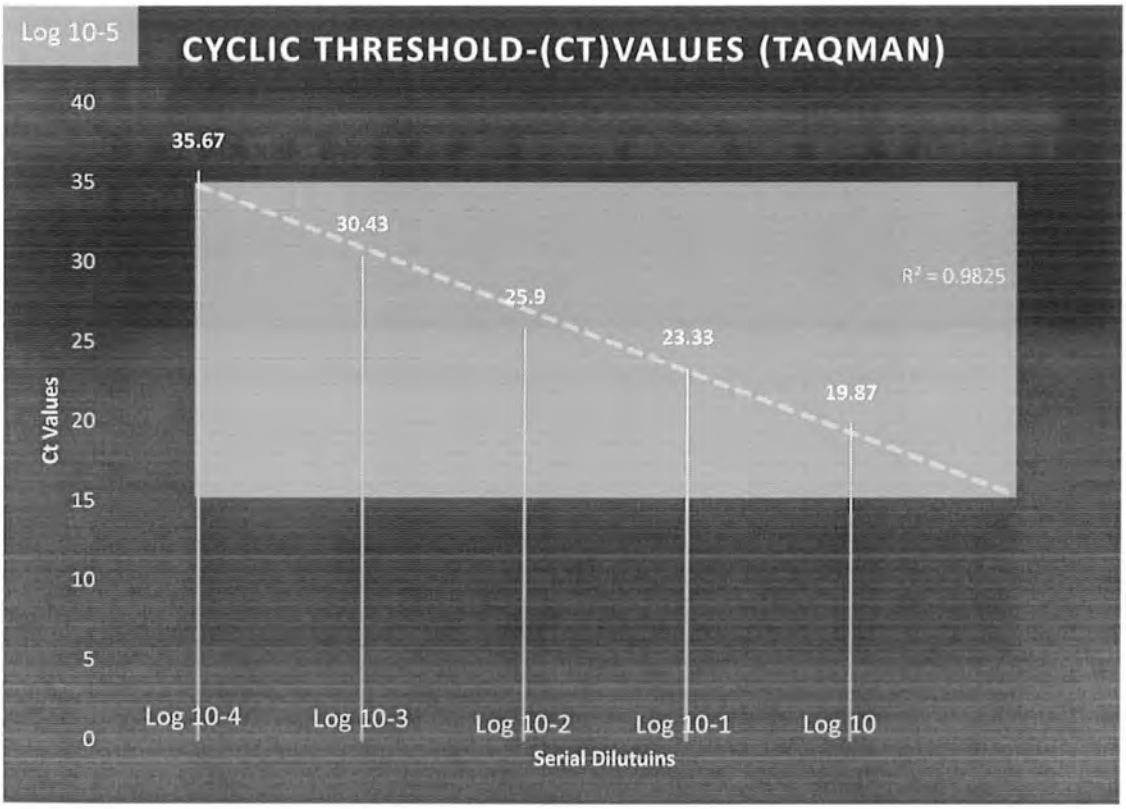


Figure 4.1: Standard curve for PPRV with RT-qPCR (Probe). Tenfold serial dilutions from 5 x 10^7 to 5 x 10 ^{2} copy numbers were tested. The regression value for it is 0.9825.

Cyclic Threshold (Ct) Values increases with the increase of dilution that is inversely proportional to the RNA concentration.

CHAPTER 4
RESULTS

4.2. Standardization of RT-qPCR (SYBR Green)

As mentioned above in Table 4.1, PPRV vaccine strain (Nigeria 75/1) RNA was also used to standardize newly developed RT-qPCR (SYBR Green) assay. qPCR of tenfold serial dilution of extracted RNA was performed.

Table 4.2: Serial dilutions results of RT-qPCR (SYBR Green)

Serial Dilutions	RNA Concentration (µg/µl)	Copy Number	Cyclic threshold (Ct)values (SYBR Green)
Log 10	7.5	$3.78x10^7$	21.63
Log 10^{-1}	0.75	$3.78x10^6$	25.62
Log 10^{-2}	0.075	$3.78x10^5$	29.17
Log 10^{-3}	0.0075	$3.78x10^4$	31.45
Log 10^{-4}	0.00075	$3.78x10^3$	35.01
Log 10^{-5}	0.000075	$3.78x10^2$	38.35
Log 10^{-6}	0.0000075	$3.78x10^1$	undetected

The concentrations were determined with the help of NanoDrop. So with increasing dilution virus titer reduced, causing an increase in the cyclic threshold values as shown in the Figure 4.2.

For the determination of assay detection limit and validity, standard curve was developed using 10-fold serial dilution of c-DNA. This assay can detect 3.78×10^2 c-DNA copies in the reaction mixture. Tenfold serial dilution was plotted on the X-axis while Ct values on the Y-axis. R^2 value was 0.9958 which shows its validity (Figure 4.2)

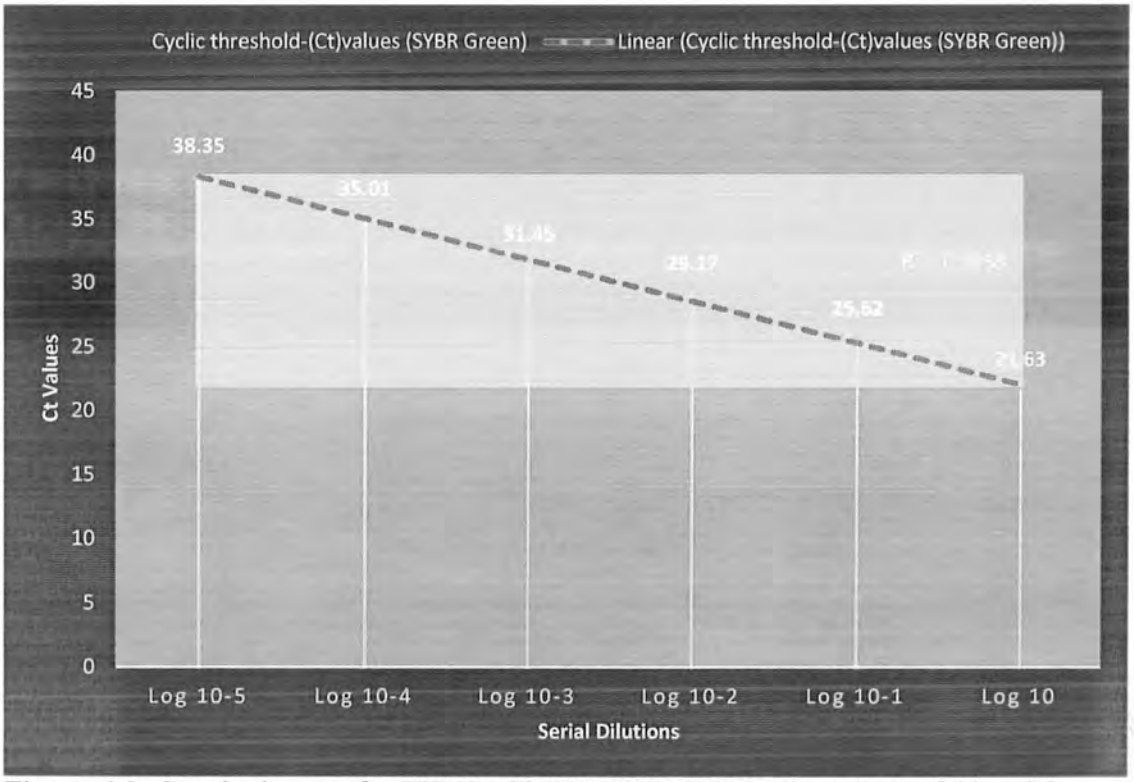


Figure 4.2: Standard curve for PPRV with RT-qPCR (SYBR Green). Tenfold serial dilutions from 3.78 x 10^7 to 3.78 x 10^2 copy numbers were tested. The regression value for it is 0.9958.

After standardization, serial dilutions of same PPRV vaccinal strain were also tested with RT-PCR, for comparison of RT-PCR, RT-qPCR (TaqMan) and RT-qPCR (SYBR Green) sensitivities. Results obtained by RT-PCR and RT-qPCR (TaqMan and SYBR Green) are shown in Table 4.3.

Table 4.3: Qualitative results of assays (RT-PCR, RT-qPCR (TaqMan) & RT-qPCR (SYBR Green)

Dilutions	RT-PCR	RT-qPCR (TaqMan)	RT-qPCR (SYBR Green)
Log 10	Positive	Positive	Positive
Log 10^{-1}	Positive	Positive	Positive
Log 10^{-2}	Negative	Positive	Positive
Log 10^{-3}	Negative	Positive	Positive
Log 10^{-4}	Negative	Positive	Positive
Log 10^{-5}	Negative	Positive	Positive
Log 10^{-6}	Negative	Negative	Negative

It is clear from above data that the sensitivity of RT-qPCR (SYBR Green) and RT-qPCR (TaqMan) was much greater than the sensitivity of conventional RT-PCR, while the sensitivity of both types of RT-qPCR (TaqMan and SYBR Green) was equivalent as both detected 10^{-5} dilution of PPRV vaccinal strain.

After standardization of RT-qPCR (TaqMan) and RT-qPCR (SYBR Green), field samples were taken from different outbreaks suspected for PPRV and were tested by RT-PCR, RT-qPCR (TaqMan) and RT-qPCR (SYBR Green) to further assess their recommendation for field conditions as well.

CHAPTER 4
RESULTS

4.3. Hemagglutination Assay (HA)

Virus titter obtained after performing HA of field samples for PPR screening are mentioned in Table 4.4. After being processed, swab samples obtained from different outbreaks were assessed by Hemagglutination assay for their initial screening. Out of total 20 swab samples, 95% were positive. Non-specific agglutination occurred in 1st and 2nd well while agglutination from 3rd well can be viewed as positive (Figure 4.3). So agglutination titer ranged from 8-128, that means minimum agglutination was at 3rd well and maximum given by sample was in 7th well in case of swab samples. Although HA results are not reliable but give a shorter range to confirm positive samples of PPR.

Tissue samples collected from different outbreaks were processed and also initially screened by Hemagglutination assay. Out of a total of 26 samples, 92% were positive. For the declaration of positive samples from the 3rd well agglutination was checked. Tissue samples agglutination titter was in the range of 8-1024.

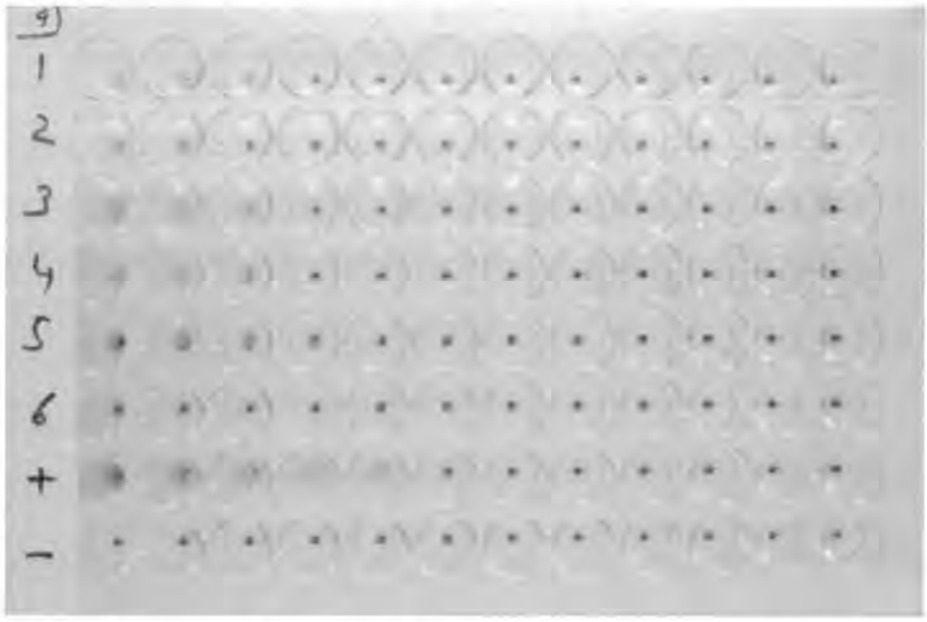


Figure 4.3: HA Plate showing agglutination and RBCs beads. Six samples in the HA plates showing agglutination. Sample No. 2 & 6 are declared negative, sample no. 2 showing agglutination just in 1st two wells so it is considered negative while sample No. 6 doesn't showing any agglutination. Remaining four samples are positive and in the 7th row of the plate is positive control while in 8th row is negative control.

CHAPTER 4
RESULTS

Out of total 46 outbreak samples, 43 samples were confirmed to be positive by HA test. So 93.47% samples were identified positive with the help of HA test.

Table 4.4: Hemagglutination Result of outbreak samples

Sample ID	Results (up to wells)	Titer	Sample ID	Results (up to wells)	Titer
1	4	01:16	24	5	01:32
2	3	01:08	25	3	01:08
3	5	01:32	26	3	01:08
4	4	01:16	27	4	01:16
5	6	01:64	28	4	01:16
6	6	01:64	29	3	01:08
7	5	00:00	30	6	01:64
8	3	01:08	31	Negative	00:00
9	Negative	00:00	32	5	01:32
10	4	01:16	33	6	01:64
11	5	01:32	34	Negative	00:00
12	3	01:08	35	4	01:16
13	6	01:64	36	5	01:32
14	6	01:64	37	4	01:16
15	3	01:08	38	3	01:08
16	5	01:32	39	4	01:16
17	4	01:16	40	3	01:08
18	4	01:16	41	6	01:64
19	3	01:08	42	4	01:16
20	5	01:08	43	10	01:1024
21	3	01:08	44	7	01:128
22	5	01:32	45	6	01:64
23	7	01:128	46	4	01:16

CHAPTER 4
RESULTS

4.4 RT-PCR

RT-PCR was performed for PPRV detection from outbreak samples, after being assessed from HA test. Amplification was done by using N gene primers. Results obtained from RT-PCR are shown in Table 4.5.

Table 4.5: RT-PCR Result of outbreak samples

Sample ID	Results	Sample ID	Results	Sample ID	Results
1	Positive	17	Negative	33	Negative
2	Positive	18	Positive	34	Positive
3	Positive	19	Positive	35	Negative
4	Positive	20	Positive	36	Positive
5	Positive	21	Positive	37	Negative
6	Positive	22	Negative	38	Positive
7	Positive	23	Positive	39	Positive
8	Negative	24	Negative	40	Negative
9	Negative	25	Positive	41	Negative
10	Positive	26	Positive	42	Negative
11	Positive	27	Positive	43	Negative
12	Positive	28	Positive	44	Positive
13	Negative	29	Negative	45	Negative
14	Positive	30	Positive	46	Negative
15	Negative	31	Negative		
16	Positive	32	Negative		

Out of 46 samples, 19 samples were negative for PPRV while 27 samples were positive for PPRV by RT-PCR. Figure 4.4 shows a representation of positive PCR results on agarose gel. So, 58.69% outbreaks samples were detected positive for PPRV with the help of RT-PCR.

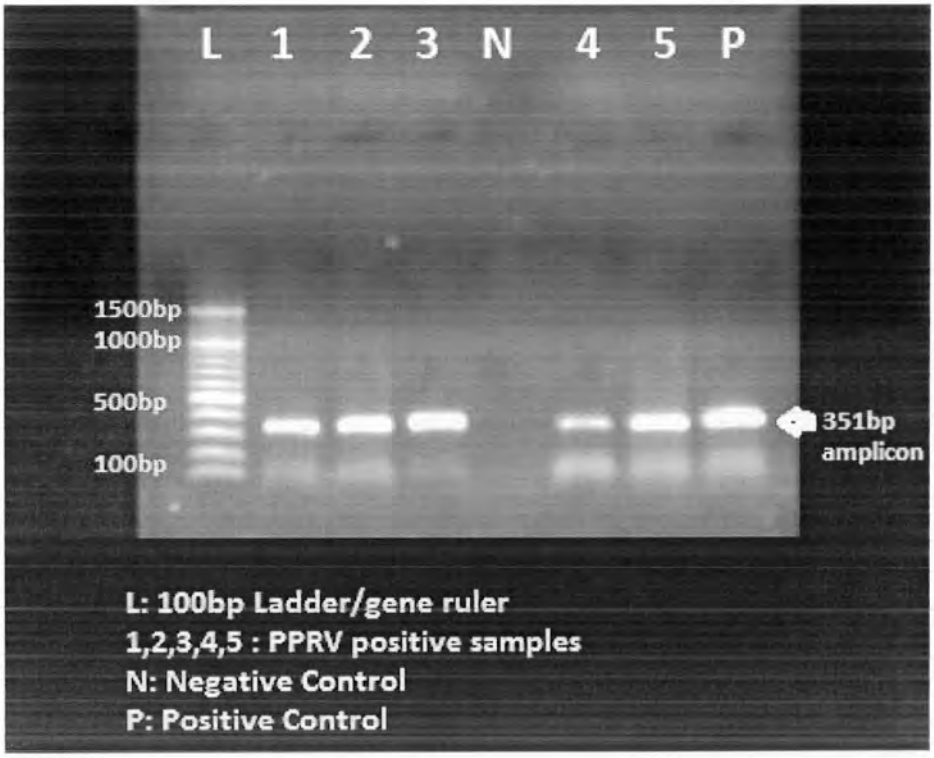


Figure 4.4: Agarose gel image showing RT-PCR positive samples. PPR positive samples after amplification are run on 1% agarose gel showing 351bp band. 100 bp DNA ladder is also run along with samples for identification of required band by comparison.

4.5. RT-qPCR (TaqMan)

All PPR suspected samples were tested with qRT-PCR (TaqMan) and results are presented in Table 4.6.

Table 4.6: qRT-PCR (TaqMan) Results of suspected PPRV samples

Sample ID	**TaqMan probe results (Ct)**	Sample ID	**TaqMan probe results (Ct)**	Sample ID	**TaqMan probe results (Ct)**
1	21.205	17	undetected	33	33.41
2	17.61	18	undetected	34	26.72
3	17.56	19	29.49	35	27.27
4	22.68	20	34.065	36	28.53
5	18.01	21	29.74	37	undetected
6	14.615	22	26.43	38	undetected
7	25.615	23	31.81	39	29.98
8	32.29	24	33.915	40	29.64
9	30.17	25	28.45	41	undetected
10	24.36	26	26.89	42	undetected
11	23.61	27	31.08	43	undetected
12	28.035	28	34.65	44	26.94
13	29.82	29	31.73	45	undetected
14	26.765	30	32.09	46	undetected
15	35.255	31	26.56		
16	undetected	32	undetected		

Out of 46 samples, 11 samples were confirmed as negative after performing RT-qPCR (TaqMan) and 35 samples were declared as positive with different Ct values. So, 76.08% samples were positive by RT-qPCR (TaqMan).

CHAPTER 4
RESULTS

4.6. RT-qPCR (SYBR Green)

Same samples tested by RT-PCR (TaqMan) were also tested by RT-qPCR (SYBR Green). Results obtained are shown in Table 4.7.

Table 4.7: qRT-PCR (SYBR Green) Results of suspected PPRV samples

Sample ID	**CYBR Green Result**	Sample ID	**CYBR Green Result**	Sample ID	**CYBR Green Result**
1	30.87	17	35.63	33	34.04
2	27	18	32.02	34	24.8
3	26.92	19	30.07	35	35.51
4	30.08	20	34.45	36	27.23
5	29.05	21	31.52	37	34.36
6	23.66	22	22.33	38	34.41
7	28.49	23	31.84	39	28.07
8	31.71	24	23.74	40	undetected
9	28.3	25	28.3	41	undetected
10	32.6	26	undetected	42	33.67
11	34.41	27	29.77	43	undetected
12	30.63	28	29	44	28.65
13	30.8	29	35.2	45	undetected
14	27.7	30	35.42	46	undetected
15	37.12	31	34.77		
16	33.85	32	35.16		

Out of 46 samples, 40 samples were confirmed as positive with distinct Ct values and 6 samples were confirmed as negative for PPRV with RT-qPCR (SYBR Green). So, 86.95% samples were found as positive.

4.7. Comparison of RT-PCR, qRT-PCR (TaqMan) and qRT-PCR (SYBR green)

Figure 4.5 shows the comparison of RT-PCR, qRT-PCR with TaqMan probes and qRT-PCR with SYBR green probes. When the results of confirmed positive results obtained by HA (93.47%), RT-PCR (58.69%), RT-qPCR (TaqMan) (76.08%) and RT-qPCR (SYBR Green) (86.95%) were compared, the positive number of cases obtained from HA method was highest, but it is not a reliable test as it has been reported in many studied that HA can give false positive results (Zahur et al. 2014). Results of outbreaks samples were further confirmed using RT-PCR, qRT-PCR (TaqMan), qRT-PCR (SYBR Green) i.e. confirmatory tests. Percentage obtained from SYBR Green method was high, as shown in graph above than RT-PCR and qRT-PCR (TaqMan).

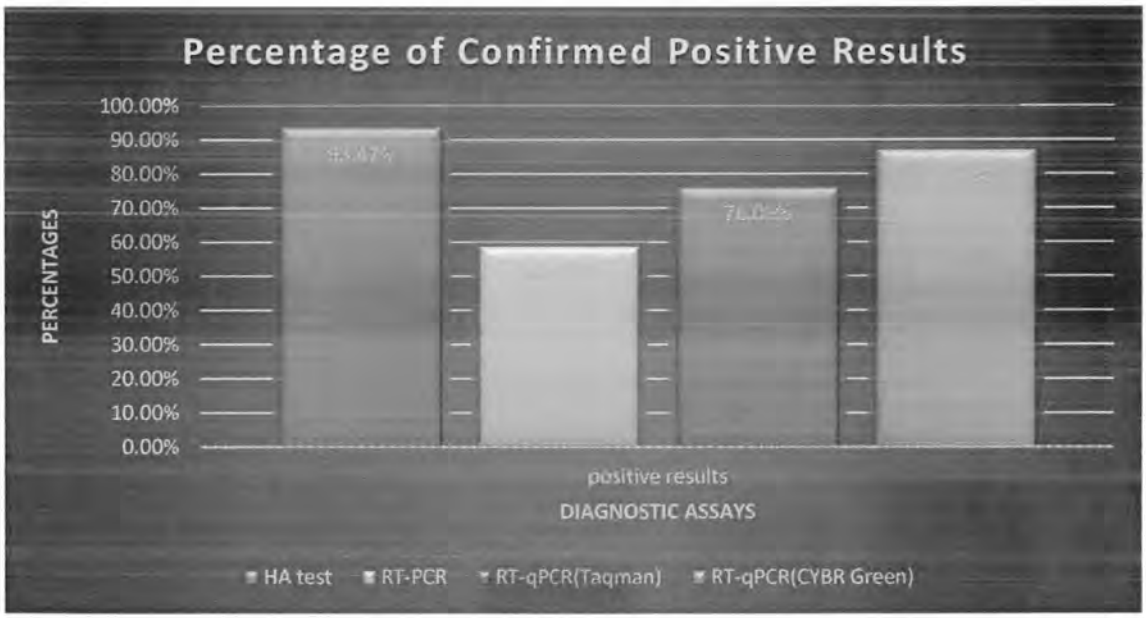


Figure 4.5: Comparison of RT-PCR, qRT-PCR (TaqMan) and qRT-PCR (SYBR green)

CHAPTER: 05

DISCUSSION

5. Discussion:

PPR is highly transmissible disease of small ruminants, with great economic influence and is also included in the transboundary diseases of animals. The causative agent for this disease is a virus i.e. PPRV with global distribution like South Asia (Pakistan, India, Nepal, Bangladesh and China), Middle East and also in Africa (Barrett, 2005). Clinical signs that appear in animals during PPR disease include ocular and nasal discharges, fever, pustules (lesions) in mouth, cough and also diarrhea with high rate of morbidity and mortality. Economic losses in PPR disease occur due to milk and meat product losses, abortion in female animals and high death rate in animals (Nawathe, 1984). Main income source of small farmers especially in developing counties are small ruminants (goats and sheep). Thus, disease control is very important to reduce poverty in developing countries (Diallo, 2006).

PPR can be diagnosed by clinical signs and symptoms in the field conditions and confirmed by various laboratory assays. However, PPR diagnosis on clinical signs and symptoms can create confusion with other related animal diseases like CCPP, sheep and goat pox, FMD, pasteurellosis etc. Many diagnostic tests including both conventional and molecular based have been developed for its diagnosis i.e. ELISA, Virus isolation, HA test and PCR with its different types. All diagnostic assays hold some advantages over others and some drawbacks of varying sensitivity and specificity. Molecular techniques are more preferred than conventional based for PPR diagnosis because their results are more reliable.

Real time RT-PCR for PPR diagnosis and quantification was standardized by various researchers using probe based technology and SYBR Green dye. First time Bao and coworkers in 2008 developed 1-step rRT-PCR using N-gene primers and probe and compared its sensitivity with conventional RT-PCR (Bao et al., 2008). Kwaitek et al in 2010 and Baten and his colleagues in 2011 developed another one step RT-PCR for the diagnosis of all four lineages of PPRV using N-gene primers and probe. He also compared this assay with conventional RT-PCR (Batten et al., 2011; Kwiatek et al., 2010). In 2012, Balamurugan et al 1st time developed real time one-step RT-PCR using SYBR Green for the detection of PPRV. He used M-gene primers and compared his assay with conventional RT-PCR, real time RT-PCR (TaqMan probe) (Balamurugan et al., 2012b). In 2014, Abera et al developed SYBR green assay using

N-gene primers and compared it with conventional RT-PCR (Abera et al., 2014). However, Abera did not compare it with real time RT-PCR (TaqMan probe) for estimation of its sensitivity. In this study we developed qRT-PCR (SYBR Green) by designing new primers using N –Gene of PPRV sequence and compared it with conventional RT-PCR, qRT-PCR (TaqMan Probe).

Field samples suspected for PPRV from outbreaks of different areas of Pakistan were collected and first HA test was performed using chicken RBCs because it is a simple, easy and less expensive test to diagnose PPR in field conditions and in lab ((Johnson and Ritchie, 1968). For HA different RBCs can be used but in this study 1% chicken RBCs were used because these are economical and easily available (Wosu, 1985). HA test was performed on swab samples, nasal swabs and also for tissue samples for PPR diagnosis because it doesn't require well equipped and modern equipment. It gave about 93.47% positive samples for PPRV but these positive results didn't match with gold standard RT-PCR results because of the limitations of HA test in that it gives false positive results and thus is not reliable (Zahur et al., 2008). So, it was used as screening test and the results obtained by this test were further confirmed by molecular based techniques like conventional RT-PCR and real time PCR (Taqman and SYBR Green).

Conventional RT-PCR is considered as gold standard test for PPRV detection (Zahur et al., 2008) and RT-PCR is widely used throughout the world for PPRV detection due to its accuracy, sensitivity and rapidity. After RNA extraction, RT-PCR was optimized by using NP3 and NP4 primers. We found 200 nmoles primers dilution and 56°C annealing temperature were the standard optimized conditions. These condition were almost close to the Couacy et al i.e. almost 220 nmoles primers concentration in final volume and 55°C annealing temperature (Couacy-Hymann et al., 2002). Then optimized conditions were applied in testing the suspected samples of PPRV. Only 58.67 % samples were found positive and showed remarkable difference from HA test as HA test can give false positive results (Zahur et al., 2008)

Real time RT-PCR is a rapid, more sensitive technique for the diagnosis of PPRV (Bao et al., 2008; Batten et al., 2011). So in this study, real time PCR (TaqMan) was standardized and its sensitivity was also determined by finding its detection limit. Standard curves was developed for this assays (Fig. 4.1) using 10-fold dilutions of

extracted RNA. Detection limit of this assay was 5.0 x 10^2 RNA copies which is lower than Bao et al results i.e. 8.1 RNA copies (Bao et al., 2008). This was probably due to difference in reagents and real time machine.

Further, we selected the new sequences of N gene for the designing of new primers. These newly developed primers were used in real time RT-PCR analysis by using SYBR Green mixture instead of TaqMan probe. This assay was optimized and standard curve was developed using 10-fold dilution of c-DNA (PPRV vaccinal strain). Detection limit of this assay was 3.78 x 10^2 c-DNA copy numbers. Sensitivities of both types of RT-qPCR were almost equivalent to each other but greater than conventional RT-PCR. These methods facilitate rapid detection of PPRV and require advanced equipment and special expertise unlike conventional RT-PCR.

After optimization of both assays, PPRV suspected samples were tested. qRT-PCR (TaqMan Probe) showed 76.08% positive while qRT-PCR (SYBR Green) showed 86.95 % positive results. This shows that qRT-PCR (SYBR Green) is more sensitive than TaqMan probe as also indicated by Balamurugan for M-gene (Balamurugan et al., 2012b). In this study, the sensitivities of both RT-qPCR (TaqMan and SYBR Green) were found to be comparable and also other studies have reported that the sensitivity of SYBR Green method is greater than TaqMan for PPRV detection but it is less expensive than TaqMan probe. So, this method can be used as an alternative of TaqMan method (Abera et al., 2014).

Both RT-qPCR methods (SYBR Green and TaqMan) are rapid, specific and sensitive than conventional RT-PCR. In both methods, 96 well plate is used for making reaction mixture thus larger number of samples can be tested at one time with more feasibility than conventional RT-PCR. Chances of cross contamination are also less in these methods. These methods revealed high recommendation for field samples of PPRV, as it is clear from the results obtained after testing outbreak samples by using them.

Thus real time PCR (SYBR Green) is suggested as fast techniques with great specificity, sensitivity and reliability than conventional RT-PCR in PPRV detection. This assay is comparatively less expensive than the qRT-PCR (TaqMan Probe) and it is more sensitive, thus this method can be used as substitution of conventional RT-PCR for rapid PPR diagnosis in animals.

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