Development and Evaluation of Stable Vero Cell Line Constitutively Expressing Signaling Lymphocyte Activation Molecule (SLAM) and Nectin-4 Receptors for Replication Efficacy of *Peste-des-petits-ruminants* and *Canine distemper viruses*

Thesis

Submitted to the
DEEMED UNIVERSITY
ICAR-Indian Veterinary Research Institute
Izatnagar - 243 122 (U.P.), India

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IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Doctor of Philosophy

(Veterinary Virology)

2019

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भाकृअनुप–भारतीय पशु चिकित्सा अनुसंधान संस्थान
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This is to be certified that the research work embodied in this thesis entitled **"Development and evaluation of stable Vero cell line constitutively expressing Signaling Lymphocyte Activation Molecule (SLAM) and Nectin-4 receptors for replication efficacy of *Peste-des-petits-ruminants* and *Canine distemper viruses*"** *submitted by* **Dr. Ajay Kumar Yadav, Roll No. P-1729,** *for the award of* **Doctor of Philosophy** *Degree in* **Veterinary Virology** *at Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate himself under my supervision and guidance.*

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ACKNOWLEDGEMENTS

The completion of this thesis would not have been possible without the help, support and guidance of many people who in one way or another contributed and extended their valuable assistance in the preparation and completion of this study. I deem it a proud privilege and feel immense pleasure to convey my gratitude to all those who helped me during the pursuit of study.

With heartfelt and immense pleasure, I would like to manifest my utmost reverence to my advisor **Dr K.K. Rajak**, senior scientist, Division of Biological Products, ICAR-Indian Veterinary Research Institute, Izatnagar for his scientific, patient counseling, talented direction, constant encouragement, and untiring help amidst of his scientific schedule. I consider myself fortunate and lucky enough to work under the guidance of my beloved guide. I discovered the pleasures of pursuing an investigation in to the realm of scientific phenomenon, with total devotion and untiring efforts.

I wish to record my sincere gratitude to **R.P. Singh**, Head, of Biological Products, ICAR-Indian Veterinary Research Institute, Izatnagar and also member of my advisory committee for his valuable suggestions, timely, constant encouragement and providing facility in the lab.

I would like to express my gratitude from the core of my heart to **Dr. M. Sankar.**, for being like a teacher, an elder brother and friend throughout my study. I must thank him for providing me with all the helpful information and materials and for his talented direction, close supervision, constructive criticism, hearty encouragement and great help when things were not going right during my research work.

I am forever grateful to **Dr. Soumendu Chakarvarti** for his invaluable guidance, technical support and expertise. His truly scientific intuition has made him as a constant oasis of ideas and passions in science, which exceptionally inspired and enriched my growth as a researcher.

I am highly indebted to **Dr. Sagar Khulape** for his valuable suggestions, cooperation and constant prying into my research work and in the preparation of ARS exam. This all would not have been possible without his timely help. I express deep sense of gratitude to him for always being ready to help me. His inspiring guidance, constant encouragement is worth thanking.

Words cannot describe the help and good association by **Dr. P. K. Gupta,** principal scientist, Division of Animal Biotechnology for his critical and logical skepticism to my work. I

gratefully thankful for his advice, supervision and crucial contribution during my struggle in research programe.

I feel immense gratitude in extending my sincere regards and thanks to **Dr. D. Muthuchelvan, Dr GVPPS Ravi Kumar, Dr Bablu Kumar**, **Dr Rajendra Singh, Dr Praveen Singh** and other learned members of my advisory committee for their timely help, valuable suggestions and guidance.

I take this opportunity to express my deep sense of gratitude to **Dr. Vikramaditya Upmanyu, Dr Vishal Chander**, **Dr Chandan Prakash Gupta**, **Dr Y.P.S. Malik, Dr P. Dhar** for providing facility in their lab at any time and their involvement in my work and advice till completion of my thesis.

I am greatly thankful to my seniors **Raja Wasim sir, Adil Sir, Shanthamani Ma'am, Vikas sir, Jobin sir, Alok sir** for their immense help, encouragement and providing friendly environment during the present study and help in work and providing homely environment during my stay at Izatnagar.

No doubt I deserve my enemies, but I do not believe I deserved my friends. Mere words can't explain my feelings for **Amit, Nihar, Bhupesh**, **Arvind, Surendra, James, Bobby, SSD Sacchan, Swati, Hina and Laxmi** have done for me is worth remembering. It is because of their support that I was able to strike the arrow home. I am thankful to them for their support and encouragement. I can never forget the love and affection I got from them.

I am also grateful to my beloved juniors, **Mukesh, Ashok, Arijit, Mousumi, Nitish, Deepika, Gulam, Tarun, Shushobhit, Jugal, Sudipta, Satish, Ravi Mandal, Vishal Rai, Samriddhi, Sandeep, Meesam, Deepak, Sumit, Manu, Dhanesh, Sushil** for their help in work at any time, encouragement during my work and providing lively & homely environment during my stay at Mukteswar as well as Izatnagar.

I am thankful to **Director** and **Joint Directors** (Academic, Research and CADRAD), IVRI, Izatnagar for providing the necessary facilities and financial support.

I am also thankful to **ICMR** for their financial support in the form of "Junior Research Fellowship" during my PhD.

It is a very good opportunity for me to pay special thanks and love to SRF, JRF and RA for their help and moral support during my course of study. I would like to special mention **Dheeraj, Arpit, Shubhankar Sarkar, Shikha, Poonam, and Atul Verma** for their unreserved selfless help, moral support and encouragement.

The staff members of Division of Biological Products deserve special mention for their assistance and co operation during my work. I am grateful to **Shri Bhramha Shankar ji, Sh. Basant Pant ji, Sh. Victor ji, Sh. M.R. Rathore,** and also the staff of Division of Biological Standardization, NPRE and CADRAD for their timely help and co-operation.

I am thankful to **Pantnagar-mess** (PNM) and **Mukteswar-mess** and their workers for providing delicious food during my stay at IVRI, Izatnagar and Mukteswar campus.

I am thankful to **Dharmendra (Chachu), D.P. (Pradeep)** and **Kuldeep** for the compilation and beautification of my thesis.

This thesis would not have been possible without the support of my **family** and friends. With deep emotions I acknowledge the love and affection of my family for what I am today. Words sound hollow and phrases lose their meaning when heart is full of sentiments and my vocabulary utterly fails in expressing my accolade to my revered **parents**. I acknowledge the support of my **mother** and **father** for being with me all the time like a shadow of my own and the guidance from them through all these years. The suffering they faced for me are raison d'être for me being at present stage. I am indebted to **my elder** brother **Arjun** who is my ideal and inspiration for life and whatever I am today I owe to him. I am thankful to god for blessing me with younger brother **Vijay** and **Sonu and with younger sister Neelam** who have been very loving, helpful and kind all the time. My special love to two of my nephews **Aman** and **Aditya.** Mere words can't explain my feelings to my family members for their moral support and encouragement during my long time absence realizing this work far away from the family. I am very proud of all of you are the source of power to my life.

Last but not least, my special thanks to are due to all who have contributed, in many ways to achieve and make this work possible and to be extended to those I forgot to mention in this space notion.

I must record my sincere thanks to all those whom I have not mentioned (unintentionally) but who have helped me during my entire period of study at IVRI.

Above all I thank God and Nature Almighty for giving me patience and strength to overcome the difficulties, which crossed my way in accomplishment of this endeavor.

(Ajay Kumar Yadav)

Date: 28/06/19
Place: ICAR-IVRI, Izatnagar

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ABBREVIATIONS

A_{492}	:	Absorbance at 492 nm
APS	:	Ammonium per-sulphate
AGID	:	Agar Gel Immuno-Diffusion
bps	:	Base pairs
BSA	:	Bovine Serum Albumin
CPE	:	Cytopathic Effect
^{0}C	:	Degree centigrade
cDNA	:	Complementary deoxyribonucleic acid
CDV	:	Canine distemper virus
c-ELISA	:	Competitive ELISA
DEPC	:	Diethyl Pyrocarbonate
CDV	:	Canine distemper virus
CNS	:	Central nervous system
CKD	:	Chronic Kidney disease
DMV	:	Dolphin Morbillivirus
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxy nucleotide tri-phosphate
dpi	:	Day post infection
DW	:	Distilled Water
E.coli	:	*Escherichia coli*
EDTA	:	Ethylene diamine tetraacetic acid
ELISA	:	Enzyme Linked Immunosorbent Assay
EMEM	:	Eagle's Minimum Essential Media
FBS	:	Fetal Bovine Serum
FITC	:	Fluorescein isothiocyanate
Fig	:	Figure
G	:	Guanine
g	:	Earth's gravitational field
h	:	hour
HN	:	Haemagglutinin Neuraminidase
HEPES	:	[(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)]
H_2O_2	:	Hydrogen Peroxide
HN	:	Hemagglutinin-neuraminidase
HRPO	:	Horse radish peroxidase
IVRI	:	Indian Veterinary Research Institute
Kbp	:	Kilo base pair
kDa	:	Kilo Dalton

LB : Luria Bertani (medium)
MAb : Monoclonal Antibody
$MgCl_2$: Magnesium Chloride
MCP : Membrane co-factor protein
Min : Minute
mL : Milliliter
mg : Milligram
mM : Millimolar
mg : Microgram
moi : Multiplicity of infection
ml : Micro-liter
NFW : Nuclease Free Water
ng : Nanogram
nm : Nanometer
NP : Nucleoprotein
OD : Optical Density
OPD : Orthophenylene-diamine Dihydrochloride
ORF : Open Reading Frame
PAGE : Polyacrylamide Gel Electrophoresis
PBMCs : Peripheral Blood Mononuclear Cells
PBS : Phosphate Buffer Saline
PCR : Polymerase Chain Reaction
PDV : Phocine Distemper virus
pmol : Picomole
P-4 or P-5 : Passage-4 or Passage-5
PPRV : *Peste-des-petits-ruminants virus*
PVRL4 : Polio-Virus-Like-Receptor-4
RE : Restriction Endonuclease
RNA : Ribonucleic Acid
rpm : Revolutions per minute
RT-PCR : Reverse Transcription-Polymerase Chain Reaction
RT-qPCR : Reverse Transcription- quantitative Polymerase Chain Reaction (Real time PCR)
SD : Standard Deviation
SED : Standard Error of the Difference
SLAM : Signaling Lymphocyte Activation Molecules
s-ELISA : Sandwich ELISA
TAE : Tris Acetate EDTA
TBS : Tris Buffered Saline
TBS-T : TBS-Tween

Taq	:	*Thermus aquaticus*
$TCID_{50}$	:	50% Tissue Culture Infective Dose
TE	:	Tris EDTA
Tris	:	Tris (hydroxymethyl) amino methane
UV	:	Ultra Violet
Vero-DST	:	Vero-DogSLAMtag
Vero/SLAM	:	Vero cells constitutively expressing SLAM of either species
VGS	:	Vero/goat/SLAM cells
VSS	:	Vero/sheep/SLAM cells
VDS	:	Vero/dog/SLAM cells
WHO	:	World health organization

LIST OF TABLES

Table No.	Title	Before Page No.
Table 1	Oligonucleotide primers details used in PCR	45
Table 2	Percent identity at nucleotide and amino acids level between SLAM and Nectin-4 genes of Caprine with other species	68
Table 3	Analysis of V-Region of SLAM of Goat, Sheep and Dog SLAM at Amino Acids Level	70
Table 4	Comparative Titre ($TCID_{50}$/mL) of PPRV both in supernatant (4a) and cell-lysate (4b) in one-step Growth Curve in different Cell Lines	76
Table 5	Comparative Titre ($TCID_{50}$/mL) of CDV both in supernatant (5a) and cell-lysate (5b) in one-step Growth Curve in different Cell Lines	76

LIST OF FIGURES

Fig. No.	Title	After Page No.
Fig.1a & 1b	Schematic diagram of a PPR Virus (a); Genome arrangement of PPRV (b).	10
Fig.2a & 2b	Schematic diagram of a Canine Distemper Virus (CDV) (a); Genome organization of CDV (b).	12
Fig.3	Schematic diagram of CD46 molecule	24
Fig.4	Schematic diagram of SLAM receptor molecule	24
Fig.5	Schematic diagram of Nectin-4 receptor molecule	28
Fig.6	Schematic diagram of pJET1.2/blunt vector map	45
Fig.7	Schematic diagram of pcDNA3.1(+) mammalian expression vector map	45
Fig.8	Strategy employed for selection of transfection reagent for Vero cells in 4-well plate	59
Fig.9	Step-wise transfection protocol of SLAM gene into Vero cells using Lipofectamine3000	59
Fig.10	Generating Stable Vero cell line by antibiotic (G418) selection method	59
Fig.11	Flow-chart describing technical programe of present study	65
Fig.12	Amplification of full-length SLAM gene	67
Fig.13	Colony PCR of SLAM gene	67
Fig.14	Amplification of full-length Nectin-4 genes	67
Fig.15	Colony PCR of Nectin-4 gene	67
Fig.16a	Alignment report of SLAM sequences of PPRV host	67
Fig.16b	Alignment report of SLAM sequences of CDV host	67
Fig.16c	Alignment report of Nectin-4 gene	67
Fig.17	Amplification of full-length SLAM gene of goat with expression primers	69

Fig. No.	Title	After Page No.
Fig.18	Colony PCR of SLAM gene by expression primers	69
Fig.19	RE digestion analysis of recombinant pcDNA3.1 Goat, Sheep and Dog SLAM plasmids with XhoI and HindIII enzymes	71
Fig.20	Sequence details of goat SLAM in pcDNA3.1(+) vector	71
Fig.21	Sequence details of sheep SLAM in pcDNA3.1(+) vector	71
Fig.22	Sequence details of dog SLAM in pcDNA3.1(+) vector	71
Fig.23	Linearization of goat, sheep and dog expression cassette	71
Fig.24	Kill curve analysis using G418 antibiotic in Vero cells	71
Fig.25	Selection of transfection reagent	71
Fig.26	Generation of stable Vero/goat/SLAM (VGS) cell line	73
Fig.27	Generation of stable Vero/sheep/SLAM (VSS) cell line	73
Fig.28	Generation of stable Vero/dog/SLAM (VDS) cell line	73
Fig.29	Characterization of stable Vero cells at transcript level by RT-PCR	73
Fig.30	Characterization of stable Vero cells at genomic level	73
Fig.31	Revival of VGS, VSS and VDS cell lines after cryopreservation and checking of SLAM transcript in revived stable Vero cells by RT-PCR	73
Fig.32	Immunofluorescence staining of Vero/goat/SLAM cells expressing FLAG tagged goat SLAM protein with anti-FLAG monoclonal antibodies and anti-mouse FITC conjugate	73
Fig.33	Immunofluorescence staining of Vero/sheep/SLAM cells expressing FLAG tagged goat SLAM protein with anti-FLAG monoclonal antibodies and anti-mouse FITC conjugate	73
Fig.34	Immunofluorescence staining of Vero/dog/SLAM cells expressing FLAG tagged goat SLAM protein with anti-FLAG monoclonal antibodies and anti-mouse FITC conjugate	73
Fig.35	Relative expression of SLAM gene by RT-qPCR in VGS, VSS and VDS cells	75

Fig. No.	Title	After Page No.
Fig.36	One-Step Growth Curve of PPRV in different cell lines	75
Fig.37	Determination of antigenic mass of one-step growth curve samples of PPRV by monoclonal antibody based sandwich ELISA	75
Fig.38	Comparative Titre of PPR Virus in Different Cell Lines	75
Fig.39	One-Step Growth Curve of CDV in Different Cell Lines	75
Fig.40	Comparative Titre of CDV in Different Cell Lines	77
Fig.41	Comparative mean titre of PPRV and CDV in VGS, VSS and VDS cell lines as compared to native Vero	77
Fig.42	PPR Virus isolation in Vero/goat/SLAM (VGS) and native Vero cells	77
Fig.43	CDV isolation in Vero/goat/SLAM (VGS) and native Vero cells	77
Fig.44	PPR Virus isolation in Vero/dog/SLAM (VDS) cells	77

CONTENTS

Sl. No.	CHAPTER	PAGE NO.
1.	INTRODUCTION	01-04
2.	REVIEW OF LITERATURE	05-41
3.	MATERIALS AND METHODS	42-65
4.	RESULTS	66-77
5.	DISCUSSION	78-94
6.	SUMMARY AND CONCLUSIONS	95-99
7.	MINI ABSTRACT	100
8.	HINDI ABSTRACT	101
9.	REFERENCES	102-139
10.	APPENDIX	

Introduction

1 INTRODUCTION

Peste-des-petits ruminants (PPR) is also known as 'goat plague', 'Kata','syndrome of stomatitis-pneumoenteritis' or 'ovine rinderpest'. It is an economically important infectious viral disease of domestic and wild small ruminants that threatens the food security and sustainable livelihood of farmers across Africa, the Middle East and Asia (Banyard et al., 2010). PPR is caused by *Peste-des-petits ruminants virus* (PPRV) which belongs to genus *Morbillivirus* under family *Paramyxoviridae* (Gibbs *et al.*, 1979). The other members of same genus are *Rinderpest virus* (RPV), *Canine distemper virus* (CDV), *Measles virus* (MV), *Phocine distemper virus* (PDV) and morbillivirus of porpoise and dolphins. Clinically, PPR is characterized by pyrexia, necrotic stomatitis, catarrhal inflammation of the ocular and nasal mucosa, enteritis and bronchopneumonia followed by death or recovery from the disease. The disease was first reported in Ivory Coast (West Africa) in 1942 (Gargadennec and Lalanne, 1942) and made geographical extension in Arabian Peninsula (Taylor, 1984), Middle East (Lefevre *et al.*, 1991), India (Shaila *et al.*, 1989), Bangladesh, Pakistan, Nepal, Israel and Saudi Arabia (Shaila *et al.*, 1996) and more recently to Central Asia (Kwiatek *et al.*, 2011; Libeau *et al.*, 2014) and Europe (Parida *et al.*, 2016). In India, PPR was first reported in Tamil Nadu state in 1987 after which it was reported throughout the country (Dhar *et al.*, 2002; Nanda *et al.*, 1996) and is now endemically present (Singh *et al.*, 2004d; Singh *et al.*, 2011). PPRV has been classified into four distinct lineages

Canine distemper (CD) is an important cosmopolitan infectious disease and considered highly fatal disease of dogs only next to rabies (Swango *et al.*, 1995). CD is caused by *Canine distemper virus* (CDV) which was first isolated by Carre´ in 1905 (Carre´, 1905). CDV infects a broad range of animals such as Canidae (domestic dogs, foxes, wolves),

Mustelidae (ferrets, minks, skunks, weasels, badgers), Procyonidae (raccoons), Ursidae (bears and pandas), Viverridae (civets, genets, and linsangs), Hyaenidae (hyenas), Felidae (lions and tigers) and fresh-water seals (Appel *et al*., 1994; Harder and Osterhaus, 1997; Guo *et al*., 2013). CDV causes generalized disease with prominent respiratory, gastrointestinal and nervous signs (Scagliarini, *et al*., 2003). Infected animals develop fever, cough, coryza and conjunctivitis (Summers *et al*., 1994). CDV being a member of morbillivirus also encodes haemagglutinin 'H' and fusion 'F' surface envelope glycoproteins which are responsible for virus attachment and fusion process respectively and important for virus replication (Sawatsky and von Messling, 2012). The 'H' protein of CDV is highly variable (Iwatsuki *et al*., 2000) and forms basis for molecular epidemiology of the virus. The worldwide isolates of CDV have been grouped phylogenetically into 14 clades/clusters and named according to geographical distribution: America 1 and 2, Arctic-like, Asia 1–4, Europe 1/South America 1 (EU1/SA1), European wildlife, Rockborn like, South America 2 (SA2) and 3 (SA3), and Africa 1 and 2 (Loots *et al*., 2017). The America 1 clade is believed to be the ancestor of currently reported epidemics all over globe and might have played a key role in initial dissemination of virus to other geographies (Panzera *et al*., 2015). Molecular evolutionary analyses has revealed diversity of 'H' gene and identified two critical substitutions at positions 530 and 549 of signaling lymphocytic activation molecule (SLAM) receptor binding site of 'H' gene (McCarthy *et al*., 2007). This is likely to be responsible for host switch-over, severity of signs, viral fitness and determinant of host cell tropism (Bieringer *et al*., 2013; McCarthy *et al*., 2007; Sattler *et al*., 2014).

Cellular receptors are considered to be major determinants of host range and tissue tropism. After entry, PPR virus first replicates in local lymph nodes like tonsils, mandibular and pharyngeal lymph nodes and causes viriaemia subsequently. Most morbilliviruses have established lymphotropism and epitheliotropism that are receptor-dependent (Cosby *et al*., 2002; De Vries *et al*., 2012; Tatsuo *et al*., 2000). The epithelial cells, activated lymphocytes and macrophages have been reported to be the target of RPV (Rey-Nores *et al.*, 1995; Rossiter *et al.*, 1993; Wohlsein *et al.*, 1993) but confirmed target cells for PPRV is yet a mystery. However, profound lymphopenia and immunosuppression during acute phase of PPR and close nature of viruses suggest that lymphocytes and other cells are probably targets for PPR virus as well. Specific interactions between cellular receptors and the viral 'H' protein facilitate virus entry into host cells by inducing virus-cell and cell-cell membrane fusion in

cooperation with the fusion protein 'F' (Birch *et al*., 2013; Muhlebach *et al*., 2011; Noyce *et al*., 2011; Pratakpiriya *et al*., 2012; Takimoto *et al*., 2002).

Morbilliviruses are highly lymphotropic viruses and use signaling lymphocyte activation molecule (SLAM/CD150) as an immune cell entry receptor that is expressed on the surface of activated T- and B-lymphocytes, macrophages and dendritic cells (Hsu *et al*., 2001; Tatsuo *et al*., 2001; Von Messling *et al*., 2004. The signaling lymphocytic activation molecule (SLAM) or CD150 has been reported as receptor for MV (Tatsuo *et al.*, 2001), CDV (Tatsuo *et al.*, 2001; Seki *et al.*, 2003) and RPV (Tatsuo *et al.*, 2001) and it is likely that SLAM too acts as a receptor for PPR virus. SLAM is a 70 kDa glycoprotein belonging to the CD2 subset of the immunoglobulin superfamily and is expressed on the surface of a proportion of primary B cells, activated T cells, memory T cells, T cell clones and immature thymocytes (Tangye *et al.*, 2000). It is rapidly induced on naïve lymphocytes after activation and cross-linking antibodies to SLAM stimulate B and T-cell proliferation (Aversa *et al.*, 1997b; Cocks *et al.*, 1995; Punonen *et al.*, 1997). Activated human T cells also express soluble form of SLAM (sSLAM) which differs from the membrane form of SLAM (mSLAM) in lacking 30 aa encompassing the entire 22 aa transmembrane region (Cocks *et al.*, 1995). The predicted amino acid sequences of SLAM of human, mice, dog, cattle and marmoset have 60-70% identity with exceptionally high identity 86% between human and marmoset SLAM (Castro *et al.*, 1999; Tatsuo *et al.*, 2001; Tatsuo and Yanagi, 2002).

Recently, polio virus like receptor-4 (PVLR-4/nectin-4) was recently identified as an epithelial tissue receptor where SLAM is not expressed for MeV, CDV and PPRV (Birch *et al*., 2013; Noyce *et al*., 2013,). The nectin-4 belongs to cell adhesion molecule family (nectin-1 to 4), and only nectin-4 acts as an epithelial receptor (Mühlebach *et al*., 2011; Noyce *et al*., 2011). Nectins are also involved in the establishment of apical–basal polarity at cell–cell adhesion sites and the formation of tight junctions in epithelial cells (Ogita *et al*., 2010; Takai *et al*., 2008). Human Nectin-4 receptor, a major component of the adherens junctions found in the epithelium where it is believed to facilitate spread of MeV (Muhlebach *et al*., 2011; Niessen *et al*., 2007).

The vaccine strains of CDV (Onderspoort) and MV (Edmonston) have been passaged on SLAM negative cells and are believed to use an alternate receptor(s) besides host SLAM,

probably due to *in vitro* adaptation (Dorig *et al.*, 1993; Naniche *et al.*, 1993; Tatsuo *et al.*, 2001). A marmoset lymphoid cell line, B95a expressing high levels of marmoset SLAM has been found to be very sensitive system for morbillivirus isolation (Kobune *et al*., 1991; Tatsuo *et al*., 2000; Sreenivasa *et al*., 2006). Furthermore, transfected vero cell line expressing SLAM (Vero dog SLAM) is more sensitive than vero cells for isolation of wild type virus (Seki *et al*., 2003; Neilson *et al*., 2008). Interestingly, monkey cell lines vero and CV1 expressing goat SLAM were found to be more sensitive than those expressing cattle SLAM for production of PPR vaccine virus or isolation of wild type PPRV from pathological specimens (Adombi *et al*., 2011; Sarkar *et al*., 2007). The possible explanation for differential sensitivity of caprine and bovine SLAM tag cell lines may probably be due to closeness of caprine SLAM to ovine SLAM rather than bovine SLAM (Sarkar *et al*., 2007). It has also been found that CD150 negative cell line-expressing host SLAM is a good host for wild-type as well as vaccine strain of measles virus (Erlenhofer *et al.*, 2002). Similar findings have been reported for RPV and CDV (Tatsuo *et al.*, 2001). Vero cell line expressing canine SLAM has been reported to be highly susceptible to wild type strains of CDV than B95a cells (Tatsuo *et al.*, 2001). This was proved by rapid isolation of CDV in Vero cells expressing canine SLAM from majority of clinical specimens as early as just one day after inoculation unlike 7 to 10 days in B95a cells with very less isolation rate from clinical specimens (Seki *et al.*, 2003).

Keeping all this in background, the present study was designed with the following objectives-

1. **To develop and characterize the Vero cell line constitutively expressing goat, sheep and dog-SLAM and or Nectin-4 receptors.**
2. **To evaluate and study on comparative sensitivity of developed cell lines for isolation and propagation of PPRV and CDV.**

✍✍✍

Review of Literature

2 REVIEW OF LITERATURE

Morbilliviruses are highly contagious pathogens that cause some of the most devastating viral diseases of humans and animals across the world. These morbilliviruses are lymphotrophic and induce multisystemic infections in their highly susceptible natural hosts, causing disease with high morbidity and mortality worldwide. The genus, Morbillivirus, belongs to the *Paramyxoviridae* family in the order, *Mononegavirales*. Members of the genus *Morbillivirus* include *Measles virus* (MV) of primates, *Canine distemper virus* (CDV) of carnivores, *Rinderpest virus* (RPV) of cloven-hoofed large animals and *Peste-des-petits ruminants virus* (PPRV) in small ruminants. The genome is a negative-sense, non-segmented, single-stranded RNA, and its structure is conserved with the nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), attachment (H) and large (L) genes present in all morbilliviruses (Griffin *et al*., 2007). The peculiarities of the Morbillivirus genus are its lack of neuraminidase activity (hemagglutinating activity is maintained) of the attachment protein (H) and its expression of an additional C-protein in the P locus (the P/C/V gene) by an RNA-editing process. The H gene encodes a key protein for morbillivirus and its animal hosts: the virus uses this protein to attach to cell receptors during the first step of infection (Griffin *et al*., 2007). The size [15,690 nt for for canine distemper virus (CDV)] and structure of the genome of morbilliviruses are similar to those of members of the Respiroviruses genus, but the two viruses are not phylogenetically related. The morbilliviruses typically are formed of inclusion bodies (with a nucleocapsid like structure) in the cytoplasm and nucleus of infected cells. Currently, seven species in the genus morbillivirus are known that can infect mammalians from different orders. The six are CDV (infecting animals of order Carnivora), *measles virus* (primates), peste-des-petits-ruminants

virus (animals of order Artiodactyla, predominantly ruminants and swine), *cetacean morbillivirus* virus (CeMV, infecting animals of order Cetacea), *phocine distemper virus* (PDV, infecting seals), *rinderpest virus* (infecting dolphins). The seventh member is feline morbillivirus (FmoPV) first described in 2012 as a morbillivirus of cats, involving with chronic kidney disease (CKD) (Kumar *et al*., 2014; de Vries *et al*., 2015). Phylogenetically, PDV is closely related to CDV. In contrast, other morbilliviruses from marine mammals are more closely related to the rinderpest and peste-des-petits-ruminants viruses. They cause fever, coryza, conjunctivitis, gastroenteritis, and pneumonia in their respective host species. The major sites of viral propagation are lymphoid tissues, and acute diseases are usually accompanied by profound lymphopenia and immunosuppression, leading to secondary and opportunistic infections (Appel *et al*., 1972, Griffin *et al*., 1996, Murphy *et al*., 1999). Natural infections by all of these, share common features, including lymphopenia and inhibition of lymphocyte proliferation in the acute phase (Griffin, 2007; Heaney *et al*., 2002; Von Messling *et al*., 2003; Yanagi *et al*., 2006). Although live vaccines have effectively reduced the incidence of disease, the highly contagious morbilliviruses are still a major problem in human and veterinary medicine (Greene & Appel, 2006; Rima & Duprex, 2006). The search for the receptor for morbillivirus began in vaccine strains of MeV, and subsequently identified receptors for wild-type strains have revealed the closely related receptor usage and unique pathogenicity of the viruses.

2.1 The Disease

(a) Peste-des-petits ruminants (PPR)

Peste-des-petits-ruminants (PPR) is an acute and highly contagious viral disease of sheep and goats (Dhar *et al.,* 2002). Clinically, PPR is characterized by pyrexia, necrotic stomatitis, catarrhal inflammation of the ocular and nasal mucosa, enteritis and bronchopneumonia (Gargadennec and Lalanne, 1942) followed by death or recovery from the disease. The term 'Peste-des-petits-ruminants' is a French phrase for "disastrous disease of small ruminants" (Snow *et al*., 2011). The disease has been variously called by early workers as: "Goat catarrhal fever" (McCulloch, 1951; Campbell, 1958); "Kata" (Whitney *et al*. (1967); "Goat pneumoenteritis complex" (Isount and Mann, 1972; Nduaka and Ihemelendu, 1973)

and pseudo-rinderpest (Durtnell and Eid, 1973). PPR which reflect clinical pictures of the disease has a high rate of morbidity and mortality and effective control of this disease will be of significant economic importance in endemic areas.

PPR occurs as peracute to subacute in domestic small ruminants depending on clinical outcome of the disease (OIE, 2013). Clinical symptoms appear more often after an incubation period of 2-6 days, followed by sudden onset of pyrexia with rise in rectal temperature to 106°F. The affected animals show dullness and depression and their body hair coat stands erect. A serous discharge runs out from eyes, nostrils and mouth turning mucopurulent in later stages which causes matting together of eyelids and difficulty in breathing by obstruction of nares. After 1-2 days of onset of pyrexia, oral mucosa becomes hyperemic and small grayish necrotic foci appear inside oral cavity including gums, dental pad, and upper surface of tongue. These foci may coalesce forming a layer of dead cells underneath which lay shallow erosions that are foul smelling and painful with the progression of the disease. Diarrhea sets in 2-3 days after onset of pyrexia and stools may vary from soft, watery, fetid, blood stained and occasionally contain gut tissue shreds depending on the severity of the disease. The affected animals have painful and noisy abdominal breathing with rapid respiration rates with bouts of soft painful cough in severe cases indicating obvious pneumonia. Such severely affected animals undergo dehydration with eye balls deep inside their sockets, emaciation and hypothermia which eventually succumb after 7-10 days of onset of clinical reactions (OIE, 2013). The pregnant animals may abort (Kulkarni *et al*., 1996; Abubakar *et al*., 2012). The animals with less severe clinical reaction may recover after protracted convalescence and are immune to subsequent PPRV infection for whole life (Hamdy *et al*., 1976).

Peracute form is manifested in immuno-naïve populations of sheep and goat lacking herd immunity characterized by high fever, depression and higher mortality with 100% case fatality. The subacute form of disease commonly occurs in local susceptible breeds and in experimental animals. Such animals do not exhibit typical clinical disease rather show inconsistent signs of affection and low mortality. Around 6th day, pyrexia and serous nasal discharge is seen and fever drops with onset of diarrhoea which in severe cases causes dehydration and prostration. Recovery from the disease usually occurs within 10–14 days.

Large ruminants like cattle and buffalo undergo subclinical infection without any presenting signs of disease but often seroconvert, generating strong neutralizing antibody response which are cross protective against Rinderpest as well (Holzer *et al*., 2016).

(b) Canine distemper (CD)

Canine Distemper virus (CDV) also belongs to genus Morbillivirus, family *Paramyxoviridae* and is the causative agent of a severe infectious disease affecting a broad range of wild and domestic carnivores. CDV is also known as a highly prevalent viral infectious disease of carnivores, posing a conservation threat to endangered species around the world (McCarthy *et al*., 2007, Beineke *et al*., 2009, Martella *et al*., 2010, Gilbert *et al*., 2014). It was first isolated by Carré in 1905. Even recently, it was proposed to rename the virus "Carnivore Distemper Virus" due to its constant threat for different carnivorous species and to commonly documented outbreaks in non-dog carnivores (Terio *et al*., 2013). CDV has also been found in naturally infected non-canine hosts (Kapil *et al*., 2011) and recent dramatic outbreaks in breeding colonies of rhesus macaques (Macaca mulatta) and cynomolgus macaques (Macaca fascicularis), has drawn attention of a possible zoonosis in the post-measles eradication times (de Vries *et al*., 2014). Generally, CDV exhibits lympho-, neuro- and epitheliotropism resulting in systemic infection of almost all organ systems including respiratory, digestive, urinary, lymphatic, endocrine, cutaneous, skeletal and central nervous system (CNS) (Von Messling *et al*., 2004, Lempp *et al*., 2014). The disease course and pathogenesis in canine distemper resemble those of human measles virus infection including, fever, rash, respiratory signs, lymphopenia, and profound immunosuppression with generalized depletion of lymphoid organs during the acute disease phase (Von Messling *et al*., 2006). In addition, CDV infection shows a high incidence of neurological complications (Lempp *et al*., 2014).

Two clinical forms of CDV can be distinguished in animals with minimal or no immune response: *an acute systemic form* and a *chronic nervous form* (Krakowka *et al.,* 1985; Baumgärtner, 1993). Acute systemic disease occurs 2–3 weeks post-infection (Williams, 2001). The virus continues to replicate and spread throughout the body causing severe clinical signs, which include biphasic fever, mucopurulent oculonasal discharge, coughing, dyspnoea, depression, anorexia, vomiting and diarrhoea (which may be bloody) (Thulin *et al.,* 1992;

Summers and Appel, 1994). During this stage of infection, the virus is found in every secretion and excretion of the body. Hyperkeratosis and parakeratosis with vesicles and pustule formation may also be noticed in dogs. CDV infection during early developmental stages, before the eruption of permanent dentition, can also infect tooth buds and ameloblasts causing clear enamel hypoplasia (Bittegeko *et al.,* 1995). Neurological signs may be concurrent or follow systemic disease within 2–3 weeks. Signs are progressive and varied depending on the area of the brain affected but commonly include abnormal behaviour, convulsions or seizures, chewing-gum movements of the mouth, blindness, cerebellar and vestibular signs, paresis or paralysis, incoordination and circling (Appel *et al.,* 1991; Williams, 2001). Twitching of a muscle or group of muscles in the leg or face (Andrea, 1995) and involuntary urination as well as defecation are among the many neurological manifestations of CDV infections (Greene and Appel, 2006). Infection in the central nervous system results in acute demyelinization, and most animals die 2–4 weeks after infection (Winters *et al.,* 1983; Appel *et al.,* 1984). Due to the immune compromising nature of CDV, clinical signs are often exacerbated by secondary bacterial infections of the skin and respiratory tract (Greene and Appel, 1990).

2.2 Causative agents

(a) *Peste-des-petits-ruminants virus (PPRV)*

The causative agent of the disease Peste-des-petits-ruminants (Goat plague), PPR virus (PPRV) is classified under the order Mononegavirales, family *Paramyxoviridae* and genus *Morbillivirus* (Van Regenmortel, 2000). Other members of this genus are, *Rinderpest virus* (RPV), *Canine distemper virus* (CDV), *Measles virus* (MeV), *Phocine distemper virus* (PDV) and morbillivirus of marine mammals such as porpoise and dolphins, which leads to distemper in several seal species and *cetacean morbilliviruses* (CeMV), which cause disease in dolphins and whales (Banyard *et al*., 2010). Off late, sequences analysis studies revealed a bat morbillivirus (endogenous to neotropical vampire bats found in Brazil), potentially representing member of the genus *Morbillivirus* (Drexler *et al.,* 2012; Vries *et al.,* 2015). All these viruses are immunologically cross reactive. There is only one serotype of PPR, but there are at least 4 lineages distinguishable by nucleic acid sequencing (Muniraju *et al.,* 2013). The virus is not very resistant and is hastily inactivated at environmental temperatures by solar

radiation and desiccation (Geerts *et al*., 2009). PPRV is enveloped and has helical nucleocapsid **(Fig. 1).**

The genome of PPRV is a single-stranded RNA, approximately 16kb long with negative polarity (Haas *et al.,*1996). The nucleocapsid have characteristic "herring-bone appearance". PPRV genome is organized into six transcriptional units encoding six structural proteins viz., the nucleocapsid (N) protein, the phosphoprotein (P), the matrix (M) protein, the polymerase or large (L) protein, two envelope proteins [haemagglutin (H) and fusion (F) protein] and two nonstructural proteins (C,V). The gene arrangement from 3' to 5' on the genome is N-P-M-F-H-L separated by intergenic region (Baron and Barret, 1995; Diallo 1989 and Bailey *et al*., 2005), which is CTT in most cases (Crowley *et al.,* 1988; Baron and Barrett, 1995). At each end of the genome there is a short sequence of 52 and 37 nucleotides (but 38nt for CDV and PDV) respectively at 3' and 5' ends and named leader for 3' end, and trailer for 5' end (Haffar *et al.,*1999). For all morbiliviruses as for many paramyxoviruses, the number of nucleotides composing the entire genome is in agreement with the so-called "rule of six" (Calain *et al.,* 1992). This rule reflects the fact that each N protein molecule associates with exactly 6 nucleotide of the genomic RNA. Full length sequence have been reported from vaccine strain [Mutuchelvan, 2004 (Accession number AJ849636), Siddappa *et al.,* 2014 (accession no. KF727981)] as well as virulent strain [Bailey *et al.,* 2005 (Accession number AY560591)]. The genome was 15948 nucleotides in length for PPRV Sungri/94 (an Indian vaccine strain) (Siddappa *et al.,* 2014) and same number of nucleotides in length for the Tu/00 isolate (Bailey *et al.,* 2005). Full length genome sequence for other morbiliviruses are also available: MeV (Cattaneo *et al.,* 1989), RPV (Baron and Barrett, 1995), CDV (Barertt *et al.,* 1987), and the *dolphin morbilliviruses* (DMV) (Rima *et al.,* 2003).

(b) *Canine distemper virus (CDV)*

Canine distemper virus is about 150 to 300 nm in diameter and contains ~15.7 Kb long non-segmented, single-stranded negative sense genome surrounded by a lipid envelop (Murphy *et al.,* 1999). Similar to other paramyxoviruses the CDV has approximately 15.7 Kb RNA genome and an enveloped virus particle that is 150 to 300 nm in diameter (Murphy

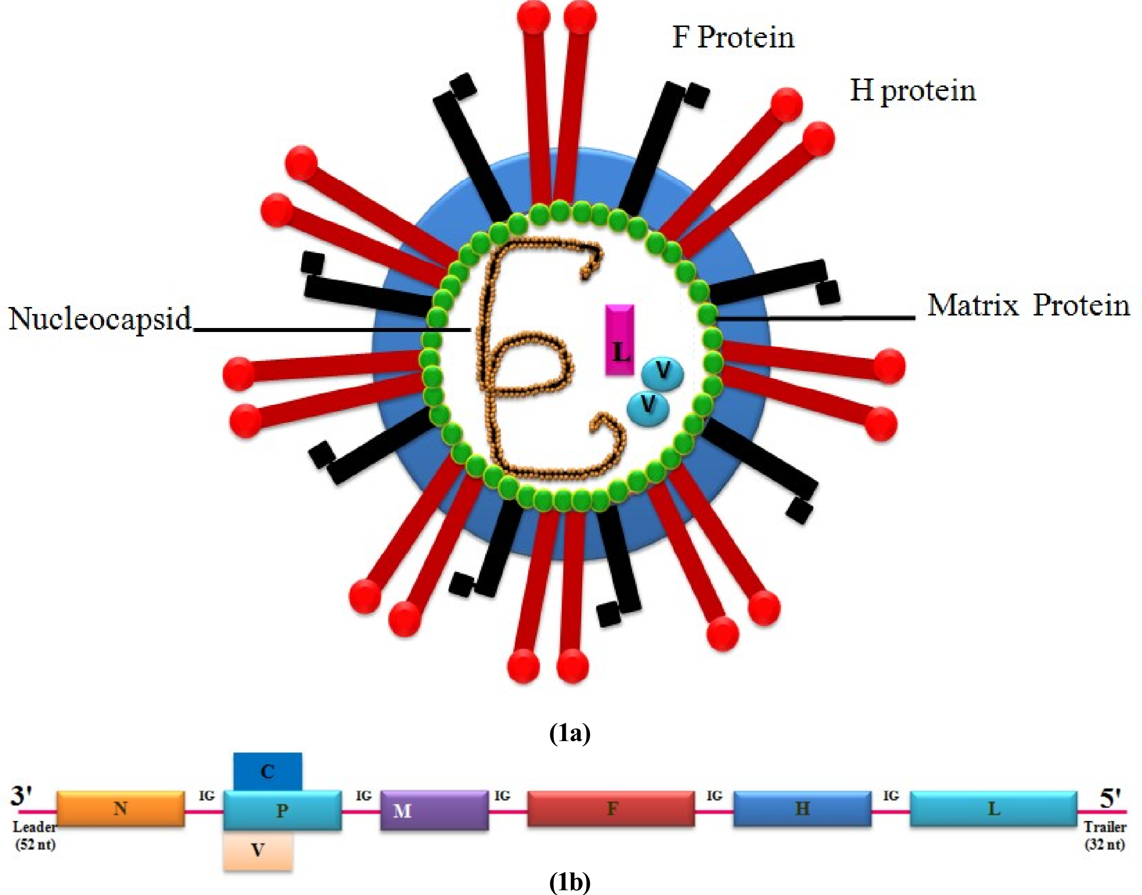


Fig. 1 a-b: Schematic diagram of a PPR Virus (a); Genome arrangement of PPRV (b). PPRV virions are enveloped and pleomorphic in shape which varies in size from 150 to 700 nm (Mean size 500nm). The virions contain a negative-strand RNA genome enclosed in a ribonucleoprotein (RNP) core. The genomic RNA is packaged by nucleoprotein (N) to form nucleocapsid along with phosphoprotein (P) and large protein (L). The virus genome is ~16kb in length (15948 nts) which consists of six structural (N, P, M, F, H and L) and two non-structural (V and C) proteins. At the 3' and 5' ends, there are untranslated regions of 52 nt and 37 nucleotide, respectively. IG = Intergenic Region, N = Nucleocapsid Protein, P= Phosphoprotein, M = Matrix Protein, F = Fusion Protein, H = Hemagglutinin Protein and L = Large Protein (Polymerase).

et al., 1999) and contains six structural proteins, termed nucleocapsid (N), phosphoprotein (P), large polymerase (L), matrix (M), hemagglutinin (H) and fusion (F) protein, and one accessory non-structural proteins (C) that were found as extra transcriptional units within the P gene (Orvell *et al*., 1980, Diallo, 1990) **(Fig. 2).** It is a labile virus and is sensitive to heat, UV irradiation, lipid solvents, detergents and oxidising agents (Grone *et al*., 1998). It can survive at room temperature in tissues and exudates for between 20 minutes and 3 hours. In environmental temperatures below zero it will survive several days if protected by organic material (Greene & Appel, 2006).The lipoprotein envelope is readily destroyed by lipid solvents which renders the virus non-infectious. It contains the H and F glycoproteins that induce a neutralizing antibody response (Appel & Summers, 1999). The H glycoprotein is responsible for viral attachment to the host cell and may also play a role in induction of protective immunity (Pardo *et al*., 2005). It also shows the greatest genetic variation that allows for the distinction of various lineages according to a geographical pattern of distribution irrespective of the species of identification (Martella *et al*., 2006). Phylogenetic studies based on the complete sequence of the hemagglutinin gene (HA) of several CDV strains isolated in distinct geographical locations around the world have revealed a phylogeographic pattern of genetic diversity. According to this pattern, there are fourteen distinct lineages known as America 1 (that includes almost all commercially available vaccines), America 2, America 3, America 4, Arctic-like, Rockborn-like, Asia 1, Asia 2, Africa 1, Africa 2, European Wildlife, Europe/ South America 1, South America 2 and South America 3 (Espinal *et al*., 2014; Panzera *et al*., 2012; Wilkes *et al*., 2914), although recently, a new criterion establishes that strains with <2 % divergence at the HA protein level within a single clade should be classified as sub-genotypes (Budaszewski *et al*., 2014).

2.3 Transmission, host range and pathogenesis

2.3.1 Transmission

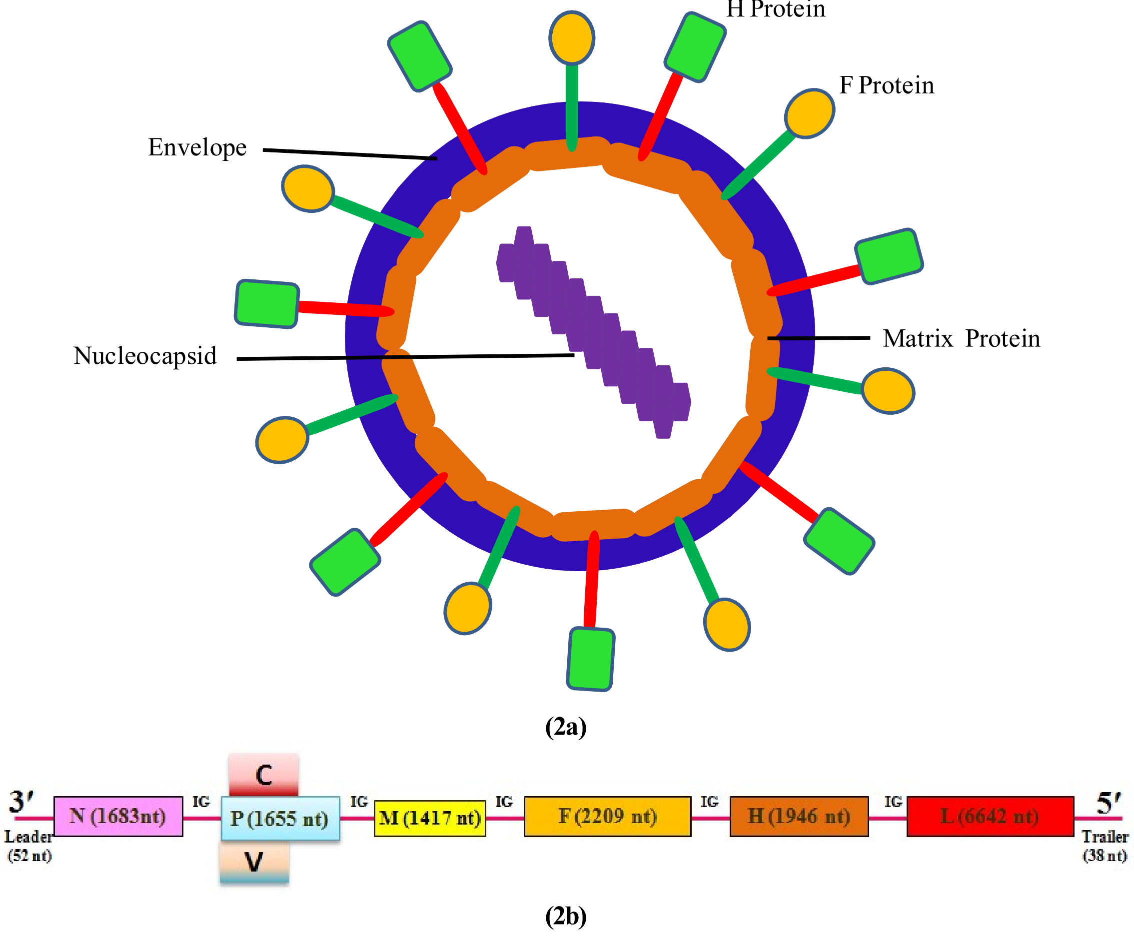
PPR is mainly transmitted by close contact of susceptible animals with infected ones (Braide, 1981). Transmission of PPR from infected to susceptible animals is achieved by direct contact, by contact with secretions or excretions of infected animals (nasal or ocular discharges, saliva, faeces, urine) contact or through respiratory and oral routes (Chauhan *et*

al., 2011). All the secretions and excretions of the sick animal are laden with virus and retain their infectivity for at least seven days after onset of disease (Abegunde *et al.,* 1977). Fine infective droplets are released into the air from these secretions and excretions, particularly when affected animals cough and sneeze (Taylor, 1984; Bundza *et al.,* 1988). Animals in closed contact inhale the droplets and are likely to become infected. Although close contact is the most important way of transmission of the disease, it is suspected that infectious materials can also contaminate water and feed trough and bedding, turning them into additional source of infection. There is currently no evidence for vertical transmission of PPRV (OIE, 2013).

The major mode of CDV transmission is through aerosolization of respiratory exudate containing virus, although other body excretions and secretions (e.g., urine) can result in infection in susceptible hosts if aerosolized. Canine distemper is highly contagious, and viral shedding may follow infection for 60–90 days (Greene *et al*., 1990).Transplacental infection has been documented in domestic dogs (Krakowka *et al*., 1977). The epidemiologic role of vertical transmission in CD and whether or not such transmission can occur in nondomestic species are unknown. Although usually short-lived in the environment, the virus can survive at lower temperatures (e.g., 48 hr at 25°C and 14 days at 5°C) (Shen *et al*., 1980) and may be transmitted either by direct contact or by fomites.

2.3.2 Host range

PPR mainly affects sheep and goat, though severity of the clinical symptoms is more predominant in goats (Singh *et al.,* 2004). Goats have been shown to be more susceptible than sheep (Dhar *et al.,* 2002; Truong *et al.,* 2014; Wernike *et al*., 2014). Outbreaks in other species like, Indian Buffalo (Govindarajan *et al*., 1997), zoological collections, which include gazelles and gemsbok (Furley *et al.,* 1987), have also been recorded. Bovine may be sub-clinically infected without any apparent clinical illness (Anderson and McKay, 1994).Cattle and pigs develop serum neutralizing antibody following experimental PPR virus infection but not susceptible to clinical disease (Gibbs *et al.,* 1979; Anderson and McKay, 1994). The PPRV antigen has also been detected in an outbreak of respiratory disease in camels of Djibouti in Africa (Roger *et al.,* 2000).Antibody against PPRV has been reported in camels and cattle from Ethiopia (Abraham *et al.,* 2005). In 2010 Khalafalla *et al* isolated PPRV samples from



(2a)

(2b)

Fig. 2 a-b: Schematic diagram of a Canine Distemper Virus (CDV) (a); Genome organization of CDV (b). CDV virions are pleomorphic in shape, surrounded by lipid envelop and size varries from 150 to 300 nm. The virions contain a negative-strand RNA genome enclosed in a ribonucleoprotein (RNP) core. The genomic RNA is packaged by nucleoprotein (N) to form nucleocapsid along with phosphoprotein (P) and large protein (L). The virus genome is ~16kb in length (15690 nts) which consists of six structural (N, P, M, F, H and L) and two non-structural (V and C) proteins. At the 3' and 5' ends, there are untranslated regions of 52 nt and 38 nucleotide, respectively. IG = Intergenic Region, N = Nucleocapsid Protein, P= Phosphoprotein, M = Matrix Protein, F = Fusion Protein, H = Hemagglutinin Protein and L = Large Protein (Polymerase).

camel. Saeed *et al,* revealed the existence of PPRV infection in dromedary camels in Sudan in 2015. More recently, the PPRV identified in bharal (*Pseudois nayaur*) in Tibet and was found to be closely related to other circulating PPRV variants recently identified in sheep and goats from Tibet (Bao *et al.,* 2011). Newborn animals become susceptible to PPRV infection at three to four months of age (Srinivas and Gopal, 1996). In endemic areas, most of the sick and dying animals are over four months and up to 18 to 24 months of age (Roeder and Obi 1999). Morbidity and mortality rates are high. Thus, this disease is a serious problem for the small ruminant industry. The disease is most prevalent in animals less than one year of age.

All Morbilliviruses except CDV have a tightly restricted host range, whereas CDV has a broad host range infecting many carnivores including dogs, wolves, hyena, foxes, raccoons and ferrets. In addition, CDV strains substantially differ in virulence (Beineke *et al*., 2009). Expansions of the CDV host range took place several times during the last decades. For example, CDV from wolves, foxes, or dogs caused an epidemic among seals in the Lake Baikal and the Caspian Sea, and also a devastating disease within the lion population in the Serengeti (Osterhaus *et al*., 1989, Roelke-Parker *et al*., 1996, Harder *et al*., 1997,). Moreover, infections of monkeys (Macaca fuscata and Macaca mulata) with CDV resulting in quite high case fatality rates have been observed (Yoshikawa *et al*., 1989, Qiu *et al*., 2011, Sakai *et al*., 2012).

2.3.3 Pathogenesis

Like other morbilliviruses, PPR virus has an established lymphatic and epithelial tropism (Coucy-Hymann *et al*., 2007; Hammouchi *et al*., 2012). Consequently, it induces the most severe lesions in organ systems rich in lymphoid and epithelial tissues. The respiratory route is the likely portal of entry. Following entry of the virus through the respiratory tract, it is taken up by immune cells within the respiratory mucosa. Multiplication starts in the tonsillar tissue and lymph nodes draining the site of inoculation then transportation of virus to lymphoid tissues take place, where primary virus replication occurs. Viremia may develop 2-3 days after infection and 1-2 days before the first clinical sign appears. Subsequently viremia results in dissemination of the virus to spleen, bone marrow and mucosa of the gastro-intestinal tract and the respiratory system (Scott 1981; Pope *et al.,* 2014). Lymph nodes, lymphoid tissue and digestive tract

organs are the predominant sites of virus replication (Truong *et al.,* 2014). Inflammation of the alimentary mucosa sets up enteritis and diarrhoea that could lead to dehydration and subsequent death in young animals. Inflammation of the respiratory cells leads to pneumonia and also to severe respiratory distress. The young goats may die due to pneumonia. Bacteria like *E. coli* and *Pasturella* and protozoa like Coccidia have been found to be associated with this problem causing aggravation of pneumonia, diarrhoea and death. Of late, an attempt to address the effects of immunosuppression on pathogenesis of PPR virus infection was undertaken by Jagtap *et al.,* (2012), study indicated that immunosuppression increased the extent and severity of the pathological lesions associated with PPRV infection. No lineage-specific difference in the pathogenicity of the virus isolates is observed (Baron *et al.,* 2014).

Natural CD pathogenesis in domestic dogs has been well characterized and may be similar in nondomestic species. A systemic infection with viraemia is often present. Central nervous system (CNS) involvement is variable and dependent on the host's immune response. Within 24 hr of entering the respiratory tracts, virus spreads in macrophages via local lymphatics to tonsils and bronchial lymph nodes. Replication of the virus occurs in the tonsils and bronchial lymph nodes 2–4 days postinfection; concurrently, low numbers of CDV-infected mononuclear cells are found in other lymphoid organs. Within 4–6 days, virus proliferates widely in lymphoid organs (e.g., spleen, mesenteric lymph nodes, Kupffer's cells in the liver, and the lamina propria of the stomach and small intestine) and spreads, probably through blood, to epithelial and CNS tissues within 8–9 days of infection. The pathogenesis within 9–14 days depends on the humoral and cell mediated host immune response. Dogs with adequate antibody titers and cell-mediated cytotoxicity will clear the virus from most tissues with no clinical signs, whereas dogs with a poor immune response experience viral spread to many tissues. Dogs with an intermediate cell-mediated response and delayed humoral response will have most virus in epithelial tissues cleared as antibody titers rise. In these latter dogs, delayed CNS signs and hyperkeratosis of the foot pads ("hard pad disease") may result when virus persists in uveal, neural, and integumental tissues (Appel *et al*., 1987, Greene *et al*., 1990).

2.4 Disease Epidemiology and geographic distribution

The disease was first reported in Ivory Coast of West Africa by Gargadenec and Lalanne (1942). Later, the disease was reported from a number of countries including Sultanate

of Oman (Taylor *et al.,* 1990), Jordan (Lefevre *et al.,* 1991), Pakistan (Amjad *et al.,* 1996), Turkey (Ozkul *et al*., 2002) and various African countries (Anderson *et al.,* 1994; Matrencher *et al.,* 1995). In recent past, the disease has also been reported from China and Morocco (OIE, 2008; Wang *et al*., 2009) which raises the threat of its introduction into Europe. The Asian lineage has recently been introduced in some African countries, including Egypt ,Cameroon and Central African Republic (Banyard *et al.,* 2010), Sudan and Morocco (Kwiatek *et al.,* 2011), Algeria (De Nardi *et al.,* 2012) and Uganda (Luka *et al.,* 2012). F- gene based molecular epidemiology of virus from all over the world has defined the presence of four different lineages of virus (Banyard *et al.,* 2010; Muniraju *et al.,* 2013). Lineage I in west Africa, Lineage II in Nigeria and Cameroon, lineage III in East Africa and lineage IV in Asia. Based on the same (F gene) sequencing, there is a solitary report of lineage III PPRV occurrence in southern India (Shaila *et al.,* 1996). However, since then there is no further isolation of virus of this lineage. Of late, analysis based on F and N- gene of PPRV isolated from India reveals that all the isolates belongs to lineage IV and no other lineage has introduce in last decade (Ahmad, 2014). In 2015, Wang *et al* investigated 11 outbreaks of PPR in China and found that the PPRV involved in the outbreak belonged to lineages II and IV.

In India, PPR was first reported in 1987 from Arasur village of Villupuram district of Tamilnadu (Shaila *et al.,* 1989). Later on, disease moved to northern India (Nanda *et. al.,* 1996) and other parts of the country (Joshi *et al.,* 1996; Majumdar, 1997; Nayak *et al.,* 1997; Kumar *et al.,* 2001). Seroprevalence carried out more than a decade ago confirmed the overall antibody prevalence about 33% in sheep and goats (Singh *et al*., 2004). The finding indicated that an average of one out of the three small ruminants is exposed to PPRV. Later on, serosurveillance in sheep and goats of samples between 2003 and 2009 was carried out. Antibody prevalence in sheep and goats was found to be 41.01% and 46.11% respectively with an overall prevalence of 43.56% (Balamurugan *et al*., 2011).This indicates the increased and widespread infection that finally gives the endemic status of the disease. In 2014, Balamurugan *et al.,* screened a total of 1498 serum samples of cattle, buffalo, sheep and goat from five different states of India for PPRV-specific antibodies by ELISA. The study revealed that an overall seroprevalence of 21.83 % with 11.07 % in cattle, 16.20 % in buffaloes, 45.66

% in sheep and 38.54 % in goats. This report presented the results of PPRV-specific antibodies in situations where the subclinical, in apparent or nonlethal or recovery of infection was suspected in cattle, buffaloes, sheep and goats. The presence of PPRV antibodies demonstrate that bovines are exposed to PPRV infection and it implies the importance of cattle and buffaloes as subclinical hosts for the virus besides widespread presence of the disease in sheep and goats.

CDV in non-dog hosts has been reported in almost all continents with the exception of Australasia and in 43 countries. Most reports of CDV came from the United States (24.3 %), followed by Japan, Canada and Germany (11.8, 5.5 and 5.1 % respectively). CDV has been reported as a pathogen of dogs for more than one century; however, early records of CDV as an infectious disease for non-dog species was not reported until 1937 during an outbreak among silver jackals (Vulpes chama) in a zoo of Johannesburg (Armstrong *et al*., 1942) and then in the Americas: the first case of CDV in the American badger (Taxidea taxus) occurred in Colorado in 1942 (Armstrong *et al*., 1942). The first inter-Order report of CDV occurred in the early 60's with the development of the hamster model of infection; however due to the non-natural intra-cerebral inoculation route, this cannot be considered a natural infection. The first report of CDV in clinically healthy wildlife was found during serological evaluation of wolves of Northern Canada, published in 1974 (Choquette *et al*., 1974). Naturally occurring CDV in captive non-human primates (Order Primates) were reported in a Japanese monkey (Macaca fuscata) in the late 80´s; however experimental infection of macaques could be traced to 1977 (Yamanouchi *et al*., 1977). In felids, in 1994, approximately one third of the lion population in the Serengeti National Park died or disappeared during the best known outbreak of CDV (Roelke-Parker *et al*., 1996); however, naturally occurring CDV infection in felids was first reported in USA.

2.5 Diagnosis and control of PPR and Canine distemper

Initially, the majority of PPR outbreaks were diagnosed based on typical clinical signs. However, the signs of PPR are often difficult to distinguish from those caused by a number of other diseases, such as foot-and-mouth disease and bluetongue disease (Munir *et al.,* 2013). Therefore specific laboratory clinical diagnostic tests are necessary for diagnosis of the PPR disease. Researchers are working towards the development of diagnostics for rapid and specific

diagnosis of PPR. The recommended specimens from live animals are swabs of conjunctival discharges, nasal secretions, buccal and rectal mucosa, and anticoagulant-treated blood for PPR detection (OIE, 2013). The most basic test for detection of PPRV antigen is agar gel immunodiffusion (AGID) (Diallo *et al.,* 1989). Counter immunoelectrophoresis for PPRV is more sensitive than AGID (OIE, 2013). Latex agglutination tests are suitable for field detection of PPRV (Keerti *et al.,* 2009). For viral antigen detection rapid ELISA technique was developed, using two monoclonal antibodies (mAb) raised to the N protein (Libeau *et al.,* 1994; Singh *et al.,* 2004b). Sandwich ELISA is widely used in India for PPRV antigen detection, developed by Singh *et al.,* in 2004. Dot ELISA was also developed for PPR antigen detection (Saravanan *et al*., 2006). Virus neutralization test (VNT) is the most sensitive and specific test for detection of antibody against PPRV. Haemagglutination (HA) and Haemagglutination inhibition (HI) tests, being simple, cheaper and comparatively sensitive, can be used for routine screening purposes in control programmes (Munir *et al.,* 2009; Ezeibe *et al.,* 2008; Dhinakar Raj *et al.,* 2000). Competitive ELISA (cELISA) is one of the most extensively used tests for serological screening and diagnosis of PPRV infected animals (Anderson & McKay, 1994; Singh *et al.,* 2004a). In India, routine serosurveillance and seromonitoring is being carried out by mAb based cELISA (Singh *et al.,* 2004a). For detection of viral nucleic acids, DNA hybridization was the first method used for detection of viral nucleic acid. Reverse transcription polymerase chain reaction (RT-PCR) test is now considered as powerful as well as novel means of detection and quantification of the nucleic acids of PPR virus in various types of clinical samples (Couacy-Hymann *et al.,* 2002 and Balamurugan *et al.,* 2006). A Loop-mediated isothermal amplification technique (Li *et al.,* 2010; Dadas *et al.,* 2012) was developed and is less time consuming than conventional PCR for PPR diagnosis and results can be read by naked eyes. In 2010, Kwiatek and group developed one-step real-time Taqman RT-PCR assay to detect the four lineages of PPRV by targeting the nucleoprotein gene of the virus. Abera *et al* in 2014 developed highly sensitive N gene based SYBR green real time RT-PCR (two step) for specific detection and quantification of PPRV nucleic acid in clinical samples.

The methods for detection, prevention and control of PPRV vary widely depending on local facilities, techniques adopted and the provision of veterinary services and vaccine,

respectively. Control strategies that have been successful in ensuring the eradication of RPV are also valid for PPRV (Barrett *et al.,* 1993) although small ruminant population structures differ greatly to that of cattle. In areas where PPRV is not endemic, outbreaks are controlled most efficiently through a number of methods including: slaughter of infected herds, good sanitation, import controls, movement restrictions and quarantine. Vaccination is considered as the most effective way of controlling PPR. In the past, when a homologous vaccine against PPR was not available, a heterologous live attenuated tissue culture rinderpest vaccine (TCRP) (based on the antigenic similarity of PPRV with RPV) was used to control (Rowland *et al.,* 1970). The TCRP vaccine was shown to provide protection against PPR for about one year (Taylor *et al.,* 1979, Mariner *et al.,* 1993). Later, the use of heterologous PPR vaccine was banned because it might have interfered with the Global Rinderpest Eradication Programme (to achieve status of Rinderpest free zone) and to avoid handling of live RPV (Anderson *et al.,* 1994). In endemic areas, the virus is currently controlled either through administration of a live-attenuated PPRV vaccine such as the Nigeria 75/1 strain (Nig 75/1) (Diallo, 2003). In India three live attenuated vaccines are currently in use: Sungri/96, Arasur/87 and Coimbatore/97 (Saravanan *et al.,* 2010) for controlling the disease. Recombinant adenovirus expressing F and H fusion proteins of PPRV induces both humoral and cell-mediated immune responses in goats (Wang *et al.,* 2013). Animals that are vaccinated and those that recover from infection with PPRV generate a long lasting immunity that may last the lifetime of the animal (OIE, 2013). Availability of effective and safe live attenuated cell culture PPR vaccines and diagnostics have boosted the recently launched centrally sponsored control programme in India and also in other countries (Balamurugan *et al.,*2014).

Global rinderpest eradication was achieved in the year 2011, by the effort of research and development and their application on various aspects of the disease and virus. Yet, no doubt that the factors such as safe and reliable vaccine covering all strains of disease, the availability of simple and effective diagnostic tests, no persistence or carrier state, transmission only by close contact, an economic incentive for local and national participation and for compliance with the programme (Rweyemamu *et al*., 2006; Singh, 2011) made the eradication easier.

If we consider the current scenario of PPR, there are availability of safe and potent vaccines (Sreenivasa *et al*., 2000), sensitive and specific reliable diagnostics (Anderson and McKay, 1994; Libeau *et al*., 1995, 1994; Singh *et al*., 2004c, 2004d) owing to the single serogroup of PPRV. The development of DIVA vaccine with suitable companion diagnostic test would hasten the eradication program. In addition, generating data on role of wild animals in epidemiology of PPR is the crucial part to be taken into consideration and require necessary action (Wohlsein and Singh, 2015). Like PPR, rinderpest was also reported in buffalo, giraffe and several species of antelope (Kock *et al*., 1999). But the disease in wild animal population disappeared following vaccination of cattle; otherwise eradication would have been difficult. However, the determination of epidemiological role of wild life is necessary for establishment of PPR control program. The immense economic impact of disease made the participation of national and international bodies in the control of PPR. In India, the availability of infrastructures which were used in National Program on Rinderpest Eradication (NPRE) could be used for PPR control and eradication (Singh *et al*., 2009). Altogether, 'there are good reasons to believe that the eradication of PPR is an achievable goal like rinderpest' (http://www.fao.org/ag/againfo/resources/documents/AH/PPR_flyer.pdf).

The diagnosis of canine distemper is primarily based on clinical signs and history. A typical haematological profile of a dog with acute CDV infection includes anaemia, thrombocytopaenia, absolute lymphopaenia, neutropaenia and monocytopaenia (Shell, 1990). Hypoalbuminaemia, hyperglobulinaemia, or hypocalcaemia have been occasionally reported in CDV infections (Appel, 1969). Canine distemper virus titers in serum may help support the diagnosis of CDV infection. The detection of anti-CDV IgM antibody indicates recent infection or recent vaccination (Guy, 1986). The neutralization test is still considered the gold standard for measuring protection against infection, and serum titers correlate well with the level of protection. Neutralizing antibodies directed against the membrane proteins (H and F) of the virus, appear beginning 10 to 20 days post infection, and may persist for the life of a recovered animal (Appel & Robson, 1973). The indirect fluorescent antibody test detects anti-CDV IgG in the serum and CSF, or anti-CDV IgM in the serum. Whole-virus ELISA has been used to detect serum IgG and IgM antibodies to CDV (Greene & Appel, 1984). Recently, an ELISA

test which uses a recombinant protein N, has been recommended for the serodiagnosis of CDV infection in dogs in India (Latha *et al.*, 2007a). Blood, CSF, urine sediment, bone marrow, and cells from conjunctival, genital and respiratory epithelium are good samples for the diagnosis of CDV by direct immunofluorescence (Fairchild *et al.*, 1967). The direct fluorescent antibody test is used for antigen detection. The cells from test samples are smeared on clean slides and stained with a fluorescein dye conjugated with CDV antibody. A negative response does not prove the absence of CDV; antibody produced by the dog may coat the viral antigen and produce a false-negative result. False-negative results may also occur if samples are taken late in the course of infection (Guy, 1986). Recently, an immunochromatographic assay which employs two monoclonal anti-CDV antibodies has been developed for rapid antemortem diagnosis of dogs with CDV infection (An *et al.,* 2008). ELISA has been used to detect viral antigen in whole blood and CSF of natural and experimentally infected dogs (Gemma *et al.*, 1996; Soma *et al.*, 2003). Monoclonal antibodies are commercially available and used to detect CDV by immunohistochemical methods. The detection of CDV antigen in the nasal mucosa, footpad epithelium, and haired skin of the dorsal neck has been used consistently for the antemortem diagnosis of CDV by immunohistochemistry (Haines *et al.*, 1999). Immunohistochemistry has been used to study CDV antigen among wild carnivores in southwest Germany (Van Moll *et al.,* 1995). Reverse transcription has been used to detect CDV RNA in buffy coat cells from dogs with acute CDV infection, and CDV nucleoprotein RNA has been detected by reverse transcription- polymerase chain reaction (RT-PCR) using serum, whole blood and CSF from dogs with distemper (Frisk *et al.,* 1999). Regardless of the duration and form of distemper, a positive result was highly specific for diagnosis. Viral mRNA has been detected in footpad specimens from infected dogs (Grone *et al.*, 2003). Polymerase chain reaction (PCR) and nucleic acid hybridization studies using single stranded RNA probes have been performed to detect virulent virus in tissue culture and histologic sections (Zurbriggen *et al.*, 1993). A real-time RTPCR assay was developed for detection and quantitation of CDV. The assay exhibited high specificity and a quantitative TaqMan was validated on clinical samples, including various tissues and organs collected from dogs naturally infected by CDV (Elia *et al.*, 2006). Reverse transcription-PCR, nested-PCR and southern blot hybridization

has been used for the detection of the phosphoprotein gene of CDV in peripheral blood mononuclear cells and internal organs of dogs and fur animals in Poland (Rzezutka & Mizak, 2002). Recently, a hemi-nested multiplex PCR which provides a rapid approach for the investigation of CDV outbreaks has been reported. The system was used to genotype the major CDV lineages and is advocated to be useful for large-scale molecular epidemiological studies of CDV and for the diagnosis of vaccine-related disease (Martella *et al.*, 2007).

Anti-CDV maternal antibodies in the serum of newborns are the major protective factors in the initial few months of life. The amount of maternal antibodies transferred to a pup is directly proportional to the amount possessed by the bitch. These maternally transferred antibodies cause a period of temporary immunity that varies in duration of only a few days to 3-4 months. During this period of passive immunity, CDV antibodies from the colostrum prevent both infection and successful immunization with CDV vaccines (Robson *et al.,* 1959; Baker *et al.,* 1968). In some pups, maternal antibody is still protective at 14 weeks of age, thus preventing immunization by CDV vaccines that were previously administered. Because practitioners cannot routinely determine when the concentration of maternal antibody in each patient, vaccinations should be done every 2 to 4 weeks beginning at 6 to 8 weeks of age and continuing until 14 to 16 weeks of age to protect the majority of pups (Shell, 1990). Inactivated CDV vaccines were used some 60 years ago and were unable to control the disease in dogs and are no longer commercially available (Appel and Summers., 1999).

Current licensed CDV vaccines contain viral strains that have been attenuated by serial passage, either on canine kidney cells (Rockborn), hen eggs (original Onderstepoort) or chicken fibroblast cultures (Lederle strain) (Chappuis, 1995). Modified live vaccines (MLV) against CDV were introduced in early 1960s. The first, Onderstepoort vaccine, was developed from a natural isolate which was passaged in ferrets and then adapted to chicken embryos, later replaced with chicken cell culture (Haig *et al.,* 1956). The other MLV was generated by adaptation of the Rockborn strain to canine kidney cell culture (Rockborn *et al.,* 1959). These two vaccines have significantly reduced CDV infection in domestic dog population, although MLV induced outbreaks have been reported in the wildlife population. A CDV vaccine generated in canine cells causes disease in grey foxes (*Urocyon cinereoargenteus*) and ferrets

(*Mustela nigripes*) (Halbrooks *et al.,* 1981; Carpenter *et al.,* 1986), while an avian attenuated vaccine can be fatal for European mink (*Mustela lutreola*) and ferrets (Carpenter *et al.,* 1986; Sutherland-Smith *et al.,* 1997). Generally, the avian cell adapted vaccine is considered safer for wildlife species, and is tolerated by both grey and red foxes (*Vulpes vulpes*), bush dogs (*Speothos venaticus*), maned wolves (*Chrysocyon brachyurus*) and fennec foxes (*Vulpes zerda*) (Halbrooks *et al.,* 1981; McInnes *et al.,* 1992). To address the problem of MLV related outbreaks, the recently generated recombinant CDV vaccine, incorporating the fusion (F) and haemagglutinin (H) proteins of CDV in a strain of canarypox virus was shown to be safe to all susceptible species tested to date, including dogs, European ferrets (*Mustela putorius furo*), giant pandas (*Ailuropoda melanoleuca*), fennec foxes, meerkats (*Suricata suricatta*) and Siberian polecats (*Mustela eversmanni*) (Loots *et al.,* 2017). Whilst the canarypox vectored vaccine is safe in numerous target species, it is replication incompetent and as such induces a milder immunological response than MLV vaccines (Schultz *et al.,* 2006). However, this latter feature highlights its applicability for immunisation of young animals in the presence of maternal antibodies (Pardo *et al.,* 2007).

2.6 Cultivation of virus

PPR virus should be isolated from field samples in cell culture for further identification, even when the detection of PPR viral antigen has been carried out by rapid immunocapture ELISA (Lefevre and Diallo, 1990; OIE, 2013). Virus isolation in cell culture can also be attempted with several different cell lines, although recovery of virus is not always successful. Previously, a marmoset-derived cell line (B95a) was primarily used (Sreenivasa *et al.,* 2006) although primary lamb kidney or African green monkey kidney (Vero) cell cultures have also been successful (Mahapatra *et al.,* 2006). African green 41monkey kidney cells (Vero) have been for a long time the cells of choice for the isolation and propagation of PPRV. However, some isolates may not grow well in these cells. Recently, transformed monkey cells expressing sheep/goat signaling lymphocytic activation molecules (SLAM or CD150), the virus cellular receptors, have been shown to possess increased sensitivity (Adombi *et al.,* 2011). Generally, cultures are examined for cytopathic effect in the days following infection of a monolayer with suspect material; the identity of the virus can be confirmed by virus neutralization or molecular

techniques (Singh *et al.,* 2009). Techniques for virus isolation cannot be used as routine diagnostic tests as they are time-consuming and cumbersome (OIE, 2013). Moreover, the preservation of samples collected under field conditions is not always adequate for successful laboratory results. Virus isolation does, however, play an important role from a research perspective.

CDV isolation is usually done by cocultivation of lymphocytes from suspect dogs with mitogen-stimulated dog lymphocytes (Appel *et al*., 1992). Field isolates of CDV also replicate in dog or ferret macrophages (Appel *et al*., 1992) as well as in primary dog brain cell cultures (Lincoln *et al*., 1971). Cell lines such as Vero (African green monkey kidney) cells do not allow the propagation of field isolates, whereas cell culture adapted CDV strains such as the Onderstepoort vaccine strain are able to replicate in many cell lines (Cherpillod *et al*., 1999). It is known that virulence for the natural host may be lost when CDV is adapted to cell culture (Seki *et al*., 2003). Between the Vero and MDCK cell lines, which are known to be susceptible to CDV, the MDCK cell line is used for virus separation as it is susceptible to CD but not for host cells for mass culture (Lednicky *et al*., 2004). In particular, the MDCK cell line is derived from canine kidney and is regarded to have high susceptibility and yield compared with other cell lines, but its susceptibility test results are actually low (Tan *et al*., 2011). Therefore, to culture CDV using cell lines, new cell lines need to be produced. Various cell culture systems have been used for the isolation of CDV.

Virulent CDV can be readily cultured in macrophages or activated lymphocytes, but it grows only with adaptation in epithelial or fibroblast cell lines. In alveolar macrophage cultures, giant cell (syncytia) formation, a characteristic cytopathic effect of CDV in many other tissue cultures, is detected within 2 to 5 days, at which time the virus can be isolated by overlays made on other cells. Macrophage cultures have been replaced by dog lymphocyte cultures for the isolation of CDV. Buffy coat cells or tissues from infected animals can be cultivated with mitogen stimulated canine blood lymphocytes, and cultures are examined 72 to 144 hours later by immunofluorescence (Appel *et al.*, 1992). A marmoset lymphoid cell line (B95a) has also been used for CDV isolation (Kai *et al.*, 1993). Ferret peritoneal macrophages has been used for the isolation of CDV (Poste, 1971; Whetstone *et al.*, 1981). Less commonly, CDV

isolation on the chorioallantoic membrane of embryonated chicken eggs has been used (Ezeibe, 2005; Haig, 1956). Canine distemper virus has been isolated effectively from naturally infected free-ranging raccoons using cell lines such as MDCK (canine epithelial kidney cells), MV 1 LU (mink lung), and Vero (African green monkey kidney cells) (Lednicky *et al.*, 2004a). Vero cells expressing canine signalling lymphocyte activation molecule (Vero.DogSLAM) has been established to aid the isolation of CDV from clinical material within 24 hours of inoculation (Seki *et al.,* 2003).

2.7 Cellular Receptor vis-à-vis morbilliviruses

There are three main receptors identified till date for morbilliviruses viz;(a) CD46, (b) SLAM/CD150 and (c) Nectin-4.

(a) CD46 (Membrane co-factor protein-MCP)

The search for the receptor for morbillivirus began in vaccine strains of MeV, and subsequently identified receptors for wild-type strains have revealed the closely related receptor usage and unique pathogenicity of the viruses. There is a correlation between receptors and tropism of the viruses. It is one of the major determinants, which determine the host range and play crucial role in pathogenesis. Majority of the study is done on MeV as model of morbilliviruses. MeV has three known receptors viz., CD46 (membrane co-factor protein, MCP), CD150 (Signalling Lymphocyte Activation Molecule, SLAM) and Nectin-4(poliovirus receptor like 4, PVRL4). CD46 is a cell-surface, type I transmembrane 57–67 kD glycoprotein that belongs to the family of complement activation regulators and is ubiquitously expressed in all nucleated human cells **(Fig. 3).** The CD46 is a specific receptor for vaccine strain of MeV (Dorig *et al.,* 1993), whereas CD150 supports the entry of all the strains of MeV including wild-type (Erlenhoeffer *et al.,* 2001). CD46 is expressed ubiquitously in human nucleated cells; whereas CD150 is expressed on activated T and B cells, memory cells and immature thymocytes.

(b) SLAM (Signaling Lymphocyte Activation Molecule) Receptor

Signaling lymphocyte activation molecule (SLAM/CD150) is a 70 kDa cell surface glycoprotein which is located on human chromosome 1q 22- q 23 (Cocks *et al.,* 1995;

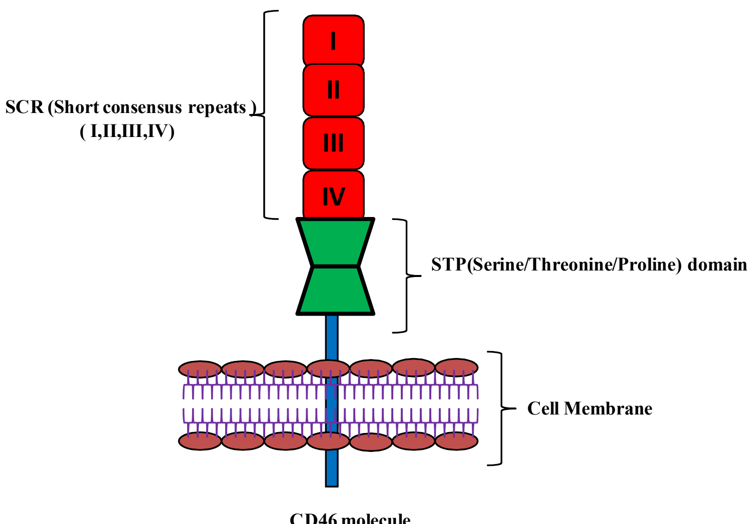


Fig. 3: Schematic diagram of CD46 molecule: it has four SCRs at the amino terminus of its ectodomain. SCR1 and SCR2 interact with virus, whereas SCR3 and SCR4 interact with complement proteins C3b and C4b.

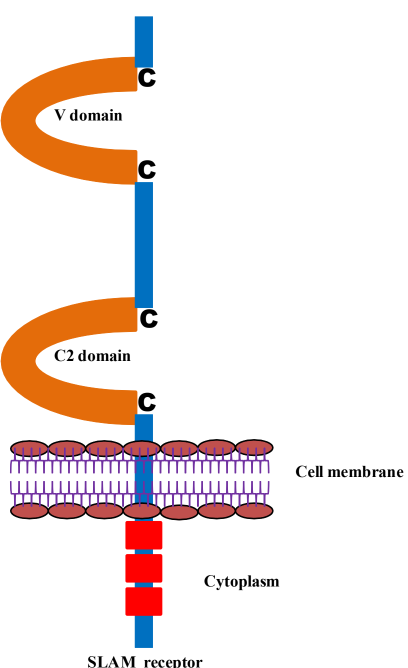


Fig. 4: Schematic diagram of SLAM receptor molecule: The extracellular domain is composed of a variable (V) and a constant (C2) Ig-like repeat. Its cytoplasmic domain contains three tyrosine residues (red block) that are surrounded by SH2 domain binding sequences. The morbillivirus H protein binds to the V domain on the target cells, which triggers viral infection

Tangye *et al.,* 2000; Veillette and Latour, 2003). SLAM was first identified as a human T-cell activation receptor in 1995 (Cocks *et al.,* 1995). Human SLAM is constitutively expressed on immature thymocytes, CD45RO memory T cells, and a proportion of B cells and induced all T and B cells following activation (Sidorenko and Clark, 1993; Cocks *et al.*, 1995; Aversa *et al.*, 1997). SLAM is differentially expressed on CD4 T cells, whereas high levels of SLAM are found on Th1 cells but only small amounts are detectable in Th2 cells (Hamalainen *et al.*, 2000). Among cultured cells, it is expressed on antigen-specific T cell clones and EBV transformed B-cell lines, but not on most T cells and monocyte/macrophage lineages. SLAM is not detected on monocytes, granulocytes and cells from non-lymphoid organs (Sidorenko and Clark, 1993; Cocks *et al.*, 1995; Aversa *et al.*, 1997). Although such tissue distribution of SLAM is consistent with lymphotropism of MeV (Kobune *et al.*, 1996; McChesney *et al.*, 1997), infection of monocytes, another major target *in vivo*, cannot be explained.

SLAM is a member of CD2 subset of the immunoglobulins super family and has two extracellular domains, V loop and C2 loop (**Figure. 4**). Its cytoplasmic domain contains three tyrosine residues that are surrounded by SH2 domain binding sequences. In fact, SLAM has been shown to associate intracellularly with SH2 domain-containing molecules such as the SLAM-associated protein (SAP), protein tyrosine phosphatase SHP-2 and inositol phosphatase SHIP (Sayos *et al.*, 1998; Shlapatska *et al.*, 2001). SLAM is reported to be a self ligand (Mavaddat *et al.*, 2000). The extracellular domain of SLAM associates with another SLAM molecule present on adjacent cells. In CD4+ T-cells, ligation of SLAM induces its binding to SAP, and combined with T-cell- receptor(TCR)-mediated signals, triggers downstream signaling for the production of Thelper2 (Th2) cytokines such as IL-4 and IL-13 (Veillette *et al*., 2007). Engagement of SLAM by a monoclonal antibody A12 leads to IL-2-independent T cell expansion and IFNg production by activated T cells, including Th2 cells (Cocks *et al.*, 1995; Aversa *et al.*, 1997; Yanagi *et al.,* 2006). Ligation of SLAM with a monoclonal antibody IPO-3 augments B-cell proliferation induced by anti-CD40 and IL-4 (Sidorenko and Clark , 1993). Soluble and membrane bound forms of SLAM induce proliferation and immunoglobulin synthesis by activated human B-cells (Punonen *et al.*, 1997). SLAM signaling also augments CD95-mediated apoptosis of B-cells (Mikhalap *et al.*, 1999).

(c) Nectin-4/Polio-Virus-Like- Receptor-4 (PVRL4)

PVRL4 (nectin-4) is recently identified as the epithelial receptor for members of the Morbillivirus genus (Muhlebach *et al.,* 2011, Pratakpiriyaa *et al*., 2012 and Noyce *et al.,* 2013). This discovery provides a better understanding of morbillivirus pathogenicity and establishes a new paradigm for the spread of virus from lymphocytes to epithelial cells. The nectin family is a cell adhesion molecule family comprising four members (nectin-1–4), and only nectin-4 functions as the EpR (epithelial receptor) (Mühlebach *et al*., 2011; Noyce *et al*., 2011). Nectin 4/PVRL4 is a member of the nectin family of cellular adhesion molecules which belongs to the immunoglobulin (Ig) superfamily. Nectin-4 is a 66-kDa protein which is involved in Ca^{2+}independent cellular adhesion (Reymond *et al*., 2001). Nectins are ubiquitously expressed and have adhesive roles in a wide range of tissues such as the adherens junction of epithelia or the chemical synapse of the neuronal tissue. This family of nectin proteins share homology with the poliovirus receptor (PVR), so they are also known as poliovirus receptor like proteins (PVRL). In humans, Nectin 4/PVRL4 is expressed abundantly in the placenta and weakly in the trachea. However, in the adult mouse, Nectin4/PVRL4 transcripts were also found in the brain, lung, and testis (Reymond *et al*., 2001). Nectin-4 is expressed on both the apical and basolateral surfaces of a number of polarized adenocarcinoma cells (Delpeut *et al.,*ý 2014). The Human Protein Atlas Project (www.proteinatlas.org) has reported that Nectin 4/PVRL4 is expressed abundantly in placental trophoblasts, glandular cells of the stomach, and adenocarcinomas of the lung, breast, and ovary. It is weakly expressed in the epithelium of tonsils, oral mucosa, and oesophagus, as well as in the respiratory cells of the nasopharynx (www.proteinatlas.org). In many cancer cell lines, Nectin 4/PVRL4 is highly over expressed and the protein is also found on the apical surface of the cell (Noyce *et al*., 2011). There have also been a number of reports investigating the efficacy of PVRL4 as a diagnostic marker for breast, lung and ovarian cancer (Derycke *et al.,* 2010; Nabih *et al.,* 2014) and a recent study has shown that upregulation of PVRL4 promotes anchorage-independent growth of tumor cells (Pavlova *et al.,* 2013).

The characterization of virus-receptor interactions is particularly important to better understand virus entry and offers new opportunities for developing antiviral therapies. PVRL4

is a member of the nectin family of adhesion molecules, which belongs to the immunoglobulin (Ig) superfamily, comprised of nectin-1, -2, -3, -4 and the prototypic poliovirus receptor (PVR) (Takai *et al.,* 2008). PVR mediates the entry for poliovirus (Mendelsohn *et al.,* 1989), while PVRL1 and 2 serve as a receptor for herpes simplex viruses (Campadelli-Fiume *et al.,* 2000). Nectins are normally localized to the adherens junctions and are components of the cell-cell adhesion system, where they play a key role in limiting cell movement, facilitating intercellular communication and regulating proliferation (Takai *et al.,* 2008). PVRL-4 initially interacts with other PVRL4 molecules in *cis*-, followed by *trans*-interactions with PVRL4 on adjacent cells, while PVRL4-PVRL1 interactions only appear *in trans* on adjacent cells (Fabre *et al.,* 2002, Reymond *et al.,* 2001, Yasumi *et al.,* 2003). As a consequence of other binding partners for PVRL4, it will be of interest to determine if such PVRL protein interactions might influence morbillivirus entry.

PVRL-4 is a type I transmembrane glycoprotein with three Ig-like ectodomains (V and two C2 domains), a transmembrane region and a cytoplasmic tail (**Figure. 5**). The V domains of nectin-4 are involved in homotypic or heterotypic interactions with nectin-1, while C2 domains enhance the affinity of these interactions (Takai *et al.,* 2008, Fabre *et al.,* 2002). The intracellular domain of nectins can bind a scaffold protein named afadin. Nectins can regulate several cellular activities, including cell movement, polarization, differentiation, and have been implicated in the entry of several viruses (Ogita *et al*., 2010). Nectin 4 is normally localized to the adherens junctions together with the cadherins. Nectin-4 interacts with itself and the V domain of Nectin-1 and its cytoplasmic tail associates with the intracellular actin-binding protein, afadin (Nishimura and Takeichi, 2009)

PVRL-4 biochemically shown to support binding to MeV Haemagglutinin protein through its V domain, leading to virus entry (Noyce *et al*., 2011). This is similar to the scenario where the V domain of SLAM also interacts with MeV H (Ono *et al*., 2001). Interestingly PVRL4-MeV H interactions are stronger than MeV H binding to SLAM, although PVRL4-MeV-H induced syncytia formation is less extensive compared to SLAM (Muhlebach *et al*., 2011). In cell culture, the PVRL-4 V domain was shown to be essential for CDV infection using whole chimeric nectins, where the V domains of PVRL-4 and PVRL-1 were exchanged

(Delpeut *et al*., 2014). A crystallographic structure of the ligand-binding domains between PVRL-4 and MeV H has identified three binding interfaces (Zhang *et al*., 2013). Sites II and III are respectively located in the B-C and C'-C" loops of the PVRL-4 V domain and contribute to receptor-ligand interactions (Zhang *et al*., 2013). In contrast, the F-G loop containing Site I interacts with two hydrophobic patches in MeV H, conferring strong stabilizing forces (Zhang *et al*., 2013). Importantly, mutations F101S, P102S, A103S and G104Y in Site I of the PVRL-4 V domain almost completely abolished MeV H recognition *in vitro* (Zhang *et al*., 2013). This FPAG motif (F101, P102, A103 and G104) within the V domain of PVRL-4 is conserved in the related PVRL-4 V domains from ovine and dog species (Noyce *et al*., 2013, Birch *et al*., 2013, Zhang *et al*., 2013) and is critical for CDV entry and virus spread (Delpeut *et al*., 2014). PVRL1, PVRL2 and PVRL3 are not used as a receptor by MeV (Noyce *et al*., 2011, Muhlebach *et al*., 2011). These studies demonstrate that morbillivirus epithelial cell infections are highly conserved, since both MeV and CDV H proteins share key PVRL4 binding residues (Sawatsky *et al*., 2012, Leonard *et al*., 2008, Delpeut *et al*., 2014). In contrast to MeV and PPRV, canine distemper virus infects a wide range of hosts, highlighting its ability to jump across species. PVRL4 sequences from human and dog origin are almost identical at the amino acid level, and both V domains contain the FPAG motif (Noyce *et al*., 2013, Birch *et al*., 2013). Due to these similarities, CDV has the intrinsic ability to use both human and dog PVRL-4 as an epithelial receptor, without a requirement for adaptive mutations in H (Bieringer *et al*., 2013, Sakai *et al*., 2013). Thus, CDV can replicate in human epithelial cells after blocking the host innate immune response (Otsuki *et al*., 2013). Although a single mutation in the hemagglutinin gene is still required for CDV to use the human SLAM entry receptor (Bieringer *et al*., 2013, Sakai *et al*., 2013), canine distemper virus has the potential to emerge as a novel human pathogen (Sakai *et al*., 2013). Indeed, natural infections with CDV have already been reported in non-human primates (Yoshikawa *et al*., 1989, Sun *et al*., 2010).

2.8 SLAM receptor versus morbilliviruses

SLAM is the principal cellular receptor for morbilliviruses allowing entry and propagation (Tatsuo *et al.,* 2001). Signaling lymphocyte activation molecule-blind CDV infected primary

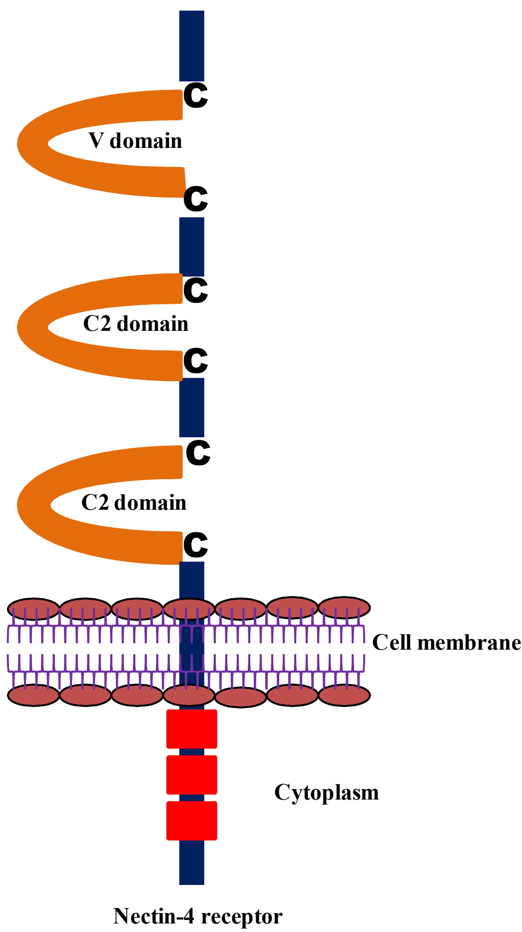


Fig. 5: Schematic diagram of Nectin-4 receptor molecule: The extracellular domain is composed of a variable (V) and a two constant (C2) Ig-like repeat. Its cytoplasmic domain contains three tyrosine residues (red block) that are surrounded by SH2 domain binding sequences. The morbillivirus H protein binds to the V domain on the target cells, which triggers viral infection .

ferret epithelial cells as efficiently as the parental wild type CDV but was incapable of entering ferret peripheral blood mononuclear cells in vitro. Experimentally infected ferrets indicated that the SLAM-blind virus is completely avirulent in ferrets; infection with this virus caused only a small, short-lived decrease in the blood leukocyte count (Von Messling *et al*., 2006). SLAM contains two immunoglobulin-like domains, the V and C2 domains, in the extracellular region. The morbillivirus H protein binds to the V domain on the target cells, which triggers viral infection (Ono *et al.,* 2001). The V domain of SLAM is necessary and sufficient for MeV receptor function and three amino acid residues, at positions 60, 61, and 63 of human SLAM, are crucial for its function (Ohno *et al*., 2003). After infection of cells with MeV, SLAM receptor was downregulated from the cell surface of activated peripheral blood lymphocytes, BJAB cell lines and also in SLAM was rapidly downregulated from the surface of CHO-SLAM cells (Erlenhoefer *et al.,* 2001). Although Vero cells had been used for MeV isolation for a long time, the isolation was not highly efficient and usually required several blind passages. Kobune *et al*. in 1990, found that an Epstein–Barr-virus-transformed marmoset B-cell line, B95a, is 10,000-fold more sensitive to the MeV present in clinical specimens than Vero cells.

Welstead *et al*., 2004, reported that expression of the hemagglutinin (H) protein of MeV cause down regulation of SLAM gene. They also demonstrated that interactions between H and SLAM at the host cell surface can also contribute to SLAM down regulation.

Interaction of MeV to dendritic cell (DC) has been seen to cause immunosuppression. Transgenic (tg) mice expressing hSLAM (human SLAM) on DCs when infected with wild type MeV demonstrated less B7-1, B7-2, CD40, MHC class I, and MHC class II molecules on their surfaces and displayed an increased rate of apoptosis when compared to uninfected DCs. Further, MV-infected DCs failed to stimulate allogeneic T cells and inhibited mitogendependent T-cell proliferation. Individual expression of human SLAM, interferon a/h receptor, tumor necrosis factor-a, and lymphotoxin-a or h from T cells was not required for MV-infected DCs to inhibit the proliferation of T cells (Hahm *et al.,* 2004).

Leonard *et al.,* 2010 reported that when a group of six rhesus monkeys (Macaca mulatta) was inoculated intranasally with the SLAM-blind virus (recombinant MeV unable to recognize human SLAM), no clinical symptoms were documented. Only one monkey had

low-level viremia early after infection, whereas all the hosts in the control group had high viremia levels. Despite minimal, if any, viremia, all six hosts generated neutralizing antibody titers close to those of the control monkeys while MV-directed cellular immunity reached levels at least as high as in wild-type-infected monkeys. These findings prove formally that efficient SLAM recognition is necessary for MV virulence and pathogenesis.

2.9 SLAM receptor and PPR virus

PPRV also uses SLAM as one the receptor for entry and play significant role in pathogenesis. Study carried out in our lab by Sarkar (2007) on comparative susceptibility of Vero and Vero/SLAM (Vero cell expression cprine SLAM) reflected that Vero/SLAM cells appeared to be the better host than Vero for PPR vaccine virus. Phylogenetic relationship based on the aminoacid sequences of SLAM protein revealed that caprine, ovine, cattle, and buffalo fall under a defined cluster but caprine SLAM is more closely related to ovine, followed by bovine (Sarkar *et al*., 2007).

Pawar *et al.,* 2008, showed the basal expression of SLAM in the PBMCs of cattle, buffalo, sheep, and goats correlated with PPRV replication assessed by real-time PCR and virus titers. PBMCs isolated from goats had the highest level of SLAM mRNA followed by sheep, cattle, and buffalo. Different breeds of goats had different basal levels of SLAM mRNA. In the PBMCs of studied animals, basal SLAM expression had high correlation with their ability to replicate PPRV. After stimulation of PBMCs isolated from different breeds of goats with concanavalin A (mitogen), the SLAM expression have increased 4 to 16 fold, which resulted in a 1.7 to 3.8-fold increase of viral mRNA and the virus titer increased by 0.4-1.3 log units. These findings revealed that SLAM expression and PPRV replication are highly correlated and different levels of SLAM mRNA could influence the virus replication in different animals.

Wild-type strains of morbillivirusese can be isolated and propagated efficiently in non-lymphoid cells expressing SLAM protein. Adombi *et al.,* 2011 demonstrated that monkey CV1 cells expressing goat SLAM gene are highly efficient for isolating PPRV from pathological samples. This finding suggests that SLAM, as is in the case for MV, CDV and RPV, is also a receptor for PPRV.

Meng *et al.,* 2011 investigated the tissue distribution and expression of signaling lymphocyte activation molecule (SLAM) in 40 tissues and organs of goats by real-time RT-PCR, in order to determine the role of these receptors in tissue tropism. SLAM mRNA was detected in all the samples investigated. The expression of SLAM mRNA was detected at high levels in spleen, mesenteric lymph node, hilar lymph node, mandibular lymph node, superficial cervical lymph node, nasal mucosa, duodenum, heart, gallbladder, thymus and blood; this was similar to the tissue tropism of PPRV. However, it was surprising that expression of SLAM was low in lungs, colon and rectum which are the major sites of replication of PPRV. In addition, very low levels were detected in larynx, tongue and oesophagus, which suggest the possible presence of an alternative receptor for PPRV.

Recently, Birch *et al.,* in 2013 found that high level of mRNA of SLAM in spleen , tonsils, lymph nodes, lungs, rectum and heart of *Ovis aries*. The expression of SLAM mRNA in these predominantly non immune epithelial tissues (lungs, rectum and heart) is likely due to the presence of SLAM-positive immune cells such as macrophages and dendritic cells, rather than epithelial cells, expressing this gene.

2.10 SLAM receptor and CDV

Wenzlow *et al*., 2007 studied the expression of SLAM using immunehistochemistry in order to evaluate the presence and distribution of the receptor in dogs *in vivo*. In seven healthy dogs, the receptor was found in various tissues, mostly on cells morphologically identified as lymphocytes and macrophages. These findings suggest that SLAM, a putative distemper receptor, is expressed in dogs *in vivo*. In seven dogs infected with CDV demonstrated up-regulation of SLAM in early distemper lesions, potentially causing an amplification of virus in the host.

In 2008, Neilsen and group reported that stable transfected Vero cell line (Vero.DogSLAMtag) expressing canine SLAM receptors, was more suitable for isolating and characterizing Phocine distemper virus (PDV) when compared with Vero (American Type Culture Collection # C1008) and primary seal kidney (PSK) cells. PDV was isolated from frozen infected ferret lung tissue within 48 hr, when isolation was attempted using the

Vero.DogSLAMtag cell line. These results indicate that Vero.DogSLAMtag cells offer a substantial improvement (including faster viral replication resulting in primary viral isolation in a shorter period of time, and higher yield of virus finally obtained) over traditional cell culture methodologies for isolation and characterization of marine mammal morbilliviruses.

Bieringer *et al*., in 2013 showed that one amino acid change (position 540:D to G) in H protein of CDV-A75/17red strain favoured adaptation to human SLAM receptor after three passage. Consequently, they also showed that no adaptive mutation is required for wild-type CDV to use human nectin-4 as receptor. This finding indicate that only a minimal alteration (in this case one adaptive mutation) is required for adaptation of CDV to the human entry receptors, and help to understand the molecular basis why this adaptive mutation occurs.

Sakai *et al*., in 2013 demonstrated that canine distemper virus (CDV) strain, CYN07-dV, associated with a lethal outbreak in monkeys, used human signaling lymphocyte activation molecule as a receptor only poorly but readily adapted to use it following a P541S substitution in the hemagglutinin protein. Since CYN07-dV had an intrinsic ability to use human nectin-4, the adapted virus became able to use both human immune and epithelial cell receptors, as well as monkey and canine ones, suggesting that CDV can potentially infect humans.

2.11 Nectin-4 receptor verses morbilliviruses

Distribution and functions of SLAM provide a good explanation for the lymphotropism and immunosuppressive nature of morbilliviruses. However, morbilliviruses, in autopsied patients and in experimentally infected animals, has also been shown to infect the epithelial cells of the trachea, bronchial tubes, lungs, oral cavity, pharynx, esophagus, intestines, liver, and bladder (Griffin *et al*., 2007). These epithelial cells do not express SLAM, but the infected cells do shed virus, suggesting that entry into these SLAM-negative cells is mediated by other cellular receptors. *In vitro* studies have shown that a number of SLAM-negative cell types of epithelial or neuronal origin result in cytopathic effects and virus release. In particular, several well differentiated polarized epithelial cell lines showed high susceptibility to wild-type MeV (Takeda *et al*., 2007; Tahara *et al*., 2008).

Muhlebach *et al* (2011) identified nectin-4 as a candidate host exit receptor for MeV. Their study revealed that Nectin-4 is sustained in MeV entry and non-cytopathic lateral spread in well-differentiated primary human airway epithelial sheets infected basolaterally. It is down-regulated in infected epithelial cells. The V domain of SLAM and Nectin-4 are binds to H protein of MeV. The Nectin-4 & MeV H protein interactions are stronger than MeV H protein binding to SLAM, although Nectin-4-MeV-induced syncytia formation is less extensive compared to SLAM. They suggested that measles virus targets nectin-4 to emerge in the airway.

Pratakpiriyaa *et al*. (2012) studied the relation of CDV and Nectin-4 receptor. They found, wild-type CDV strains showed efficient replication with syncytia in Vero cells expressing dog nectin4 receptor, and the infection was blocked by an anti-nectin-4 antibody. In dogs with distemper, CDV antigen was preferentially detected in nectin-4 positive neurons and epithelial cells, suggesting that nectin-4 is an epithelial cell receptor for CDV and also involved in its neurovirulence.

Noyce *et al*. (2013) also identified nectin-4 receptors in dog for CDV that facilitates virus entry and syncytia formation. Inhibition of dog nectin-4 expression by RNAi (siRNA treatment) or nectin-4 antibodies decreased CDV titers and syncytia formation. This study confirmed that dog nectin-4 can be used by CDV to gain entry into epithelial cells and facilitate virus spread.

Melia *et al.* (2014), identified PVRL-4 is also used by wild type PDV to get entry in to the epithelial cells like other morbilliviruses.

Due to recent discovery of Nectin-4 as morbillivirus receptor (Muhlebach *et al*., 2011), work on this aspect is scanty. In a study conducted by Mühlebach *et al*., 2011 showed the down regulation of this receptor in macaque tracheas following infection with MeV.

(a) Nectin-4 and Peste-des-petits-ruminants virus

Recently, Birch *et al* (2013) identified Nectin-4 is a receptor for PPR virus in sheep (*Ovis aries)*. This epithelial cell receptor permits efficient replication of PPRV and high levels of ovine Nectin-4 transcripts were found in epithelial tissues, including the lower lip, tongue,

tonsils, upper respiratory tract, epiglottis, trachea, lungs, bronchi, oesophagus, rumen, reticulum, omasum, abomasum, duodenum and rectum. They suggested that the correlation of pathology with receptors does not explain all aspects of PPRV pathogenesis. In some of the organs (duodenum, ileum, colon, and rectum) the level of Nectin-4 and SLAM expression is relatively low despite the PPR induced lesion. In contrast, pathology in the stomach (rumen, reticulum, and omasum) is rare, despite higher levels of Nectin-4 expression in these tissues. The intestinal pathology may therefore be the result of host-mediated effects or infection via alternative routes, and this represents an obvious and interesting area for continued investigation.

Manu *et al*., in 2015, has conducted an experiment to find out the relative expression and down regulation of both SLAM and nectin-4 receptors upon experimental infection of goat and sheep. In case of SLAM gene, both infected sheep and goat showed varying degree of down regulation in different tissues. Sheep exhibited more down regulation in the respiratory tract, whereas; it was more in upper digestive and respiratory tracts and lymphoid tissues in the goat. There was slight up regulation of SLAM gene in the lower digestive tract of the goat. Relative expression of Nectin-4 gene in both the animals also demonstrated down regulation of the gene. Down regulation was more in lower digestive tract and lungs of sheep, whereas; in goat, it was more in upper digestive and respiratory tracts. Less/no change was noticed in the expression of Nectin-4 gene in the rumen, reticulum, omasum, spleen and mesenteric lymph node of both animals. Like disease severity and viral loads, intensity of down regulation of both the gene was mostly observed in goat than sheep. Susceptibility of the goat to PPRV as compared to sheep might be due to the presence of both the receptors at a greater magnitude.

(b) Nectin-4 and canine distemper virus

Pratakpiriya *et al* in 2012 studied the relation of CDV and Nectin-4 receptor. They found that wild type CDV strains showed efficient replication with syncytia in Vero cells expressing dog nectin-4 receptors, and the infection was blocked by an anti-nectin-4 antibody. In dogs with distemper, CDV antigen was preferentially detected in nectin-4 positive neurons and epithelial cells, suggesting that nectin-4 is an epithelial cell receptor for CDV and also involved on neurovirulence.

Noyce *et al*., in 2013 developed a Vero cell line expressing Nectin-4 from MDCK and from labrador retriever dog and found that both the cell lines were efficiently sensitive for CDV and dog nectin-4 facilitates CDV entry and cell-to-cell spread in cultured Cero cells that express nectin-4 receptor. Inhibition of dog nectin-4 expression by RNAi (siRNA treatment) or nectin-4 antibodies decreased CDV titres and syncytia formation. This study confirmed that dog nectin-4 can be used by CDV to gain entry into epithelial cells and facilitate virus spread.

Delpeut *et al*., in 2014 demonstrated that the V domain of PVRL4 is critical for CDV entry and virus cell-to-cell spread. Furthermore, four key amino acid residues (F101, P102, A103 and G104) within the V domain of dog PVRL4 and two within the CDV hemagglutinin were shown to be essential for receptor-mediated virus entry.

Alves *et al*., in 2015 showed that nectin-4-dependent cell-cell fusion in Vero cells, triggered by a demyelinating CDV strain, remained extremely limited, thereby supporting a potential role of nectin-4 in mediating persistent infections in astrocytes. However, nectin-4 could not be detected in either primary cultured astrocytes or the white matter of tissue sections. In addition, a bioengineered "nectin-4-blind" recombinant CDV retained full cell-to-cell transmission efficacy in primary astrocytes. These findings are suggestive for the existence of a hitherto unrecognized third CDV receptor expressed by glial cells that contributes to the induction of noncytolytic cell-to-cell viral transmission in astrocytes.

2.12 Use of receptors in morbillivirus research

2.12.1 Host-pathogen interaction and virus tropism

Expression and distribution of cellular morbillivirus receptors in a host is particularly important,because the interaction of a virus with its cellular receptor links virus tropism and pathogenesis Most morbilliviruses have established lymphotropism and epitheliotropism that are receptor-dependent (Noyce *et al*., 2013). Specific interactions between cellular receptors and the viral hemagglutinin protein (H) facilitate virus entry into host cells by inducing virus-cell and cell-cell membrane fusion in cooperation with the fusion protein (F) (Baker *et al*., 1999). SLAM gene mostly expressed on lymphoid and respiratory tissues; whereas, nectin-4 distributed

to digestive and respiratory tracts. High levels of ovine PVRL4 transcripts are found in epithelial tissues, including the mouth, the upper respiratory tract and the stomach (Birch *et al.*, 2013). PVRL4 actually plays a key role in virus spread from immune to epithelial cells (Ludlow *et al.*, 2014). Down regulation of receptors are positively correlated with viral loads (Manu M, 2015). Nevertheless, morbillivirus pathogenesis cannot be explained by the presence of SLAM and PVRL4 alone. Small ruminants infected with PPRV often exhibit lesions in the intestine that express relatively low levels of SLAM and PVRL4 (Hammouchi *et al.*, 2012). In contrast, higher levels of cytoplasmic PVRL4 expression in the stomach epithelial cells (Uhlen *et al.*, 2010) does not correlate with the gastric pathology associated with PPRV infection (Hammouchi *et al.*, 2012, Birch *et al.*, 2013). It has been seen that Mutations affecting the binding site of the H protein for virus cellular receptors which may leads to disease emergence in novel host species (McCarthy *et al.*, 2007; Bieringer *et al.*, 2013). Substitutions at residues 530 and 549 of the CDV-H protein are reportedly crucial for influencing host membrane fusion and host switches (Von Messling *et al.*, 2013).

2.12.2 Detection of mutant / variants strains

Yu-Ping *et al.*, in 2014 have generated a panel of recombinant measles viruses, having single and double mutations in the Haemagglutinin and adopted these mutants to different cell lines (Vero and CHO) expressing CD46, human SLAM and human Nectin-4, that bind to one or more of the MV receptors. All the mutations successfully yielded the intended phenotype such that virus usage of intended receptor was abolished. To determine whether ablating CD46, SLAM and nectin4 tropism from these viruses might prevent shedding, increasing its safety profile as an oncolytic, or might have any effect on CD46 binding, they also generated VSVFH (vesicular stomatitis virus (VSV) encoding the MV-Edm F and H entry proteins in place of G) viruses with H mutations that disrupt attachment to SLAM and/or nectin4. Their results suggest that mutations at nectin4, SLAM, and CD46 binding sites all effectively knock out receptor binding, but nectin4 ablation also interferes with CD46 binding. They found that amino acid at position 482 (S-serine) is specific for SLAM binding and 543(A-alanine) in Haemagglutinin is specific for nectin-4 receptor binding because these are critical amino acids presents at receptor binding groove of Haemagglutinin along with some other amino acids.

2.12.3 Increased production of antigen

Stable cell lines constitutively expressing cellular receptors are highly sensitive to increase vaccine titer and antigen production for diagnostics. Nizamani *et al*., in 2014 has proved that Vero-dog SLAM showed 0.8 log10 higher virus titer than the Vero cell line for both "wild type" and "vaccine strains" of PPRV. Fakri *et al*., in 2015 has shown that from different vaccine batches of PPRV Nigeria 75/1, the average viral titer was 0.9 log and 1.7 log $TCID_{50}$/ml higher in VeroNectin-4 cells compared to Vero cells and VeroDogSLAM cells, respectively.

2.12.4 Development of stable cell lines

These cellular receptors can be utilized for development of specific cell lines for easy isolation of different viruses. Specific receptors expressing cell lines on their cellular membrane are highly sensitive for virus isolation from clinical samples as well as antigen production. Seki *et al* (2003), has successfully developed stable Vero cell by trnasfecting the cell with dog SLAM (Vero.DogSLAMtag). The Vero DogSLAMtag cell line constitutively expressing SLAM gene was found to be more susceptible than other known cell line(s) used for virus isolation. Tatsuo *et al*., in 2000, has developed Vero cell line expressing human-SLAM for cultivation of Measles virus. Sarkar *et al*., in 2007 has been shown that Vero cell expressing the goat SLAM is the better host than naïve Vero cells for PPR vaccine virus (Sungri/96). Adombi *et al.,* 2011 has developed monkey cells expressing sheep/goat signaling lymphocytic activation molecules (SLAM or CD150). Noyce *et al.,* in 2013, developed a Vero cell line expressing Nectin-4 from MDCK cells and from labrador retriever and found that both the cell lines efficiently sensitive for CDV replication and dog nectin-4 facilitates CDV entry and cell-to-cell spread in cultured Vero cells that express nectin-4 receptor.

2.13 Development of Stable cell line harbouring cellular receptors

Seki *et al* (2003), has successfully developed stable Vero cell by trnasfecting the cell with dog SLAM (Vero.DogSLAMtag) using pCAGGS vector under G418 antibiotic selection. The Vero DogSLAMtag cell line constitutively expressing SLAM gene was found to be more susceptible than other known cell line(s) used for virus isolation.

Tatsuo *et al*., in 2000 developed Vero-hSLAM (Vero-human SLAM) cell line using Lentivirus vector strategy. They identified that SLAM as a receptor for measles. This cell lines has been used for isolation of wild type MV from clinically affected humans.

Sarkar *et al*., in 2007 developed Vero-gSLAM (Vero-goatSLAM) cell line using pTARGET vector under G418 antibiotic selection. This cell line used for propagation and isolation of vaccine strain of PPRV Sungri/96 and concluded that Vero/gSLAM is a better host for PPR vaccine virus than native Vero cells but propagation of "Wild Type" PPR virus could not be conclusively ascertained.

Adobmi *et al*., in 2011 developed monkey CV1 cell lines by FLIP-In stretegy. This cell line has been used for isolation of PPRV from clinical and pathological samples. They found that monkey CV1 cell expressing goat SLAM is highly efficient for PPRV isolation.

Nikolin *et al*., in 2012 developed Vero-Dog/SLAM, Vero-Cat/SLAM and Vero-Lion/SLAM cell lines by using pCG vector. A virus titer assay demonstrated that dog strains produced higher titers in cells expressing dog SLAM than cells expressing SLAM of non-dog hosts.

Feng *et al*., in 2016 developed Vero-Monkey/SLAM cell line using pCAGGS vector. Monkey-BJ01-DV (a rhesus monkey isolate of CDV strain) can efficiently use monkey- and dog-origin SLAM to infect and replicate in host cells in comparision to Vero cells, but further adaptation may be required for efficient replication in host cells expressing the human SLAM receptor.

Noyce *et al*., in 2013 developed a Vero cell line expressing Nectin-4 from MDCK and from labrador retriever dog using pBMN-IRES-Neo retrovirus strategy and found that both the cell lines were efficiently sensitive for CDV and dog nectin-4 facilitates CDV entry and cell-to-cell spread in cultured Cero cells that express nectin-4 receptor. Inhibition of dog nectin-4 expression by RNAi (siRNA treatment) or nectin-4 antibodies decreased CDV titres and syncytia formation. This study confirmed that dog nectin-4 can be used by CDV to gain entry into epithelial cells and facilitate virus spread. Vero-nectin infected with PPRV Nigeria/75 showed high titre (0.9log) compare to native Vero cells. Comparative virus titration of

PPRV Nigeria/75 in Vero-Nectin and Vero.dogSLAM showed higher titre by 1.7 log in Vero-Nectin cells.

2.14 Replication efficacy and growth kinetics of morbilliviruses in different cell lines

Singh *et al*., in 2008 investigated the virological and antigenic characterization of two Indian Peste des Petits Ruminants (PPR) vaccine viruses namely "PPRV-Sungri/96" and "PPRV-AR/87 in native Vero cell line." They conducted one-step growth curve of both the viruses using 1.0 multiplicity of virus/cell. PPRV-Sungri/96 showed an eclipse phase at around 12 h with a decline in detectable virus titre, which subsequently reached to peak at 72 h post-infection. Infectivity assay of cell associated PPRV-AR/87 depicted a steady increase in infectivity titre, which reached peak at around 21 h, with no defined eclipse phase. The virus infectivity assay with both vaccine viruses demonstrated that majority (>80%) of the virus remain cell associated at peak of virus replication. PPRV-Sungri/96 virus showed a distinct eclipse phase at around 12 h post-infection, where in about only 1/30 of the virus could be demonstrated. The eclipse phase was not distinct in PPRV-AR/87 probably due to fast cytopathic effect induced by this virus. One-step growth curve revealed that maximum virus titre could be obtained at 21 and 72 h post-infection in PPRV-AR/87 and PPRV-Sungri/96, respectively. This indicates that the PPRV-AR/87 isolate replicates very fast to high infectivity ($10^{8.8}$ $TCID_{50}$/ml) as compared to Sungri isolate ($10^{5.5}$ $TCID_{50}$/ml). Experiences of the authors with PPRV-AR/87 have shown that, it overtakes all other isolates of PPR virus in replication using Vero cells. The infectivity assay of cell associated virus at peak of replication was at least 10 times higher than that of cell free virus in both the viruses. This indicates that more than 90% virus remains cell associated at peak of replication.

Fakri *et al*., in 2015 conducted the growth kinetics of PPRV Nigeria 75 strains cultivated in Vero and VeroNectin-4 cells. The virus was inoculated with a multiplicity of infection (m.o.i.) of 0.01 in 25 cm2 flasks containing cell suspensions, and then incubated for 6 days at 37ºC. Every 24 h, one pool of supernatant (representing extracellular virus) was removed from the flasks and titrated for virus infectivity. Every 24 h, one flask was frozen at -80ºC and titrated after freeze-thawing the cells to obtain the supernatant (representing the total virus). Titration

was conducted on Vero-nectin-4 cell lines. They found maximum titre of PPRV in supernatant at day 4 (96h) post infection and maximum titre of total virus was found at 72h post infection. The difference of virus titre in supernatant at 96h post infection was $1.5\log_{10}$ and in total virus at 72h post infection was $1.6\log_{10}$. They have also titrated different batches of vaccine viruses and compared using Vero, VeroNectin-4, and VeroDogSLAM cells. They found that Vero-nectin infected with PPRV Nigeria/75 showed high titre (0.9 log) compare to native Vero and comparative virus titration of PPRV Nigeria/75 in Vero-Nectin and Vero.dog.SLAM showed higher titre by 1.7 log in VeroNectin-4 cell line. They found only 10% efficiency of Vero-nectin cells for isolation from frozen samples. The efficiency of isolation of Vero-nectin was 42% and a VeroDogSALM cell was 22% from fresh samples. The efficiency of isolation from fresh swabs was 23% for Vero-nectin cells and 4.5% for VeroDogSLAM cells.

Feng *et al*., in 2016 evaluated the replication ability of the Monkey-BJ01-DV virus (CDV isolated from rhesus monkey isolate of in 2008) in engineered Vero/SLAM cell lines. Monkey-BJ01-DV replicated to the highest titers on Vero/DogSLAM ($10^{5.2\pm0.2}TCID_{50}$/ml) and Vero/MonkeySLAM ($10^{5.4\pm0.1}TCID_{50}$/ml) at 48 h post-infection, whereas replication on the Vero/HumanSLAM cells was reduced approximately 100-fold ($10^{3.3\pm0.3}TCID_{50}$/ml. The dog-CDV isolate from a Tibetan mastiff replicated to high titers on the Vero/DogSLAM cells and displayed reduced titers on the Vero cells expressing SLAM from monkey-or human-origin. These results indicate that both the monkey-and dog-CDV isolates can efficiently replicate on Vero cells expressing either the monkey-or dog-origin SLAM receptor, but replicate less efficiently on cells expressing the SLAM receptor of human-origin.

Bieringer *et al*., in 2013 adapted recombinant wild type CDV-A75^{red} virus to human CD150 by a single change in amino acids at 540 in H protein (D540G) and also conducted the single step growth curve in Vero-cSLAM and Vero-hSLAM. They concluded their findings that the Human CD150 adapted CDV-A75^{red} (CDV-A75-ad) grow well in hSLAM cells than the parental virus.

Leonard *et al*., in 2008 measured the growth kinetics of MV in Vero-hSLAM and Vero cell lines. They found that MV replicates efficiently in Vero-hSLAM than native Vero cells. They found maximum titre of cell associated virus around 60 h post infections in both

Vero ($10^{4.5}TCID_{50}$/ml) and Vero-hSLAM ($10^{6}TCID_{50}$/ml) and in supernatant maximum titre was found around 60h post infection in Vero ($10^{2}TCID_{50}$/ml) and Vero-hSLAM ($10^{5}TCID_{50}$/ ml).

Birch *et al*., in 2013 developed PO cell line encoding ovine SLAM or Nectin-4 ORFs by Lentivirus transduction strategy followed by puromycin selection. They conducted High-MOI (4 moi) single-step growth curve of PPRV in PO cell lines with PPRV (Ivory Coast 1989 strain). Experimental infection of the transduced cell lines with PPRV (Ivory Coast 1989 strain resulted in large syncytia that formed 24 to 48 h post infection, whereas no syncytia or visible cytopathic effects were seen in the wild-type cells, demonstrating that lentivirus-encoded expression of ovine SLAM or Nectin-4 proteins permits efficient replication of PPRV. Comparison of the replication kinetics of PPRV in a single-step growth curve (multiplicity of infection (moi) of 4) demonstrated significantly faster replication in cell lines expressing SLAM or Nectin-4 compared to nontransduced cells.

Lan *et al*., in 2005 conducted growth profile of three types of canine distemper virus (Virulent CDV strain MD77, KDK-1 and vaccine strain of Onderstepoort) on Vero cells expressing SLAM. They found that Vero DST (DogSLAMTag) cells were appropriate for propagation of all the three types of canine distemper viruses.

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Materials and Methods

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Cell Lines

Vero cells (ATCC CCL-81) available at Division of Biological Products, Indian Veterinary Research Institute, Izatnagar were used in the study. The cells were grown at 37 °C in Eagle's Minimum Essential Medium (EMEM Cat. No#M0769-50L, M/s Sigma Aldrich, USA) supplemented with 10% fetal calf serum (FCS, Cat. No.#16000044, Gibco-BRL, Invitrogen, France) and 2mM glutamine in presence of antibiotic and antimycotic solution (Cat No. #TCL012-5X100ML, M/s Himedia Pvt. Ltd., India) and 100mM Sodium pyruvate (Cat No. #TCL015-1X100ML, M/s Himedia Pvt. Ltd., India) For maintaining the cells, maintenance medium containing EMEM with 2% FBS was used. The Vero cell line was used for transfection, propagation of vaccine virus (both PPRV and CDV) and virus neutralization assay.

3.1.2 Viruses

Two viruses, viz. *Peste-des-petits-ruminants virus* and *canine distemper virus* were used in the current study as per the work design.

(I) *Peste-des-petits-ruminants virus*

PPR vaccine virus "PPRV-Sungri/96" available at Division of Biological Products, ICAR-Indian Veterinary Research Institute was used in this study. This vaccine virus had been adapted and attenuated in Vero cells by serial passaging 60 times and extensive evaluation has found it to be highly efficacious, potent and safe in small ruminants (Sreenivasa *et al*., 2000).

(II) ***Canine distemper virus***

Canine distemper virus, Onderstepoort vaccine strain obtained from M/s Indian Immunologicals (IIL), Hyderabad and maintained at Division of Biological Products, ICAR-Indian Veterinary Research Institute was used in this study.

3.1.3 Clinical Samples

Samples in the form of blood, swabs and tissues (spleen, lungs, lymph nodes, caecum and urinary bladder) were collected from sheep, goat and dogs during the course of studies were used for virus isolation.

3.1.4 Chemicals and Reagents

All the chemicals and reagents used in the study were analytical and molecular grade and procured from Sigma-Aldrich, Fermentas, HiMedia, Amresco, Qiagen, Hyclone, Merck, SRL, Invitrogen, Applied Biosystems and other reputed firms. Detailed formulation of all the reagents and buffers used in the study are given in appendix. The source of special chemical used in this study has been mentioned at methodology section described in this chapter. The glassware and plasticware were procured from Borosil, Schott Duran, Axygen, Nunc, Nalgene, TPP and other reputed firms.

- Chloroform (Sigma-Aldrich, St. Louis, USA)
- Diethylpyrocarbonate (DEPC) (Sigma-Aldrich, St. Louis, USA)
- Histopaque-1077 (Sigma-Aldrich, St. Louis, USA)
- Tri Reagent LS (Sigma-Aldrich, St. Louis, USA)
- Moloney murine leukemia virus-reverse transcriptase (MMLV-RT) origin (Promega Corporation, Madison, WI, USA)
- SapphireAmp Fast PCR Master Mix (Takara Bio INC.)
- Phusion High-Fidelity DNA polymerase (Thermo Scientific)
- Q5® High-Fidelity DNA polymerase (New England Biolab)
- Lipofectamine 2000 (Invitrogen)
- Lipofectamine 3000 (#L3000001, M/s Invitrogen)
- 10 mM dNTP’s mix (Thermo Scientific, Maryland, USA)

- 100 mM $CaCl_2.2H_2O$ (Merck)
- Glycerol (100% w/v) (Amresco)
- Ethidium Bromide (Sigma-Aldrich, St. Louis, USA)
- Luria- Bertani (LB) Broth (Himedia, Mumbai, India)
- Luria- Bertani (LB) Agar (Himedia, Mumbai, India)
- Ampicillin (Gold Biotechnologies, St. Louis, MO)
- Agarose (Amresco)
- 100 bp plus DNA ladder (Thermo Scientific, Maryland, USA)
- 1 kb gene ruler (Thermo Scientific, Maryland, USA)
- 6X loading dye (Thermo Scientific, Maryland, USA)
- Ethylene Diamine Tetra Acetic acid, EDTA (Sigma-Aldrich, St. Louis, USA)
- Isopropanol (Sigma-Aldrich, St. Louis, USA)
- Ethanol (Amresco, Solon, Ohio)
- Acrylamide and bis- acrylamide (Himedia)
- Sodium-dodecyl sulphate (Himedia)
- Ammonium per sulphate (Himedia)
- Tetramethylethylenediamine (TEMED) (Himedia)
- Coomassie Brilliant Blue R-250 (Amresco)
- Neomycin-G418 (Gibco, Life technologies)
- Phenylmethylsulfonyl fluoride (PMSF) (Thermo fisher)
- Concanavalin A (conA) (Sigma-Aldrich)
- 3,3'-diaminobenzidine tetrahydrochloride (DAB)
- Skimmed milk powder (Merck)
- Methanol (Amresco)
- Nuclease free water (Sigma-Aldrich, St. Louis, USA)

3.1.5 Different Kits Used in the Study

- RNA Extraction Kit: RNeasy Mini Kit, (Cat no.74104, M/s Qiagen GmbH, D-40724 Hilden, Germany)
- cDNA Synthesis Kit: RevertAid™ First Strand cDNA Synthesis Kit, (Cat no.K1632, Thermo Scientific, Maryland, USA)
- Gel extraction kit: QIAquick Gel Extraction Kit, (Cat no. 28704, M/s Qiagen, Hilden, Germany)
- GeneJET Plasmid Miniprep Kit: (Cat no.K0503, M/s Thermo Scientific, Maryland, USA)
- CloneJET PCR Cloning Kit: (Cat no. K1232, M/s Thermo Scientific, Maryland, USA)

- SureSpin Plasmid Mini Kit: (Cat no. NP-37105 M/s Genetix Biotech, Asia Pvt. Ltd)
- TransformAid Bacterial transformation kit: (Cat no. K2711 M/s Thermo scientific, Maryland, USA)
- Endofree Plasmid Maxi Kit: (Cat no. 12362, M/s Qiagen, Hilden, Germany)
- DNeasy Blood & Tissue kit: (Cat no. 69504, M/s Qiagen, Hilden, Germany)

3.1.6 Vectors:

1. **pJET1.2/blunt vector (**Thermo Scientific, Maryland, USA): It is a linearized cloning vector having novel positive selection feature. This vector contains a lethal gene which is disrupted by ligation of a DNA insert into the cloning site. As a result, only cells with recombinant plasmids are able to propagate, eliminating the need for expensive blue/ white screening **(Fig.: 6).**

2. **pcDNA3.1 (+) mammalian expression vector** (Invitrogen): This vector was a kind gift by Dr P.K. Gupta, PS, Division of Vety. Biotechnology. The vector having size of 5446bp with the neomycin resistance gene for selection of stable cell lines using neomycin (Geneticin®) **(Fig.: 7)**.

3.1.7 Oligonucleotide Primers used in the study

The primers used in the study synthesized at Eurofins Genomics India Pvt. Ltd., Bengaluru, Karnataka, India are listed in Table 1.

3.1.8 Equipments

- Refrigerated Centrifuge 2-16PK (M/s Berthold Hermle AG, Gosheim, Germany)
- Microcentrifuge-Minispin (M/s Eppendorf AG, Hamburg, Germany)
- Bench top refrigerated centrifuge-HermleZ360K (M/s Berthold Hermle AG, Gosheim, Germany)
- SureCycler 8800 Thermal Cycler (Agilent technologies)
- Personal cycler (M/s Biometra, Analytik Jena AG)
- Thermo scientific CO_2 incubator-MC0175 (M/s Thermo Scientific, Maryland, USA)
- Vortex mixer, cyclomixer (M/s Remi Instruments Ltd., Mumbai, India)

Table 1: Details of oligonucleotide primers used in the study

S No.	Primer code	Sequence (5' to 3')	Size (Bases)	Amplicon Size (bp)	Reference
1	SLAMSGF(Full)	5-CTGATGGATCACAAAGGGCTCCTC-3	24	1017	In present study
2	SLAMSGR(Full)	5-GATGTCAGCACTCTGGAACCGTCAC-3	25		
3	SLAM(Dog)F	5' ATGGATTCCAGGGGCTTCCTCTCC 3'	24	1029	In present study
4	SLAM(Dog)R	5' TCAGCTCTCTGGGAACGTCACGC 3'	23		
5	NPF	5-ATGCCTCTATCCCTTGGAGCCGAG-3	24	1530	In present study
6	NPR1	5-TCAGACCAAATGCCCCCACCCGTTG-3	25		
7	NPR2	5-TCAGACCAAATGCCACCACCCGTTG-3	25		
8	DN-For-Noyce	5-CCAATGCCTCTATCCCTGGGAGCCGAG-3	27	1530	Noyce *et al.*, 2013
9	DN-Rev-Noyce	5-GGAGGCAGGCCTGGGTCAGACCAGGTG-3	27		
10	SLAM/int/for	5' CACCCTGGCCTGCATGGTGGAGAAAG 3'	26	463	Sarkar *et al.*, 2009
11	SLAM/int/rev	5' TGTGGCAGCGACGTAAATGGTG 3'	22		
12	Nectin-4 for	5' TACCTGGGACACAGAGGTCA 3'	20	150	In present study
13	Nectin-4 rev	5' GGGATACCACGCAGGTAAGT 3'	20		
14	SLAM for	5' CCCAAGTCCAGAAATCAGGT 3'	20	137	In present study
15	SLAM rev	5' GCGTCACACTGGCATAGACT 3'	20		
16	Beta Actin (BA-1)	5' GAG AAG CTG TGC TAC GTC GC 3'	20	275	Collins *et al.*, 1995
17	Beta Actin (BA-2)	5' CCA GAC AGC ACT GTG TTG GC 3'	20		
18	GS-Exp-For	5'TTAAGCTTgtgATGGATCACAAAGGGCTC 3'	29	1041	In present study
19	GS-Exp-Rev	5' AACTCGAGTCACTTGTCGTCATCGTCTTTGTAGT CacctccGCTCTCTGGAACCGTCAC 3'	59		
20	SS-Exp-For	5'TTAAGCTTGTGATGGATCACAAAGGGCTC 3'	29	1041	In present study
21	SS-Exp-Rev	5'AACTCGAGTCACTTGTCGTCATCGTCTTTGTAGT CacctccGCTCTCTGGAACCGTCAC 3'	59		
22	DS-Exp-For	5' TTAAGCTTgtgATGGATTCCAGGGGCTTC 3'	29	1053	In present study
23	DS-Exp-Rev	5'AACTCGAGTCACTTGTCGTCATCGTCTTTGTAGT CacctccGCTCTCTGGGAACGTCAC 3'	59		
24	Nectin-Exp-For	5' TTGGTACCgtgATGGCTCTATCCCTTGGAG 3'	30	1554	In present study
25	Nectin-Exp-For	5' AACTCGAGTCACTTGTCGTCATCGTCTTTGTAGT CacctccGACCAAATGCCCCCACCCGT 3'	61		

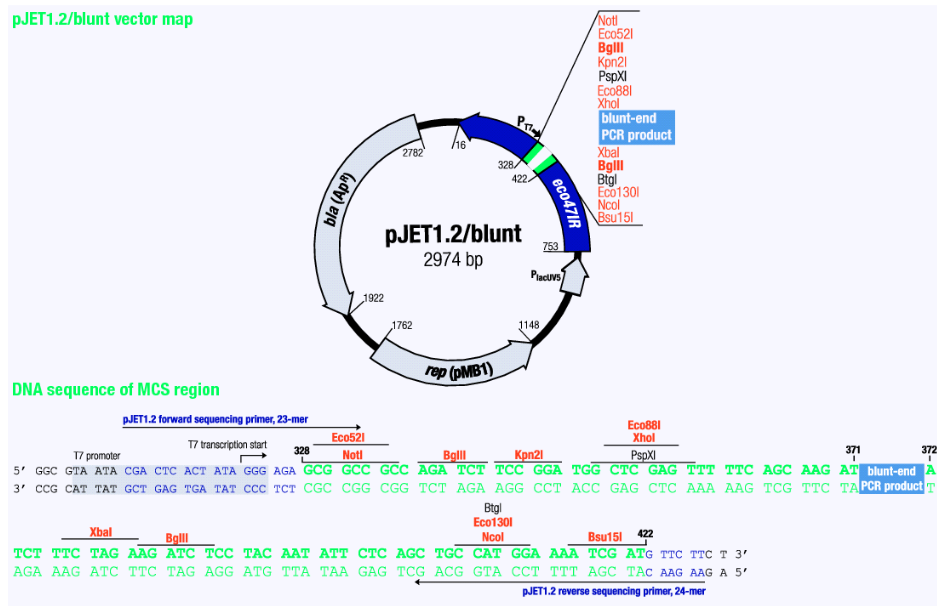

Fig. 6: Schematic diagram of *pJET1.2/blunt* vector map. Linearized cloning vector having lethal gene which is disrupted by ligation of a DNA insert into the cloning site, as a result, only cells with recombinant plasmids are able to propagate (No blue white screening is required).

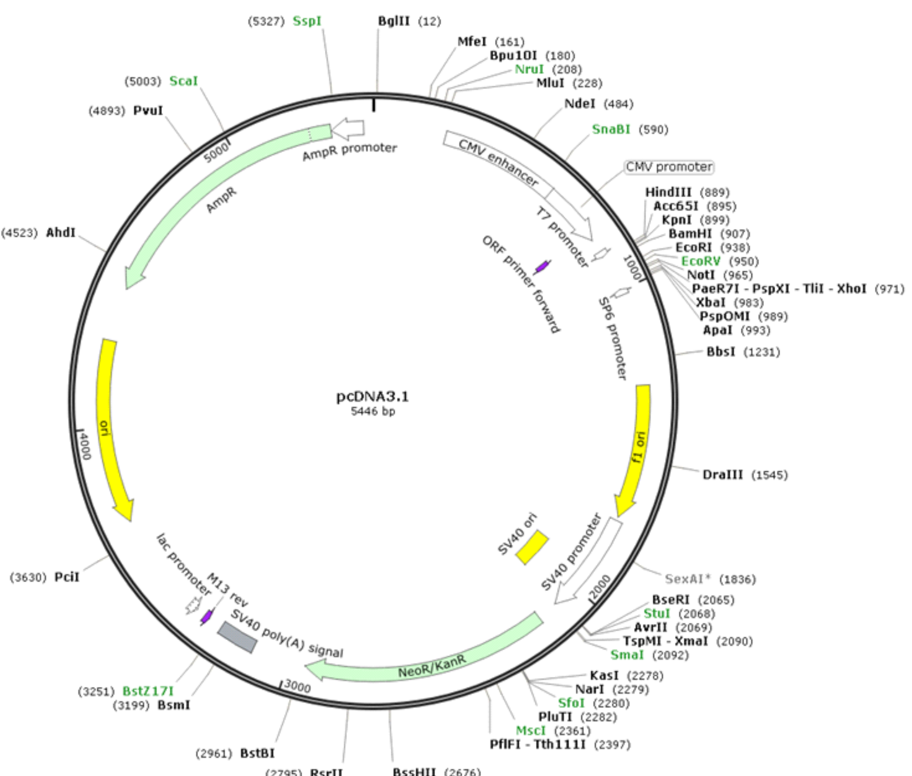

Fig. 7: Schematic diagram of pcDNA3.1(+) mammalian expression vector map. A mammalian expression vector having size 5428 bp with neomycin resistance gene for selection of stable cell lines using neomycin (Geneticin®)

- Micropippettes of various volumes (0.5ìL-1000 µL) (M/s Eppendorf India Ltd., Chennai, India)
- Horizontal electrophoresis apparatus: Amersham Pharmacia Biotech (M/s GE health care U.K.)
- BioGlow® benchtop UV transilluminators @312nm (M/s Crystal Technologies, Inc.)
- Gel Documentation system-Bio Rad (M/s Bio Rad,)
- Freezer (-80°C) (M/s New Brunswick Scientific Co. Inc, Edison, USA)
- Freezer (-20°C) (M/s Vestfrost, Denmark)
- Refrigerator (M/s Godrej Pvt. Ltd.)
- Weighing Balance, BP-121S (M/s Sartorius, Germany)
- Nanodrop (Eppendorf AG, Hamburg, Germany).
- UV Face shield/UV eye guard spectacles (UVP, USA).
- Water Bath: (Kalorstat, Dwarka Equipments Pvt. Ltd., Mumbai)
- Vertical electrophoresis apparatus (Genei Laboratories Pvt Ltd, Banglore)
- Fluorescence microscope (M/s Nikon, Tokyo, Japan)
- pH meter (Benchtop lab system)
- Inverted Binocular Microscope (Olympus Optical Co. Ltd., Japan)
- Laboratory Centrifuge (Remi Equipments, India)
- CO_2 Incubator (Thermo Scientific, Maryland, USA)
- Ice dispenser (Model No. SLF-320A-Q, Blue Star, Italy)
- Laminar Air Flow Cabinets (Klenzaids, India)
- Real-time Thermal Cycler (Aria 1.6, Agilent Technologies, USA)
- Digital Water bath (Akash Deep, Scientific Industries, Delhi, India)
- Nanodrop Spectrophotometer (6135F1402820, Eppendorf, Germany)
- Freezers (–80 °C, New Brunswick Scientific Co. USA)
- Weighing Balance (BP-12S, Sartorius, Germany)
- Microwave Oven (Daewo, Netherland)
- ELISA Reader (Expert Plus, ASYS Hitech, GmbH, Austria)
- Autoclave (DESCO, Medical, India)
- Vacuum Pump (Nippon Electricals, India)

3.1.9 Plastic and glassware

All the plastic ware used in the study were procured from national and international reputed firms *viz.,* M/s Axygen Scientific Inc., Union City, CA, USA; M/s Nalgene Nunc Int., Hamburg, Germany, Future Bio sciences, Falcon, TPP, while glassware were procured from M/s Borosil India Pvt. Ltd, New Delhi, India and M/s Schott Duran, AG Mainz, Germany**.**

3.2 Materials

3.2.1 Blood collection and isolation Peripheral Blood Mononuclear Cells (PBMCs)

PBMCs were isolated as per the method described by Boyum, (1968) with some modifications. Blood from goat and sheep was collected aseptically in a tube supplemented with heparin or EDTA anticoagulents from animal facility of Division of biological products; dog blood was collected from male Labrador retriever presented in teaching veterinary clinical complex, ICAR-Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India. Separation of PBMCs was done by density gradient centrifugation using Histopaque 1077 (M/s Sigma Aldrich St. Louis, USA). 5ml of blood was diluted with equal volume of sterile PBS in a sterile 15 ml tube and mixed gently. In another sterile 15 ml tube, 3 ml of Histopaque-1077 was taken and the diluted blood (8 ml) was slowly layered over the Histopaque by touching the wall of the tube. The tube was then centrifuged at 2000rpm for 45 min at 20^0C using swing out rotor. After centrifugation, a white ring (Buffy coat) containing PBMCs appeared in between histopaque and plasma, was collected in sterile 15 ml tube gently by a sterile pipette avoiding RBCs. Sterile PBS was added to the collected PBMCs to make it to a final volume of 10ml and centrifuged at 2000xg for 10 min in order to get pellet. RBC contamination was removed by resuspending the PBMC pellet with 3 mL of RBC lysis solution and kept in ice for 10 minutes, then sterile PBS was added to make it to a final volume of 10ml and centrifuged at 2000xg for 10 min in order to get pellet. Finally, the PBMCs pellet was resuspended in 6 mL of sterile 10% RPMI 1640 and seeded in 6 well plates $1x10^6$ cells/ml, with mitogen concanavalin-A (conA) @10 µg/mL. After 48 hrs of stimulation the PBMCs were harvested and kept in -20ºC for further use.

3.2.2 Collection of tissue samples for nectin-4 gene amplification

Placental tissues from goat were collected from local meat shop and preserved in RNA later. The canine mammary tumor tissues was collected from a female dog presented to teaching veterinary clinical complex and preserved in RNA later and kept in -20 ºC.

3.2.3 RNA Isolation

Total RNA was isolated using Tri-Reagent® (M/s Sigma Aldrich). This method was used to extract total RNA from PBMCs as well as from tissue samples. In this method, modified guanidium-phenol-chloroform procedure (Chomczynski and Sacchi, 1987) was followed. Briefly: 750 µL Tri-reagent® was added to 250 µL of cell (2×10^6 cells/ml) or tissue (10% suspension in 1X PBS) suspension, in a 2.0 mL microcentrifuge tube and was kept at room temperature (RT) for 15 min after mixing the solution thoroughly by vortexing. 200 µL of chloroform was then added to it and kept at RT for 15 min and vortexed. The mixture was further centrifuged at 10,000xg for 15 min. Clear supernatant was then carefully collected in a new 1.5 µL microcentrifuge tube without disturbing the sediments. Equal volumes of isopropanol was added to it and kept standing at -20 ºC for 1h to allow precipitation of RNA. The precipitated RNA was then pelleted at 12,000xg for 15 min at 4°C in a refrigerated centrifuge. Supernatant was discarded and the pellet was washed by adding 70% ethanol (approx. 500µL) and centrifuged at 10,000xg for 10 min. The supernatant was discarded and pellet was air-dried. Finally, RNA pellets were dissolved in 20 µL nuclease-free distilled water (Sigma-Aldrich, St. Louis, USA), quality checked and then stored at -20ºC for further use.

3.2.4 Reverse Transcription (cDNA synthesis)

Total RNA isolated from PBMCs and tissues were reverse transcribed using RevertAid™ First Strand cDNA Synthesis Kit #K1621, #K1622 (M/s Thermo Scientific, Maryland, USA) as per manufacturer's instructions. In a 0.5 mL microcentrifuge tube, 1 µL (0.2µg/µL) of oligo(dT) primer was mixed with 1000 ng RNA and total volume was made up to 12µL using NFW. Then it was incubated at 70°C for 5 min, followed by snap-cooling. After that following reagents were added to the same tube and made the final volume up to 20µl:

Component (Stock)	Amount (Volume)
5X RT Buffer	4.0 µL
10mM dNTP	2.0 µlL
MMLV RT (200U)	1.0 µL
Ribolock (20 U)	1.0 µL
	8.0 µl
	12.0 µL (Primer + RNA + NFW)
Total volume	**20.0 µL**

The reaction mixture was kept at 25^{0}C for 5 minutes, 42^{0}C for one hour, and at the end of incubation, the reaction mixture was heated at 80^{0}C for 5 minutes to inactivate residual MMLV-RT. The cDNA thus obtained was used for PCR and the remaining was kept at – 20^{0}C until further use.

3.2.5 Polymerase Chain Reaction (PCR) for full-length SLAM and nectin-4 genes amplification

RNA isolated from PBMCs and tissues were subjected for cDNA synthesis and then amplification of SLAM and nectin-4 genes. PCR for both the genes was carried out using self designed and standardized primers. The following reagents were taken in a 0.2 mL PCR tubes for full length amplification of SLAM and nectin-4 genes:

Component (Stock)	Amount (Volume)
5X Phusion HF Buffer	4.0 µL
10mM dNTPs	0.4 µL
Forward primer (10 pmol/µL)	1.0 µL
Reverse primer (10 pmol/µL)	1.0 µL
cDNA	3.0 µL
Phusion DNA Polymerase (5U/µL)	0.2 µL
Nuclease free water	10.4 µL
Total reaction volume	**20.0 µL**

The PCR components were mixed properly and set with the amplification condition as mentioned below:

Step 1			
Initial Denaturation	30 sec	98°C	
Step 2			
Denaturation	20 sec	98°C	35 cycles
Annealing	30 sec	60°C	
Extension	30 sec/kb	72°C	
Step 3			
Final extension	10 min	72°C	

The amplicons so generated by PCR amplification were resolved on agarose (Amresco) gel electrophoresis using 1X TAE buffer (Appendix) at 80 volt for 45 min. The 5 µL of the PCR product was run on 1% agarose gel and visualized in a UV transilluminator (M/s Crystal Technologies, Inc) and documented. Appropriate molecular weight DNA marker (100bp plus, MBI Farmentas, Maryland, USA) was used to confirm the product size.

3.2.6 Gel purification of PCR product

Electrophoresis was done with PCR product in 1% agarose gel and the specific band was cut out carefully with minimum exposure to the UV light so as to avoid nicking of the DNA and collected in a 2.0 mL microcentrifuge tube. PCR product was purified by QIAquick gel extraction kit (Qiagen) as per the manufacturer's instructions. The sliced gel was placed into a pre-weighed sterile 2 mL microcentrifuge tube and weighed. Three volume of buffer QG was added and the mixture was incubated at 50°C for 10 min (or until the gel slice dissolved completely). To facilitate melting process and to ensure complete mixing, tube was inverted at every 1-2 min during incubation. After the gel slice dissolved completely, 1 gel volume of 100 % isopropanol was added and mixed by inverting the tube 4-6 times. QIAquick spin column was placed into a 2 mL collection tube provided with the kit. To bind the DNA, the sample was transferred to the QIAquick spin column, and centrifuge at 12,000 rpm for 1 min. The maximum volume of the column reservoir is 800 µL. For sample volumes of more than 800 µL, the remaining sample was processed in the same way using the same column. The flow-through was discarded and QIAquick spin column was placed back into the same collection tube and again 500 µL of buffer QG was added to the column and centrifuged at 12,000 rpm

for 1 min. The flow-through was discarded and placed the column into the same collection tube. To wash the DNA, 750 µL of buffer PE was added to the QIAquick spin column let the column stand 2-5 min after addition of buffer PE and centrifuged for 1 min at12, 000 rpm. The flow-through was discarded and column was placed back into the same collection tube. To completely remove the residual wash buffer, the empty QIAquick spin column was additionally centrifuged for 1 min at 12,000 rpm. The QIAquick spin column was placed into a new 1.5 µl micro centrifuge tube and 20 µl of elution buffer was added to the centre of the purification column and stand 3-5 min and then centrifuged for 3 min. at 12,000 rpm. The eluted DNA was checked for quality and quantity by Nanodrop spectrophotometer and stored at -20°C till further use.

3.2.8 DNA isolation

DNA was isolated by DNeasy Blood & tissue kit (Cat no# 69504, M/s Qiagen, Hilden, Germany). DNA was isolated from the transfected Vero and native Vero cells as per the manufacturer's protocol. Briefly; $5x10^6$ cells were centrifuged for 5 min at 2000 rpm. The cells were resuspended in 200 µl of PBS and 20µl of proteinase K was added. Then 200 µl of buffer AL was added and mixed by vortexing. The tube was incubated at 56°C for 10min in waterbath. 200 µl of ethanol was added and mixed by vortexing. The content of the tube was transferred to DNeasy Mini spin column placed in 2 ml collection tube and centrifuged at 6000xg for 1 min. and flow-through was discarded. Column was placed in a new collection tube and 500 µl of buffer AW1 was added and centrifuged for 3 min at 20,000xg after that the flow-through was discarded. The spin column was transferred to a new 1.5 ml microcentrifuge tube and DNA was eluted by adding 100 µl of buffer AE to the center of the spin column membrane after centrifugation at 6000xg. The purity of isolated DNA was checked in Nanodrop.

3.2.9 Preparation of *Escherichia coli* DH5α competent cells

The competent cells were prepared by TSS method of Chung *et al.* (1989). A single colony of *E. coli* STBL3 cells was picked from a LB agar plate and grown overnight in 5 µL of SOB without any antibiotic at 37°C with shaking at 180-200 rpm in an orbital shaker. This overnight grown culture was diluted 1:100 in freshly prepared LB broth without antibiotic and

incubated at 37°C with shaking at 180-200 rpm to obtain 0.25-0.35 OD_{600}. The bacterial cells were pelleted by centrifugation at 5000 rpm for 10 min. The supernatant was discarded and the cells were incubated on ice. The pellet was carefully resuspended in 1/10th volume of chilled TSS (Appendix) by slow pipetting for 3 to 4 times. The tubes were incubated in ice for 1 h. After incubation, the cells were gently mixed and 200 µL of the competent cell suspension was dispensed using a cut tip in pre-chilled, sterile labeled 1.5 µL microfuge tubes and stored at -80°C till further use.

3.2.10 Preparation of pcDNA3.1 (+) mammalian expression vector

Glycerol stock of pcDNA3.1 (+) vector was revived in 5 mL of LB broth with 100 µg/mL of Ampicillin. The overnight culture was used for plasmid DNA isolation as per the standard protocol mention below.

3.2.11 Plasmid DNA isolation

Plasmid DNA was isolated using GeneJET Plasmid Miniprep Kit: (#K0503, Thermo Scientific, Maryland, USA) as per the manufacturer's instructions. An overnight culture in LB broth was centrifuged at 6000 rpm for 10 min and supernatant was decanted. The cells pellet was resuspended in 250 µL of the Resuspension solution and transferred to a microcentrifuge tube. Then 250 µL of the Lysis Solution was added and mix thoroughly by inverting the tube 4-6 times until the solution becomes viscous and slightly clear. 350 µL of the Neutralization Solution was added and mixed immediately and thoroughly by inverting the tube 4-6 times and centrifuged for 5 min at 12,000 rpm to pellet cell debris and chromosomal DNA. The supernatant was transferred to the supplied GeneJET spin column by decanting or pipetting and centrifuged for 1 min at 12,000 rpm. The flow-through was discarded and column was placed back into the same collection tube and 500 µL of the Wash Solution (diluted with ethanol prior to first use) was added to the GeneJET spin column. Then centrifuged at 12,000 rpm for 30-60 seconds was given. The flow-through was discarded and the column was placed back into the same collection tube to repeat the washing procedure using 500 µL of the Wash Solution. Again the flow-through was discarded and an additional spun at 12000 rpm for 1 min was given to remove the residual Wash solution. The GeneJET spin column was transferred into a fresh 1.5 mL microcentrifuge tube and 30 µL of the Elution Buffer was added to the center of

GeneJET spin column membrane to elute the plasmid DNA. After incubation of 2 min at room temperature the tube was centrifuged at 12,000 for 2 min. The spin column was discarded and the purified plasmid DNA was stored at -20°C.

3.2.12 Ligation protocol

(I) Blunt end cloning

Blunt end cloning of purified PCR products of SLAM and Nectin-4 gene was carried out in *pJET1.2* cloning vector. The ligation of vector and insert was carried out in 1:3 ratio of vector to insert. The amount of Vector and insert required for the ligation were calculated as per the following formula:

$$\frac{\text{Size of insert (bps)}}{\text{Size of vector (bps)}} \times \text{amount of vector (ng)} \times 3$$

The ligation reaction was set in a 0.2 mL PCR tubes: 10x ligation buffer 2µL, Insert 15 µL, Blunting enzyme 1µL, tube was spun briefly and incubated for 5 min at 70ºC and the snap chill was given. After that 1 µL of PEG4000, 1 µL of T4 DNA ligase and 1 µL of *pJET1.2* cloning vector was added and after brief spin the tube was kept at 4 ºC overnight. Next day tube was kept in -20 ºC until use for transformation.

(II) Directional cloning

Plasmid of pcDNA 3.1 (+) mammalian expression vector and SLAM and Nectin-4 gene was RE digested with *XhoI* and *HindIII* enzymes as following;

Component (Stock)	**Amount (Volume)**
10X Cut smart buffer	3.0 µL
XhoI	2.0 µL
HindIII	2.0 µL
pcDNA3.1 vector	10.0 µL
or	
SLAM (Goat, Sheep and Dog)	15.0 µL
Nuclease free water	13.0 or (7) µL
Total reaction volume	**30.0 µL**

Both the RE digested vector and insert were gel eluted as per the standard protocol and were ligated using standard protocol mention above. The ligation reaction was set in a 0.2 mL PCR tubes: 10x ligation buffer 2µL, Insert 15µL, pcDNA vector 1µL, 1 µL of PEG4000, 1 µL of T4 DNA ligase were added and after brief spin, the tube was kept at 22ºC for 1hr in PCR machine and then ligation mix was kept at 4ºC overnight. Next day tube was kept in -20 ºC until use for transformation.

3.2.13 Transformation

Competent cells (100 µL) were thawed over ice for 20 min and 5µL ligation mixture or 1µL of plasmid DNA was added, mixed and kept on ice for 30-45 min. Following heat shock at 42° C for 90 sec, the tube was immediately transferred to chilled ice for 5 min. After addition of 500 µL SOC medium (Appendix), the tube was kept for shaking in the orbital shaker incubator at 37°C for 1 hr to allow the expression of Ampicillin resistance genes and recover from stress caused by the heat shock. The transformed bacterial cultures were pelleted by centrifugation at 4000 rpm for 5 min at room temperature. Approximately, 400 µL of supernatant was discarded from each, the pellets were resuspended in rest of the supernatant by slow pipetting and plated on LB agar plate containing appropriate antibiotic i.e. Ampicillin (100 µg/mL) which was incubated at 37°C for 16 to 20 hr. When visible on plates, the recombinant bacterial colonies were selectively grown in LB broth with appropriate antibiotic.

Identification/screening of Recombinant clones

Screening of recombinant clones was done by following method:

1. Observation of recombinant colony growth in the presence of particular antibiotic (antibiotic selection)
2. Amplification of insert DNA from recombinant plasmid construct/Colony PCR
3. Restriction enzyme analysis of the plasmid DNA in agarose gel for identification of the insert and its orientation.
4. DNA sequencing

3.2.14 Colony PCR (Touch-up PCR)

Colony PCR was done to check the positive clones of full length SLAM and Nectin-4 gene. The recombinant clone(s) harbouring the plasmid DNA with insert were screened

based on their ampicillin resistance. In order to confirm the recombinant clones, randomly several colonies were selected and used as template for the PCR amplification using insert specific primers. The positive colonies screened by colony PCR was used for isolation of recombinant plasmid and to make glycerol stock for future use. To prepare glycerol stock, 700 µL of positive culture was taken in 1.5 mL microcentrifuge tube and 300 µL of sterile 50% glycerol was added to it. The mixture was mixed properly and stored at -80ºC. The reaction components and condition used were as follows:

200 µL of bacterial culture was taken in a sterile 0.5 mL tube and centrifuged at 6000 rpm for 2 min and media was decanted and bacterial pellet was resuspended in 30 µL of nuclease free water and heated at 95 ºC for 5 min and then snap chill was given. The bacterial culture was centrifuged at 3000 rpm for 3 min and supernatant was taken as template in colony PCR as below; 2X Hot Start PCR Master Mix (SapphireAmp® Fast PCR Master Mix) 10 µL, T7 forward primer 1 µL, BGH rev primer 1 µL, template (Bacterial culture supernatant after biolysis) 2 µL and NFW 6 µL. The reaction was mix properly and after short spin tubes were incubated in PCR machine in following reaction condition; 94 ºC (3min), 97 ºC (30 sec), 55 ºC (40 sec), 72 ºC (2 min) for 35 cycles, final extension 72 ºC (10 min). The PCR products thus obtained were analyzed 1% agarose gel electrophoresis using standard DNA molecular weight markers.

3.2.15 Restriction Digestion Analysis of recombinants plasmids

The recombinant plasmids were RE digested to confirm the insert in the right orientation. The goat, sheep and dog recombinant plasmids were RE digested with *XhoI* and *HindIII* RE enzymes in 0.2 mL tube as mentioned below;

Component (Stock)	**Amount (Volume)**
10X Cut smart buffer	2.0 µL
XhoI	2.0 µL
HindIII	2.0 µL
Recombinanat pcDNA3.1 SLAM plasmids	3.0 µL
Nuclease free water	11.0 µL
Total reaction volume	**20.0 µL**

The reaction mixture was mixed well and kept at 37ºC in water bath overnight. The reaction mixture was analyzed on 1% agarose after electrophoresis.

3.2.16 DNA Sequencing

The positive clones after confirmation by colony PCR and RE digestion was outsourced sequenced by Eurofins Genomics India Pvt. Ltd., Bengaluru, Karnataka, India. The sequences obtained after sequencing were analyzed by various bioinformatics softwares like Lasergene 6.0 software (DNASTAR Inc., Madison, USA), Bioedit, Megalign and NCBI BLAST.

3.2.17 Isolation of transfection grade endotoxin free plasmid

The positive clone confirmed by colony PCR and restriction digestion analysis, was sequenced and their orientation was checked by DNA STAR bioinformatics tools and they were found in correct frame and orientation. The endotoxin free plasmid was isolated by Endofree Plasmid Maxi Kit (Cat no. 12362, M/s Qiagen, Hilden, Germany) as per the manufacturer's protocol. Positive clone was inoculated in 150 µL of LB broth with Ampicillin (100 µg/mL) and kept at 37ºC overnight in a shaker incubator. The overnight culture was centrifuged at 6000 rpm for 10 min and cell pellet was resuspended in 10 mL of Resuspension buffer (P1). Then 10 mL of the Lysis Solution (P2) was added and mix thoroughly by inverting the tube 4-6 times until the solution becomes viscous and slightly clear and incubated at room temperature (15–25°C) for 5 min. 10 mL of the chilled Neutralization Solution (P3) was added and mixed immediately and thoroughly by inverting the tube 4-6 times. Lysate was poured into the barrel of the QIAfilter Cartridge and incubated at room temperature for 10 min. The cap of QIAfilter Cartridge outlet nozzle was removed and plunger was inserted into the QIAfilter Maxi Cartridge and the cell lysate was filtered into a 50 ml tube. 2.5 mL of Buffer ER was added to the filtered lysate, mixed by inverting the tube approximately 10 times, and incubated on ice for 30 min. The QIAGEN-tip 500 was equilibrated by applying 10 ml Buffer QBT, and the column was allowed to empty by gravity flow. The filtered lysate was applied to the QIAGEN-tip and allowed to enter the resin by gravity flow. The QIAGEN-tip was washed with 2 x 30 mL of Buffer QC. The eluate was collected in a 30 mL endotoxin-free or pyrogen-free tube with 15 ml of Buffer QN. DNA was precipitated by adding 10.5 ml (0.7 volumes)

room-temperature isopropanol to the eluted DNA. The tube content was mixed and centrifuged immediately at ≥15,000 x g for 30 min at 4°C and the supernatant was decanted carefully. The DNA pellet was washed with 5 mL of endotoxin-free room-temperature 70% ethanol and centrifuged at ≥15,000 x g for 10 min. The supernatant was decanted carefully without disturbing the pellet. The pellet was air-dried for 5–10 min, and redissolves the DNA in a suitable volume (100 µL) of endotoxin-free buffer TE and aliquoted in small aliquots (10-10 µL) and stored at -20°C till further use.

3.2.18 Linearization of expression cassette

The goat, sheep and dog SLAM gene after successful cloning and confirmation by sequencing into pcDNA3.1(+) mammalian expression vector, was linearize for the transfection into Vero cells. Care was taken not to disturb the MCS (multiple cloning site) and Neomycin resistance site. A panel of restriction enzymes are available to linearize the construct. The *ScaI* RE enzyme was selected to linearize the expression cassette at ampicillin site. This enzyme will cut the plasmid at a single site (Ampicillin site). The transfection grade endotoxin free plasmids of goat, sheep and dog were linearized with *ScaI* enzyme as follows;

Component (Stock)	**Amount (Volume)**
10X Cut smart buffer	3.0 µL
ScaI	3.0 µL
Endotoxin free plasmid (5 µg)	3.0 µL
Nuclease free water	21.0 µL
Total reaction volume	**30.0 µL**

The single digested recombinants plasmids were gel eluted as per the standard protocol and quantified. This linearized plasmid was used to transfect the Vero cells. Approximately 6-7 mg of the plasmid was used to transfect Vero cells in 25cm^2 flask.

3.2.19 Kill curve analysis

The first critical step for stable cell line generation is determining the optimal antibiotic concentration for selecting stable cell colonies; the optimal concentration is cell type dependent.

A kill curve is a dose-response experiment where the cells are subjected to increasing amounts of antibiotic to determine the minimum antibiotic concentration needed to kill all the cells at a given time. In this study, Vero cells (P-160) were taken to calculate the suitable Neomycin (G418) antibiotic concentration to kill the non-transfected cells. A range of different concentrations of Neomycin was tested to ensure the minimum concentration necessary to kill the non-transfected Vero cells. Kill curve analysis was done as per following procedures (Seki et ai., 2003 and pcDNA3.1 manual, Invitrogen); Vero cells were sub-cultured from 25cc flask and seeded in 6 well plate (approx 1x 10^5 cells /well). Following concentrations of antibiotic was added to duplicate wells: 0, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 µg/mL Neomycin in 2 mL of media. Media was changed with 2% EMEM every 3rd day having different concentration of antibiotic (G418), and observed for the percentage of surviving cells. After 21 days of selection, the minimum concentration of G418 was selected that is sufficient to kill the non-transfected Vero cells.

3.2.20 Selection of transfection reagent

The lineralized cassette of pcDNA3.1 (+) having goat SLAM, Sheep SLAM and Dog SLAM was transfected in Vero cells. Three liposomal transfection reagents i.e.Lipofectamine2000 (Invitrogen), Lipofectamine3000 (Invitrogen) and Viafect (Promega) were used to check the transfection efficiency and downstream of the work. Transfection was done in four well plates as shown in figure **(Fig. 8).**

3.2.21 Transfection Protocol

The Vero cells (P-162) were transfected at 70-80% of confluency. The Transfection was done by Lipofectamine 3000 (M/s Thermo scientific). Briefly 313 µLof OptiMEM was mixed with 19 µL of Lipofectamine 3000 in a 1.5mL tube. In the next step 313 µL of OptiMEM, 6.5-7 µg of endotoxin free lineralised cassette of recombinant pcDNA3.1 (+) having goat SLAM, Sheep SLAM and Dog SLAM and 13 µL of P3000 were mixed in a separate 1.5 mL tube. Then contents of both the tubes were mixed properly and kept at room temperature for 10-15 minutes. Then at the final stage the mixture of plasmid DNA and Lipofectamine 3000 with OptiMEM was transferred to 25cc flask **(Fig. 9).**

3.2.22 Generation of goat, sheep and Dog SLAM expressing stable Vero cell Line

The transfected Vero cells were kept in incubator at 37ºC and after 48 hrs of transfection cells were subcultured and 1x10^5cells/well were seeded in a 6 well plate along with non-transfected Vero cells as control. The Neomycin (G418) antibiotic was added (600µg/mL) as per the concentration determined by kill curve analysis. The plate was kept in 5% CO_2 incubator and media was changed with EMEM containing 10% serum every third day along with addition of neomycin (600 µg/mL). The non-transfected Vero cells start dying after one week and complete death was observed after two weeks of antibiotic pressure. The transfected positive clones were survived under neomycin pressure and patches of resistant clones were started growing after one week of transfection. A confluent monolayer of transfected cells was observed after 21-24 days post transfection. The monolayer was then transferred from 6-well plate to 25cm2 flask under continuous neomycin pressure **(Fig. 10).**

3.2.23 Checking the integration of SLAM gene into genome of Vero cells at different passages level

To check the integration of SLAM gene into the genome of Vero cells, DNA was isolated from both the native Vero cells and transfected Vero/SLAM by DNeasy Blood & tissue kit (Qiagen, Hilden, Germany) as per the manufacturer's protocol. Briefly, SLAM integration into genome of Vero cells was checked by PCR using DNA as a template from native Vero and transfected Vero/SLAM cells as described below;

Component (Stock)	Amount (Volume)
2X PCR master mix (Sapphire^R)	10.0 µL
Forward Expression primer (10 pmol/µL)	1.0 µL
Reverse Expression primer (10 pmol/µL)	1.0 µL
DNA template (From Vero and Vero/SLAM cells)	1.0 µL
Nuclease free water	7.0 µL
Total reaction volume	**20.0 µL**

The PCR components were mixed properly and set with the amplification condition in two step protocol as mentioned below:

Selection of transfection reagent

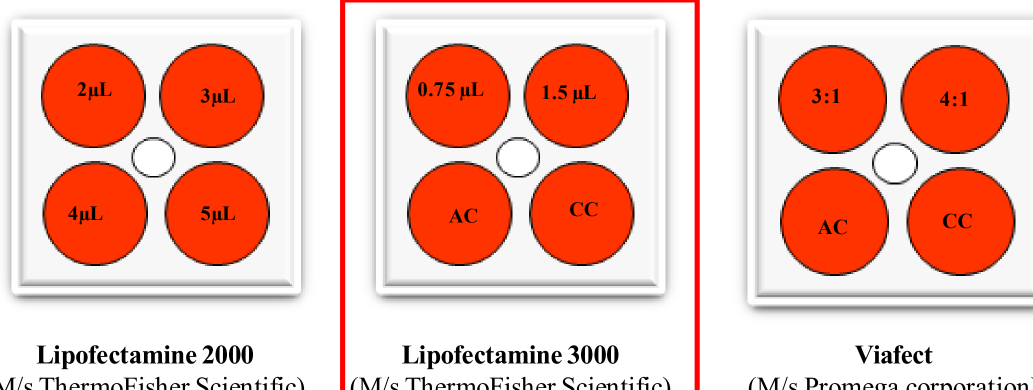

Fig. 8: Strategy employed for selection of transfection reagent for Vero cells in 4-well plate. Reagent in red square (Lipofectamine 3000) was chosen for further study. AC (Antibiotic control), CC (Cell control)

Transfection protocol

Day 0

Step-1: 70 – 90% confluent monolayer

Day 1

Step-2: Dilution of **Lipofectamine™ 3000** in Opti-MEM medium

Step-3: Preparation of master mix of DNA **(6.5-7 µg/25cc flask)** by diluting DNA in Opti-MEM™ Medium, then add P3000™ Reagent as per manufacturer's protocol

Step-4: Add Diluted DNA to each tube of Diluted **Lipofectamine™ 3000 Reagent (1:1 ratio)**

Step-5: Incubation for 10-15 min at RT

Step-6: DNA-lipid complex to cells added

Day 2-4

Step-7: Visualize/analyze transfected cells

DNA

Adding DNA –lipid complex to the Vero cells

Fig. 9: Step-wise transfection protocol of SLAM gene into Vero cells using Lipofectamine 3000

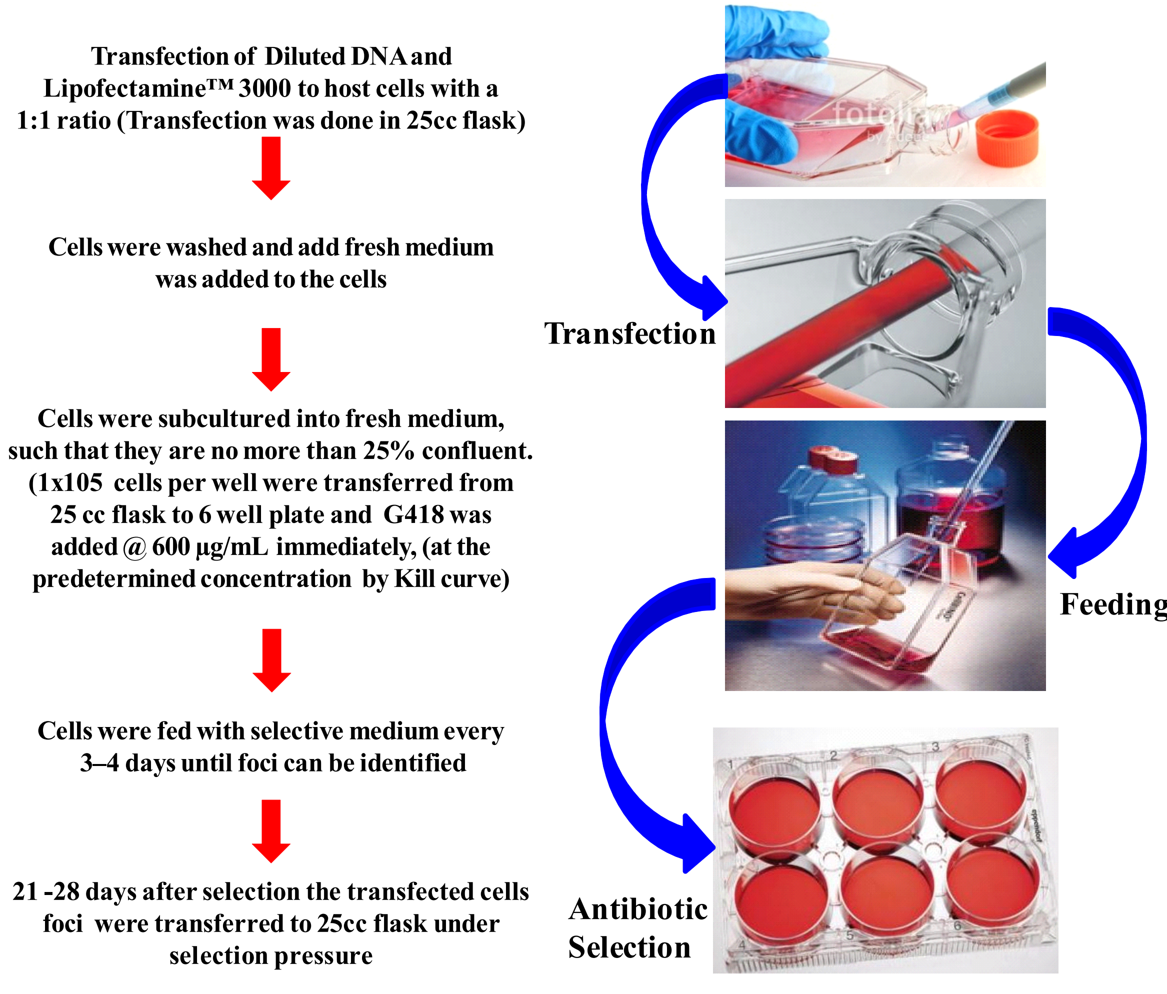

Fig. 10: Generating Stable Vero cell line: Critical steps involved in transfection of Vero cells with recombinant goat, sheep and dog SLAM and Lipofectamine™ 3000 in 25cm^2 flask

Step 1

Initial Denaturation	3.00 min	97˚C	

Step 2

Denaturation	30.0 sec	94˚C	35 cycles
Annealing	40.0 sec	60˚C	
Extension	1.00 min	72˚C	

Step 3

Final extension	10.00 min	72˚C	

The amplicons so generated by PCR amplification were resolved on agarose (Amresco) gel electrophoresis using 1X TAE buffer (Appendix) at 80 volt for 45 min. The total PCR product was run on 1% agarose gel and visualized in a UV transilluminator and documented. Appropriate molecular weight DNA marker (1 kb DNA ladder, MBI Farmentas, Maryland, USA) was used to confirm the product size. SLAM gene from goat, sheep and dog has been amplified as expected size (Around 1000 bps).

3.2.24 Checking the full length SLAM transcript in Vero cell at different passages by RT-PCR

RNA was extracted from Vero/goat/SLAM (VGS), Vero/sheep/SLAM (VSS) and Vero/dog/SLAM (VDS) cells and native Vero cells by Trizol and cDNA synthesis was carried out from total RNA by RevertAid cDNA synthesis kit as described before. The full length SLAM was checked at transcript level in different passage level by RT-PCR using full length SLAM expression primer of the respective species. Briefly,

Component (Stock)	Amount (Volume)
5X Phusion HF Buffer	4.0 µL
10mM dNTPs	0.4 µL
Forward primer (10 pmol/µL)	1.0 µL
Reverse primer (10 pmol/µL)	1.0 µL
cDNA	3.0 µL
Phusion DNA Polymerase (5U/µL)	0.2 µL
Nuclease free water	10.4 µL
Total reaction volume	**20.0 µL**

The PCR components were mixed properly and set with the amplification condition in as mentioned below:

Step 1

Initial Denaturation	30.00 sec	98˚C	

Step 2

Denaturation	20.0 sec	98˚C	35 cycles
Annealing	30.0 sec	60˚C	
Extension	30 sec/kb	72˚C	

Step 3

Final extension	10.00 min	72˚C	

The amplicons so generated by PCR amplification were resolved on 1% agarose (Amresco) gel electrophoresis and documented as described above.

3.2.25 Confirmation the expression of SLAM protein in the stable Vero cells by Immunofluorescence/Immunocytochemistry.

Native Vero cells and respective engineered cell lines were seeded in a 4 well plate at a concentration of $1x10^5$ cells per well in 10% EMEM and kept in incubator having 5% CO_2. After 70-80% of confluency of cells, plate was used for Immunocytochemistry. The protocol for this is described below; cells were fixed with 80% acetone in PBS (80% acetone: 20% PBS) and kept at 4% overnight. Next day acetone was decanted and plate was air dried. Cells were washed three times with 100 mM Glycine in PBS. 1 mL of permeabilization buffer (Appendix) was added to each well and kept for 15-20 min at room temperature after washing with PBS-T (1X PBS with 0.2% tween-20) thrice. Blocking was done with 3% BSA in 0.2% tween-20 for 1hr at 37ºC. Plate was washed thrice with PBS-T and primary antibody against FLAG (M2-monoclonal anti-FLAG antibody, Sigma Aldrich) was added in 1:500 dilution (antibody was diluted in permeabilization buffer) and kept at 37 ºC for 1 hr. Plate was washed thrice with PBS-T and secondary antibody FITC conjugated goat-anti mouse monoclonal antibody (Sigma Aldrich) was added in 1:100 dilution in permeabilization buffer and incubated further at 37 ºC for 1 hr. The plate was washed with PBS-T thrice and 50 µL of 50% glycerol in PBS was added to each well and the plate was observed under fluorescent microscope and result was documented.

3.2.25 Checking of Relative Expression of SLAM gene in different cell lines by RT-qPCR

Total RNA from VGS, VSS and VDS was extracted from different passage (P-3, P-5 and P-8) and cDNA synthesis was carried out using Quantitect reverse transcription kit (Qiagen, Germany) as per the manufacturer's protocol. The cDNA synthesized thus used as template to check the expression level of SLAM in different cell lines (VGS, VSS and VDS). The cDNA synthesized from PBMCs of different species (Goat, sheep and Dog) for SLAM expression was taken as calibrator. The 18S RNA was used as endogenous control. The relative expression of SLAM in different cell lines was quantified with respect to calibrator by ΔΔCt Method or Comparative Ct method (Livak and Schmittgen, 2001).

3.3 Studies on comparative sensitivity of native and stable Vero cell line constitutively expressing goat, sheep and dog SLAM for PPRV and CDV replication efficacy

3.3.(A) Studies on comparative sensitivity of native and stable Vero cell line constitutively expressing goat, sheep and dog SLAM for replication efficacy of PPR (Sungri/96) vaccine virus and canine distemper virus (CDV) Vaccine virus

1. One-step growth kinetics of PPRV Sungri/96 vaccine virus and CDV vaccine virus

One-step growth kinetics of PPRV was carried out as per the protocol described by Singh *et al.,* 2010 and for CDV by Lan *et al.,* 2005 .A confluent monolayer of native Vero, VGS, VSS and VDS cells in culture dishes (35 mm, 8cm^2 surface area) were infected with PPRV and CDV separately using 1.0 moi (Multiplicity of infection) in 2 mL of 2% EMEM. After one hour of incubation the inoculum was removed from the dishes and fresh medium was added and dishes were kept in 5% CO_2 at 37ºC. Culture supernatant (Cell-free virus) and cell-lysate (Cell-associated virus) were harvested separately at 0h, 6h, 12h, 18h, 24h, 48h, 72h, 96h, 120h and 144h post-infection. The adherent infected cells were dislodged in 2.0 mL fresh maintenance medium. The harvested samples were stored in -20ºC till titration. All the samples were subjected to infectivity assay after two cycles of freezing and thawing. The infectivity assay of cell associated and cell free viruses were determined on preformed monolayer

of Vero cells as per the standard method and end point titration was determined using Reed and Muench (1938).

2. Studies on comparative replication efficacy of PPRV and CDV in native Vero and different stable cell lines (VGS, VSS and VDS) using different multiplicity of infection.

Both PPRV and CDV were infected to native Vero, VGS, VSS and VDS in different multiplicity of infection to determine susceptibility of all the four cell lines. Vero, VGS, VSS and VDS cells were subcultured from 25cm^2 flask and seeded in a 24 well plate with a cell concentration of 1x10^{5}cells/well. Neomycin antibiotic was added at a concentration of 600µg/ well in the wells of VGS, VSS and VDS cells. The confluent monolayer (24 hours old) was infected with PPRV and CDV using different moi (1.0, 0.1, 0.01 and 0.001 moi), cell control for all the cell lines were also kept. Media was changed every third day with EMEM having 2% serum. VGS, VSS and VDS cells were maintained under antibiotic pressure up to the observation period of the experiment. Cytopathic effect was observed from third day post infection and final reading of visual cytopathic effect was taken after 6th day post infection.

3.3.4 Titration of PPRV and CDV harvested from Vero and Vero/SLAM cells

Replication efficacy of the PPRV and CDV was assessed by determining the end point titre both in one-step growth kinetics samples and different moi samples. Titration was carried out in 96-well-microtiter plates using Vero cells as per the technique described by Mariner *et al.* (1990). Brief procedure is described below:

Virus was diluted 10-fold ranging from 10^{-1} to 10^{-6} in EMEM containing 2% serum. An aliquot of 100 µL of virus suspension was added to each well keeping four replicates per dilution. The diluted viruses were then transferred to 96-well-microtiter plates having Vero cell monolayer (24 hours). Four wells per plate were also kept as cell-control (without virus). The plates were incubated at 37°C in presence of 5% CO_2. Final reading was taken on 6th day of titration. End point was calculated using Reed and Muench formula (1938).

3.3.5 Detection of PPRV antigen load by monoclonal antibody based PPR Sandwich ELISA

The PPR virus samples of one-step growth kinetics both in Vero and Vero/SLAM cells were detected by sandwich ELISA. The antigenic mass of PPRV in both the cells was compared in relation to OD (Optical Density) value. The assay was carried out as per Singh *et al.* (2004b). Briefly, ELISA plates (Nuc, Maxisorp) (Nalgene Nunc Int., Hamburg, Germany) were coated with capture antibody (100µL/well) diluted in PBS (1:4000; pH 7.2, 0.01 M). The plates were incubated for 1h at 37°C under constant shaking. Unbound antibody was washed thrice using washing buffer (1:4 diluted PBS containing 0.05% tween). Blocking buffer (PBS with 0.5% negative serum and 0.1% tween-20) was added to all the wells (50 µL/well) except in antigen blank well where 100 µL of blocking buffer was added. 50 µL of samples (both supernantant and cell lysate) were added to the sample wells in vertical duplicate. Controls included positive control (received 50 µL reference positive antigen), negative control (received 50 µL reference negative antigen) and antigen blank (no antigen). Then the plates were incubated for 1h at 37°C under constant shaking. Unbound antigens were washed with washing buffer followed by addition of 100mL of diluted PPRV anti N-MAb "4G6" (1:20 diluted in blocking buffer) to each well. The plates were again incubated at 37°C for 1 h under constant shaking and washed thrice. 100µL of rabbit anti-mouse HRPO conjugate (Dako Cytomation, Glostrup, Denmark) (diluted 1:1000 in blocking buffer) was added to each well and again incubated for 1 h at 37°C and washed. 100 µL of freshly constituted substrate solution containing 0.04% w/v of Orthophenyline diamine (OPD) (Sigma-Aldrich, St. Louis, USA) in distilled water was added to each well and kept at 37°C without shaking. 4 µL of 3% hydrogen peroxide (H_2O_2) per mL of substrate was also added just before use. After 10-12 min. colour reaction was stopped by adding equal volume of 1M H_2SO_4. Optical density of the wells was measured at 492nm wavelength. Mean O.D. was calculated by taking average value of the antigen blank wells. Double the mean O.D. of antigen blank wells was taken as cut off point to declare the samples as positive.

3.3.6 Use of stable Vero cells expressing the SLAM receptor for isolation of virulent PPRV and CDV from positive samples

The lung tissues from goat and sheep were collected from Division of Pathology. These tissues which were having optical density of more than 0.7-0.8 in sandwich ELISA for PPRV were chosen for PPRV isolation. Tissue was triturated and sonicated aseptically in ice. 10% tissue suspension was prepared in PBS, antibiotic-antimycotic solution was added to the tissue suspension twice the dose of normal cell culture dose and kept at 4ºC over night. Next day tissue suspension was subjected to high speed centrifugation (12000 rpm for 10 min.) and supernatant was collected in a separate sterile eppendorf tube aseptically. The prepared inoculum was added gently to cell monolayer in a 6 well plate in Vero, Vero/goat/SLAM and Vero/dog/SLAM cells and kept the plate for 1 hr in 5% CO_2 incubator, after 1 hr of incubation, the inoculums was aspirated and fresh media (10% EMEM) was added to the experimental wells. Healthy Vero, Vero/goat/SLAM and Vero/dog/SLAM cells were kept as cell control. After 4th day post infection, all the contents from each well of 6 well plates were transferred to 25 cm^2 flask along with cell control and observed daily for visual cytopathic effect.

For isolation of CDV, blood sample of CDV positive dog was used. The dog blood was diluted 1:1 with EMEM and diluted blood was added gently to the monolayer of Vero and Vero/dog/SLAM cells in 6 well plate. The plate was then kept for 1 hr in incubator to allow the virus to adsorb with cell. At the end of incubation, the blood was aspirated carefully and 10% EMEM media was added to the experimental wells. Healthy Vero and Vero/dog/SLAM cells were kept as cell control. After 4th day post infection, all the contents of 6 well plates were transferred to 25 cm^2 flask along with cell control and observed daily for visual cytopathic effect.

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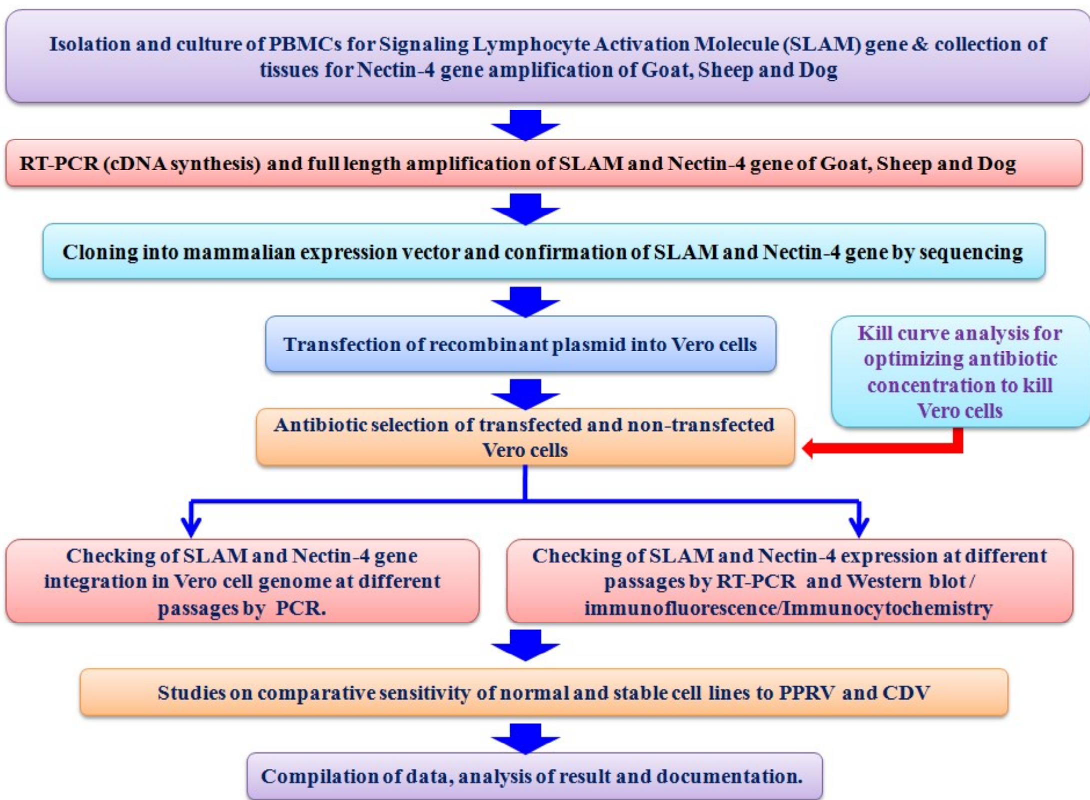
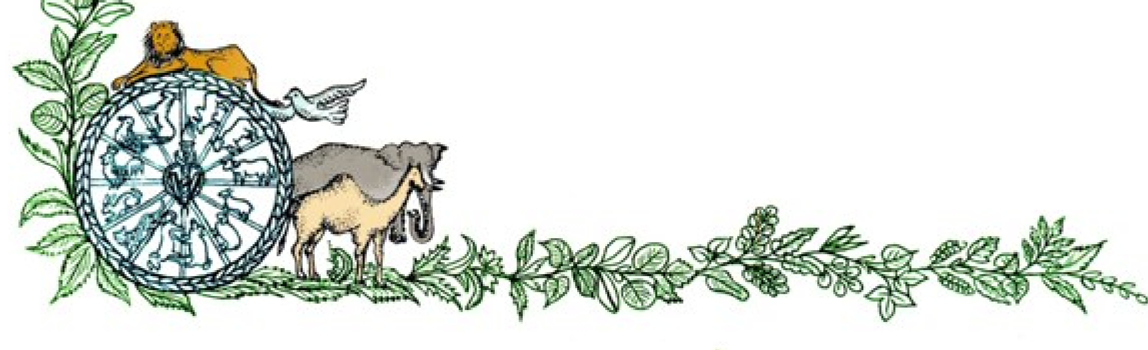


Fig. 11: Flow-chart describing technical programme of present study

Results



4 RESULTS

4.1 Isolation & culture of Peripheral Blood Mononuclear Cells (PBMCs)

Blood from sheep, goat and dog was collected aseptically in a heparinized tube and peripheral blood mononuclear cells (PBMCs) were isolated successfully using Histopaque 1077 and cultured in 10% RPMI-1640 media in a 6 well plate. The PBMCs were stimulated by mitogenic effect of ConA (10µg/mL) and proliferated like bunch of grapes **(Fig:12 a-c)**. The goat, sheep and dog PBMCs were harvested after 48 hrs of post stimulation**.**

4.2 RT-PCR of SLAM and Nectin-4 genes

Total RNA was extracted from stimulated PBMCs and tissue sample as per standard protocol by Guanidium-Phenol-Chlorofom procedure (Chomczynski and Sacchi, 1987) and its purity was checked in Nanodrop spectrophotometer. The RNA concentration and purity was found optimum and cDNA synthesis was carried out as per the standard protocol.

PCR was carried out with cDNA as template which resulted in amplification of SLAM gene of expected size of 1017bp (goat and sheep) and 1029bp (dog) respectively **(Fig:12 d-f)** as observed after resolving the amplified products in 1% agarose. The amplified product was gel purified and concentration and purity was checked in Nanodrop spectrophotometer.

Similarly, PCR amplification of nectin-4 gene of goat, sheep and dog was carried out from cDNA prepared from placental/mammary tumor tissues. Full length nectin-4 gene of all the species was amplified as per expected size of around 1530bp **(Fig:14 a-c)**. The amplified

products were resolved in 1% agarose and gel purified and concentration and purity was checked by Nanodrop spectrophotometer.

4.6 Cloning and Sequencing of SLAM and Nectin-4 gene in *pJET1.2/ blunt* vector

The gel purified products of full length SLAM and Nectin-4 gene from sheep, goat and dog were cloned into *pJET1.2* cloning vector and screened by colony PCR **(Fig: 13 a-c and 15 a-c** respectively**)**. Plasmid DNA from full length clone was isolated from positive recombinant clones. All the recombinant plasmids carrying desired inserts in *pJET1.2* vector were sequenced and aligned with gene sequence available in NCBI data base. Analysis of nucleotide and deduced amino acid sequences from the SLAM of all three species (sheep, goat and dog) and goat nectin-4 confirmed that the clones were having SLAM and Nectin-4 gene in correct frame and orientation. The sequences were submitted to GenBank with following Accession numbers: MG669626 (Goat-SLAM), MG669627 (Sheep-SLAM), MG669628 (Dog-SLAM) and MG870289 (Goat-Nectin-4). Nectin-4 of sheep and dog could not be confirmed as these samples failed in sequencing even after several attempts. Hence, downstream processing of nectin-4 gene could not be initiated and only SLAM genes were taken further.

4.7 Analysis of SLAM gene sequences of goat, sheep and dog

(i) Analysis of SLAM gene:

The SLAM gene sequences of goat, sheep and dog were aligned with human (accession number: NM 003037), Cat (accession number: AB771742) and Asiatic lion (accession number: JN812972) SLAM gene sequences [available in GeneBank] by ClustalW programme of MegAlign of Lasergene 6.0 software **(Figure 16: a & b).** The SLAM gene sequences of these species were aligned with human and dog SLAM gene sequences which showed highest homology between goat and sheep both at nucleotide level (99.8%) and protein level (99.4%). The dog SLAM showed highest homology with redfox and raccoon both at nucleotide (98.5% and 98.9%) and protein level (99.1% and 99.4%) respectively. The dog SLAM showed 84.0% homology at nucleotide level and 74.3% homology at amino acids level with Asiatic lion SLAM. The caprine SLAM distantly related to dog SLAM phylogenetically because it

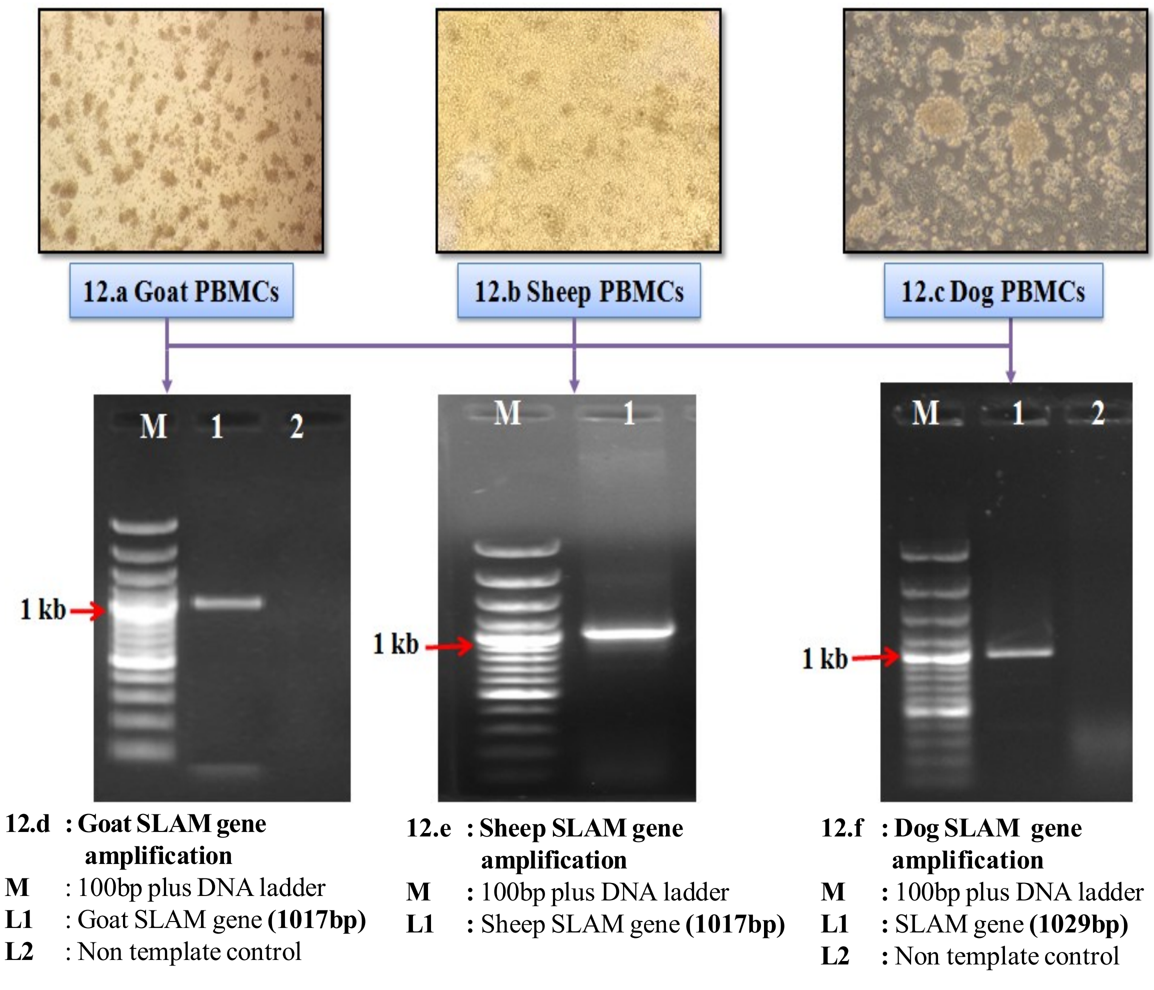
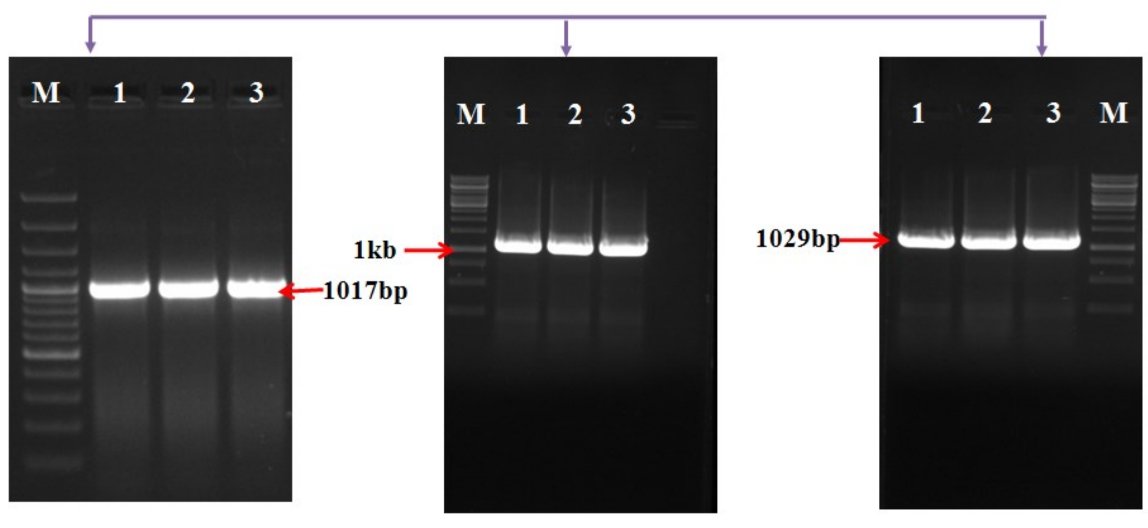


Fig. 12: Amplification of SLAM gene. PBMCs Culture of goat, sheep and dog at 48hrs post stimulation with Con-A@10µg/well (12a-c). Amplification of full-length SLAM genes from PBMCs of respective species (12d-f).

Colony PCR of SLAM gene after cloning into pJET/1.2 blunt vector



13.a	**: Goat SLAM gene**
M	**: 100bp pl**us DNA ladder
L1-3	: different clone

13.b	**: Sheep SLAM gene**
M	: 1 kb DNA ladder
L1-3	: different clone

13.c	**: Dog SLAM gene**
M	: 1 kb DNA ladder
L1-3	: different clone

Fig. 13: Colony PCR of SLAM gene. Confirmation of SLAM insert post cloning into *pJET1.2/ blunt* vector by colony PCR of Goat (13.a), sheep (13.b) and dog (13.c) SLAM genes

Amplification of Goat, Sheep and Dog Nectin-4 gene

Goat Placenta

Sheep placenta

Canine Mammary Tumor

M 1 2 3

1.5 kb

M 1

1.5 kb

M 1

1.5 kb

14.a : Goat Nectin-4 (1530bp)
M : 100bp plus DNA ladder
L1 : Nectin-4gene amplification
L2 : Nectin-4gene amplification
L3 : NTC

14.b : Sheep Nectin-4 (1530bp)
M : 100bp plus DNA ladder
L1 : Nectin-4 gene amplification

14.c : Dog Nectin-4 (1530bp)
M : 1Kb DNA ladder
L1 : Nectin-4 gene amplification

Fig. 14: Amplification of full-length Nectin-4 genes of goat (14.a), sheep (14.b) and dog (14.c)

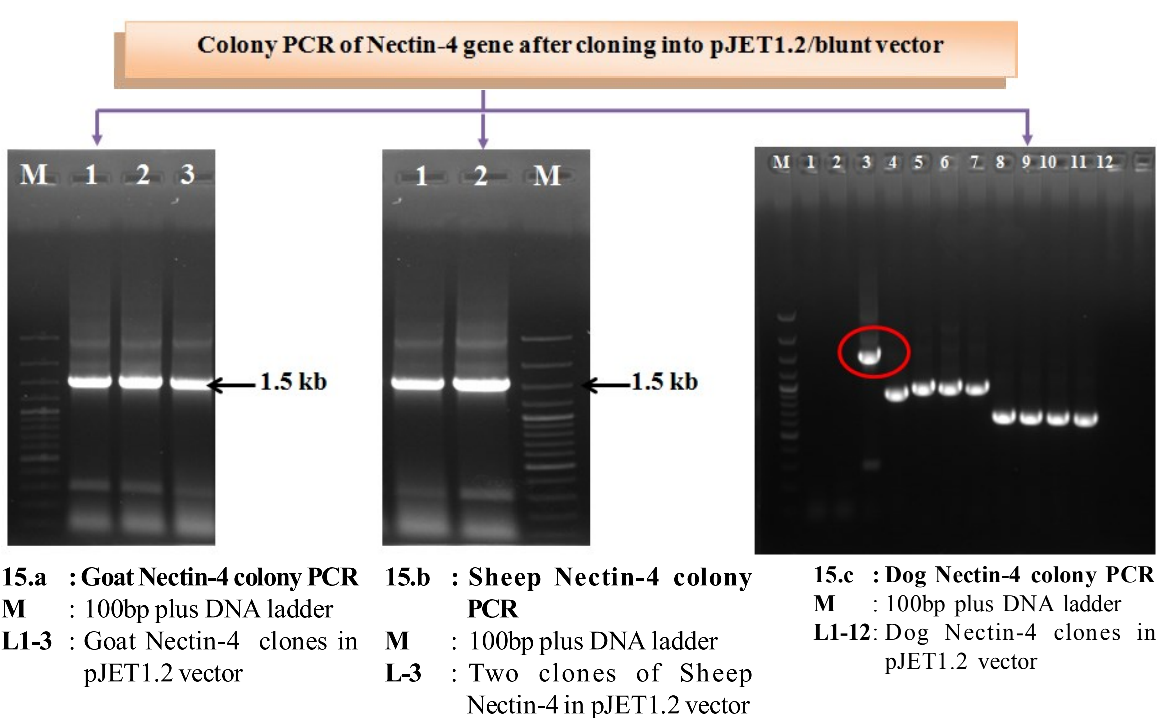


Fig. 15: Colony PCR of Nectin-4 gene. Confirmation of nectin-4 insert post cloning into *pJET1.2/blunt* vector by colony PCR of goat (15.a), sheep (15.b) and dog (15.c) nectin-4 genes

```
                                10        20        30        40        50        60        70        80        90       100       110       120       130       140
MG669626/Goat           MDHKGLLSSNVLLFFSLIIELSCRTGEGLTSSTKTIRGQLGSSVLLPLASEEISRSMNKSIHILVTMAESPRDTVKKKIVSLDLRKGDSPRLEDGYEFHLENLSLRILKSRKEDEGWYFISLEENVSVQHFSLQLKLYEQ
MG669627/Sheep          ............................................................................................................................................
DQ228869/Goat           ............................................................................................................................................
DQ228866/Sheep          ..P.....L..............................................................................G..H.................................................
AF329970/Cattle         ..P..........L......................L..................................K....................N...............................................
DQ228867/Zebu cattle    ..P..........L......................L..................................K....................N...............................................
DQ228868/Water buffalo  ..P..........L....V.................L..................................K...............G....................................................

                               150       160       170       180       190       200       210       220       230       240       250       260       270       280
MG669626/Goat           VSTPQIKVLNSTQEDGNCSLMLACVVEKGDHVTYNWSEEAGAPLLSPTNSSHLLYLTLGPQHANNVYICIASNPISNSSQTFIPWSRCSSRPPESRQWGLYTGLFLGGIVGVIMILQVVILLLRRRGKTDNYQPTMEAKS
MG669627/Sheep          ............................................................................................................................................
DQ228869/Goat           ............................................................................................................................................
DQ228866/Sheep          ........................M................T.....................................................................I...................C........
AF329970/Cattle         ..........T.............M................V............................TV..............PS...SA........A.........N..V....................T....
DQ228867/Zebu cattle    ..........T.............M................V............................TV..............PS...SA........A.........I.......................T....
DQ228868/Water buffalo  ..........T.............M................V........................I...TV..............PS...S.........V.....S...I.......................T....

                               290       300       310       320       330
MG669626/Goat           LTIYAQVQKSGSIQKRPDPLPAQDPCTTIYVAATEPVPEPIQESGSFTVYASVTVPEC
MG669627/Sheep          ..........S..............................................S
DQ228869/Goat           ............VK...........................................S
DQ228866/Sheep          ..................................................L..S
AF329970/Cattle         ........T...V..K..................................L..S
DQ228867/Zebu cattle    ........I...V..K...............V..................L..S
DQ228868/Water buffalo  ........T...V..K....A.............................L..S
```

Fig. 16a: Alignment report of SLAM sequences of PPRV host. The sequence in present study (in red box) matching with already available database sequences of SLAM of other species.

```
                              10        20        30        40        50        60        70        80        90       100       110       120       130       140
MG669628/Dog           MDSRGFLSLRCLLVLALASKLSCGTGESLMNCPEVPGKLGSSLQLSLASEGISKRMNKSIHILVTRAESPGNSIKKKIVSLDLPEGGSPRYLENGYKFHLENLTLRILESRRENEGWYFMTLEENFSVQHFCLQLKLYEQ
AB771742/Cat           ..LK.L...NF..F.S..FE..........T.Q..TEQ..RN.L.P........S..........KS...QKNV.T.....S...RA...F........P...K.E..Q.KK.......T........R........F..
FJ626692/American Mink ..PKRL...NF..F.S..FE...A.....L...KM..Q....VL.P...R....SV...V.V...........V......................T......S.G....KK................R...........
AF325357/Dog           ............................................................................................................................................
EU678639/Raccoon       ...............T............................................................................................................................
EU678638/Red fox       .....................................................R......................................................................................
JN812974/Spotted hyena --------------------------......Q..IHQ..CNVA.P........S..........K....QKN..E....VN........F..D.........S.E....KKK...............R....H......
JN812972/Asiatic lion  --------------------------......Q..TKQ..GN.L.P........S..........KS...QKNV.R..M..S...RA.L.F........P...K.E..Q.KK.......T........R........F..

                             150       160       170       180       190       200       210       220       230       240       250       260       270       280
MG669628/Dog           VSTPEIKVLNWTQENGNCSMMLACEVEKGDNVVYSWSEKLGIDPLIPAN-SSHLLHLSLGPQHVNNVYVCTVSNPVSNRSWSFNPWSKCRPESSVPRQWRLYAGLFLGGIVGVILIFEVVLLLLRRRGKTNHYKPTKEEK
AB771742/Cat           ..................LT.........H.A...R.ET.A..P.S..--.SH..........H..........I..H.QT.T.G.V.M.DPPEL.P.G..V..SS.AVI....TLK.AV........A...Q..T.A.
FJ626692/American Mink ..........L.RG.....LT...D.....Y...N...EVAT...T...N.T.....N....N.....I......I.S..RT.T.K.Q..L..FEST..G......I.........L...I........A.Q.Q..M.A.
AF325357/Dog           .................................................-..........................................................................................
EU678639/Raccoon       ...................V.............................-..........................................................................................
EU678638/Red fox       ...................V....K........................-..........................................................................................
JN812974/Spotted hyena ..........R........L.........H.A...R.EA.T..P.S..--.SH..........H.I.I......I..Q.QT.T.S...M.DPPEL.P.G..V..S..V......TL...V..F.....A...Q..T.A.
JN812972/Asiatic lion  ..................LT......R..H.A...R.ET.A..PVS..--.SH..........H..........I..H.QT.T.G.V.M.DLPEL.P.G..V..SS.AVI....V.K.AV........A...Q..T.A.

                             290       300       310       320       330       340
MG669628/Dog           SLTIYAQVQKSGSTQKKPDPLPAEDPCTTIYVAATEPVPEPAPEPVQEPHSITVYASVTFPES
AB771742/Cat           .............I.....APS.Q................----.....K.........L...
FJ626692/American Mink .............V.........Q..........A....D----....SN.M.......L...
AF325357/Dog           ...............................................................
EU678639/Raccoon       ...............................................................
EU678638/Red fox       ...............................................................
JN812974/Spotted hyena .............I.........Q................----.....N.........L...
JN812972/Asiatic lion  .............I.....APS.Q................----.....K.........L...
```

Fig. 16b: Alignment report of SLAM sequences of CDV host. The sequence in present study (in red box) matching with already available database sequences of SLAM of other species.

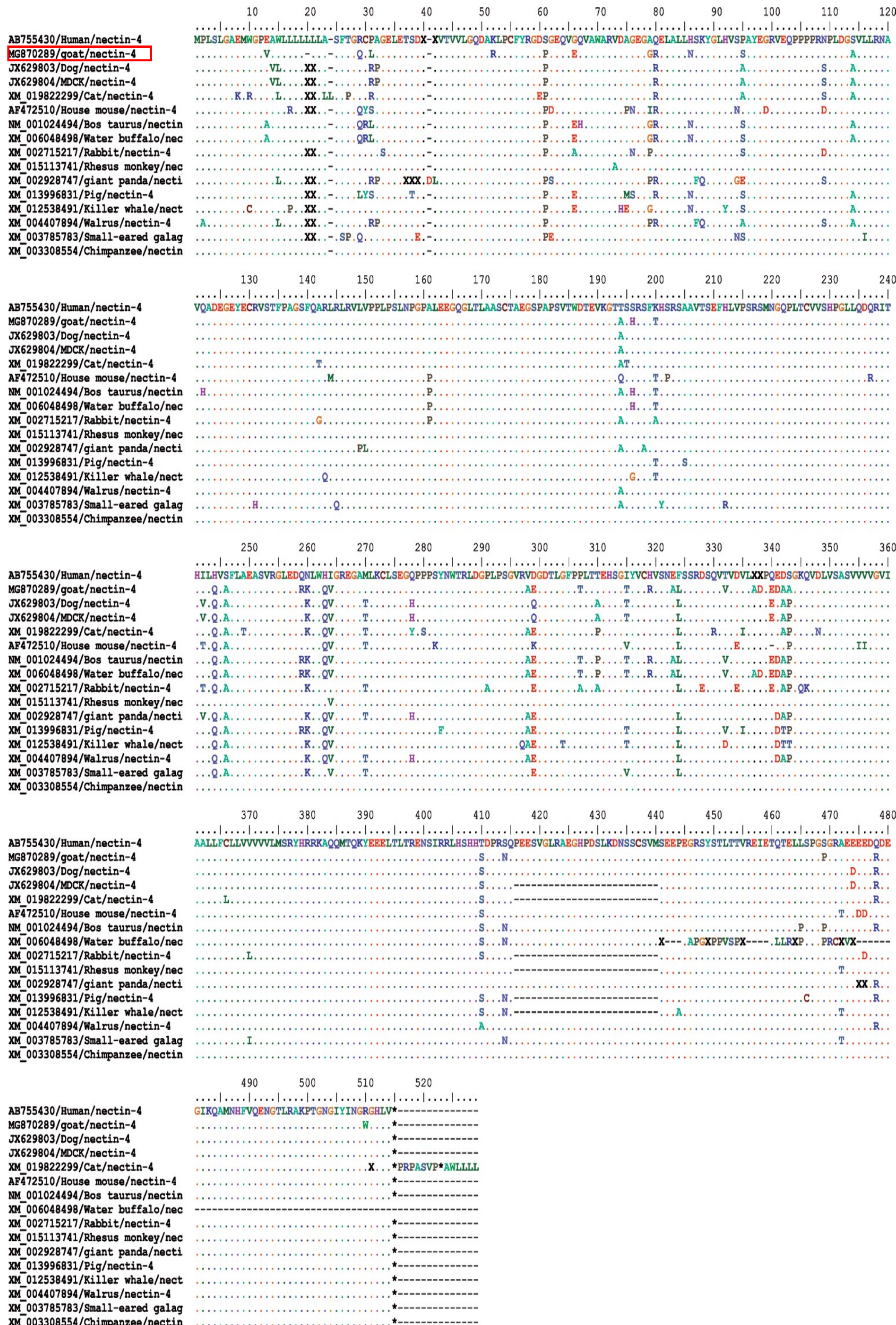


Fig. 16c: Alignment report of Nectin-4 gene. The sequence in present study (in red box) matching with already available database sequences of Nectin-4 of other species.

Table 2: Percent identity between SLAM and Nectin-4 genes of Caprine (*Capra hircus*) with other species [goat SLAM (Accession No: MG669626) and goat Nectin-4 (Accession No: MG870289) were taken as reference sequence]

Species	SLAM		Nectin-4	
	Nucleotide level	Amino acid level	Nucleotide level	Amino acid level
Sheep (*Ovis aries*)\ (Accession No.: MG669627)	99.8	99.4	86.9	86.9
Cattle (*Bos taurus*) (Accession No.: AF329970)	96.3	93.2	97.3	97.7
Zebu Cattle (*Bosindicus*) (Accession No.: DQ8867)	96.3	93.2	90.4	89.8
Water buffalo (*Bubalusbubalis*) (Accession No.: DQ228868)	94.2	92.9	85.3	85.5
Dog (*Canis lupus familiaris*) (Accession No.: MG669628)	80.2	69.8	88.6	93.7
Cat (*Felis catus*) (Accession No. AB771742)	76.4	69.1	85.1	87.8
Racoon (*Procyon lotor*) (Accession No.EU678639)	80.7	69.8	NA*	NA
Red Fox (*Vulpes vulpes*) (Accession No.: EU678638)	80.1	70.1	NA	NA
Spotted Hyena (*Crocuta crocuta*) (Accession No.: AB428368)	81.3	71.1	NA	NA
American mink (Neovison vison) (Accession No: FJ626692)	80.5	72.4	NA	NA
Asiatic lion (*Panthera leopersica*) (Accession No. JN812972)	71.4	67.0	NA	NA
House Mouse (*Mus musculus*) (Accession No.: AB196799)	71.5	57.2	86.4	89.4
Rabbit (*Orycto laguscuniculus*) (Accession No.: XM_8264211)	43.5	17.7	82.3	86.3
Cotton-top-tamarin (*Saguinuso edipus*) (Accession No.: AF302038)	75.4	63.1	NA	NA
Chimpanzee (*Pan troglodytes*) (Accession No.: NM001302657)	38.7	19.8	89.3	92.2
Rhesus macaque (*Macaca mulatta*) (Accession No.: NM_001302666)	37.1	19.8	83.9	87.1
Orangutan (*Pongo*) (Accession No.: XM_002809926)	34.2	18.9	88.7	20.4
Small eared galago (*Otole murgarnettii*) (Accession No.: XM_003795183)	40.2	8.0	87.7	91.6
Giant panda (*Ailuropodamelano leuca*) (Accession No.: XM002929914)	24.2	5.2	88.2	92.0
Killer whale (*Orcinus orca*) (Accession No.: AB428367)	88.9	83.2	87.1	88.8
Dolphin (*Delphinus delphis*) (Accession No.: AB428366)	88.7	83.4	79.2	75.7
Seal (*Phoca vitulina*) (Accession No.: AB428368)	82.4	74.6	90.0	93.3
Walrus (*Odo benusrosmarus*) (Accession No.: AB428369)	81.6	74.0	85.3	85.5
Horse (*Equus caballus*) (Accession No.: XM_005609955)	41.0	57.2	NA*	NA*
Human (*Homo sapiens*) (Accession No.: AY040554)	78.9	63.9	89.4	92.2

NA*: Sequence not available in the NCBI database

showed 80.2% homology at nucleotide and 69.8% at amino acids level. The caprine SLAM is distantly related to Human SLAM (78.9% homology at nucleotide and 63.9% at amino acids level) **(Table.2).**

Table 2: Percent identity between SLAM and Nectin-4 genes (both at nucleotide and amino acids level) of Caprine with other species

Species	SALM		Nectin	
	Nt. level (%)	AA level (%)	Nt. level (%)	AA level (%)
Sheep	99.8	99.4	94.4	95.4
Cattle	96.3	93.2	97.3	97.8
Zebu Cattle	96.3	93.2	94.3	93.5
Dog	80.2	69.8	88.8	93.9
Cat	76.4	69.1	85.1	88.0
Racoon	80.7	69.8	NA	NA
Red Fox	80.1	70.1	NA	NA
Spotted Hyena	81.3	71.1	NA	NA
Human	78.9	63.9	89.3	94.2

NA: Sequence not available

(ii) Analysis of Nectin-4 gene:

The full length goat nectin-4 was amplified first time and upon sequencing analysis revealed that it was 1533bp in length, while in NCBI database the predicted nectin-4 sequences are 1530bps. The goat nectin-4 sequence thus generated was submitted to GenBank. (Accession no: MG870289). The goat nectin-4 sequence was aligned with nectin-4 sequences of sheep (Accession no: XM_015092338), dog (Accession no: JX629803), cat (Accession no: XM_019822299), cattle (Accession no: NM_001024494), water buffalo (Accession no: XM_006048498), rabbit (Accession no: XM_002715217), monkey (Accession no: XM_015113741), giant panda (Accession no: XM_002928747), chimpanzee (Accession no: XM_003308554) and human (Accession no: AB755430) **(Figure. 16c).** The goat nectin-4 is more closely related to the predicted sheep nectin-4 and showed 94.4% homology at nucleotide level and 95.4% at amino acids level. The goat nectin-4 showed 97.3% homology at nucleotide level and 97.4% an amino acids level with predicted cattle nectin-4 sequence

(Accession no: NM_001024494). The goat nectin-4 showed 89.3% homology at nucleotide level and 94.2% at amino acids level with human nectin-4 (Accession no: AB755430) **(Table 2).**

(iii) Analysis of binding region of SLAM gene

SLAM protein has two domains C2 and V. The V region of SLAM is responsible for the interaction with H protein of morbilliviruses. The binding portion of V region of SLAM comprises of 52-136 amino acid residues (Ohishi *et al.,* 2013). The 34 amino acids of the V region are critical for the binding to the H protein of morbilliviruses. The full length SLAMs of goat, sheep, dog, cattle, lion and cat were aligned by Bioedit. The analysis of binding region amino acids of SLAM gene revealed no significant difference in the binding region amino acids of goat and sheep SLAM while cattle SLAM had lysine (K) at position 72. The dog and cat SLAM has major difference at positions 66, 67, 72, 74 and 82. The critical amino acid changes in V region of goat, sheep, dog, cat and cattle are presented in **table 3.**

4.8 Amplification of SLAM genes with expression primers and cloning into pcDNA3.1(+) vector

The SLAM gene of goat, sheep and dog was re-amplified from recombinant clones using expression primers of the respective species having *XhoI* and *HindIII* restriction endonuclease (RE) sites **(Fig:17a-c)**. The inserts (SLAM of goat, sheep and dog) following RE-digestion with *XhoI* and *HindIII* enzymes and after gel purification of both inserts and vector (pcDNA3.1), SLAM genes of goat, sheep and dog were cloned successfully in eukaryotic mammalian expression vector pcDNA 3.1 (+). Dew drop like colonies were obtained after overnight incubation of the transformants containing putative recombinant plasmid at 37ºC in LB-agar/ampicillin plates.

4.9 Confirmation of insertion of the desired DNA in pcDNA3.1(+) mammalian expression vector

(a) PCR amplification/colony PCR

The assay was performed to confirm insertion of the desired gene in to the putative recombinant plasmid using full length primer pair for SLAM (both forward & reverse) as well as vector specific primers (T7 promoter & BGH reverse) and forward SLAM primer (SLAM

Table 3: Analysis of V-Region amino acids of Goat, Sheep and Dog SLAM. Critical amino acids of V region of SLAM involved in binding to PPRV and CDV H protein was compared. Some critical changes were noticed among amino acids of PPRV and CDV host SLAM.

Residue no	58	59	61	63	65	66	67	68	69	72	74	80	82	84	117	119	121	123	125	128	130
Goat SLAM	N	K	I	I	V	T	M	A	E	R	T	V	L	L	W	F	S	E	N	V	H
Sheep SLAM	N	K	I	I	V	T	M	A	E	R	T	V	L	L	W	F	S	E	N	V	H
Cattle SLAM	N	K	I	I	V	T	M	A	E	K	T	V	L	L	W	F	S	E	N	V	H
Dog SLAM	K	S	H	L	T	R	A	E	S	N	I	S	D	P	W	F	T	E	N	V	H
Cat SLAM	K	S	H	L	T	K	S	E	S	K	V	S	S	P	W	F	T	E	N	V	H

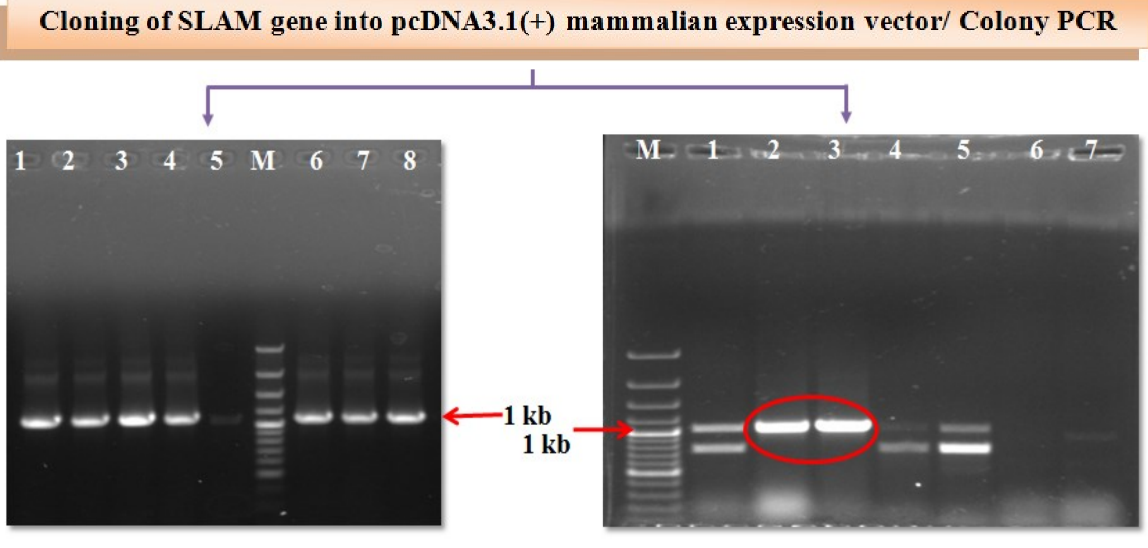
Amplification of Goat, Sheep and Dog SLAM gene with expression primer

17.a : Goat SLAM gene
M : 100bp plus DNA ladder
L1-2 : Goat SLAM gene with expression primers

17.b : Dog SLAM gene
M : 1 kb DNA ladder
L1-2 : Dog SLAM amplified with expression primers
L3 : Non template control

17.c : Sheep SLAM gene
M : 1 kb DNA ladder
L1-2 : Sheep SLAM amplified with expression primers
L3 : Non template control

Fig. 17: Amplification of full-length SLAM gene of goat (17.a), sheep (17.c) and dog (17.b) with expression primer



18.a : Goat and Sheep SLAM colony PCR
L1-5 : Goat SLAM clones
M : 100bp plus DNA ladder
L6-8 : Sheep SLAM clones

18.b : Dog SLAM colony PCR
M : 100bp plus DNA ladder
L1-L7 : Dog SLAM clones (Clone in red circle indicated as positive in colony PCR)

Fig. 18: Colony PCR of SLAM gene by expression primers. Amplification of full-length goat and sheep SLAM (18.a) and dog SLAM (18.b) after cloning in to pcDNA3.1(+) a mammalian expression vector

forward) and reverse vector specific (BGH reverse) primer. The cloned SLAM gene of goat, sheep and dog was successfully amplified from the recombinant pcDNA 3.1(+) vector. Subsequent electrophoretic migration of PCR amplicons in agarose gel along with known molecular weight DNA marker revealed bands just above the 1kb reference band of the marker DNA for goat, sheep and dog SLAM **(Fig:18 a & b)** indicating the specific nature of the amplified product of 1017bp (goat & sheep) and 1029bp (dog).

(b) Restriction Enzyme digestion

Insertion of the desired DNA into the recombinant pcDNA 3.1(+) plasmid vector was further confirmed by RE digestion. *XhoI* and *HindIII* restriction enzymes were used for this assay since they cleave the plasmid at positions flanking coding region gene without cutting the insert in between. On electrophoresis, the RE digested products in 1% agarose gel revealed two bands of approximate 1kb and 6kb **(Fig:19 a-c).** The DNA fragment of more than 1kb size specifies elution of the desired SLAM DNA from the recombinant plasmid.

(c)Sequencing of the cloned DNA into pcDNA3.1(+) vector

The sequencing of cloned products outsourced from Eurofins Genomics Pvt. Ltd. Bangalore, revealed right orientation of goat, sheep and dog SLAM genes in pcDNA3.1(+) plasmid vector upon analysis with Lasergene 6.0 software (DNASTAR Inc., Madison, USA). All the sequences were analyzed for the correct orientation of FLAG tag present at C-terminus of the goat (**Fig:20**), sheep (**Fig:21**) and dog SLAM protein (**Fig:22**).

4.10 Linearization of goat, sheep and dog SLAM-pcDNA3.1(+) expression cassette

The linear plasmid has more chance of integration than circular plasmid. So before going to the actual transfection in the Vero cells with desired construct it has be linearized to increase the efficiency of integration. The goat, sheep and dog SLAM expression cassette were linearized by single digestion with *ScaI* RE enzyme which cut the recombinant plasmids at the ampicillin resistance site. The RE digested products were electrophoresed and single band at approx. 5 Kb was gel purified subsequently **(Fig:23).**

4.11 Kill curve analysis for Vero cells using neomycin (G418) antibiotic

In the present study, pcDNA 3.1(+) mammalian expression vector (M/s Invitrogen) having Neomycin resistance gene and CMV (Cytomegalovirus) promoter was used. A range of different concentrations (0-1000μg/mL) of Neomycin was tested to ensure the minimum concentration necessary to kill the non-transfected Vero cells. The cells became round and started dying after one week of antibiotic selection. After 21 days of selection the minimum concentration of G418 which killed all of the cells was found to be 600μg/mL (**Fig: 24**).

4.12 Development of stable Vero cell constitutively expressing caprine, ovine and canine SLAM

(i) Selection of transfection reagent:

Three liposomal transfection reagents (Lipofectamine 2000, Lipofectamine 3000 and Viafect) were used to check the transfection efficiency and it was found that both Lipofectamine2000 and 3000 worked well but Viafect didn't work properly as indicated by death of the transfected cells after 15 days alike healthy Vero cells (**Fig:25**). Among two lipofectamines, the Lipofectamine3000 was selected for downstream work as the cells looked brighter and healthier and seems to be less toxic than the Lipofectamine2000.

(ii) Generation of stable cell lines:

After confirming the right orientation of all the three caprine, ovine and canine SLAM gene in recombinant pcDNA3.1(+) plasmid by sequencing, the linearized cassette of pcDNA3.1 (+) having goat, sheep and dog SLAM was separately transfected in Vero cells. The SLAM genes were successfully transfected to Vero cells in 25cm^2 tissue culture flask using Lipofectamine3000 transfection reagents. The transfected cells from 25cm^2 flask were subcultured after 48 hrs of transfection and 1x10^{5}cells/well were transferred to 6 well plates along with non-transfected Vero cells and were subjected to G418 selection pressure (600μg/mL) keeping native Vero cells as control. The plate was kept in 5% CO_2 incubator and media was changed containing 10% serum every third day along with addition of neomycin (600μg/mL). During day-to-day monitoring of the cells, it was found that the cells started dying (as indicated by rounding of cells and granulation of cell cytoplasm) from 5-7th day onward of

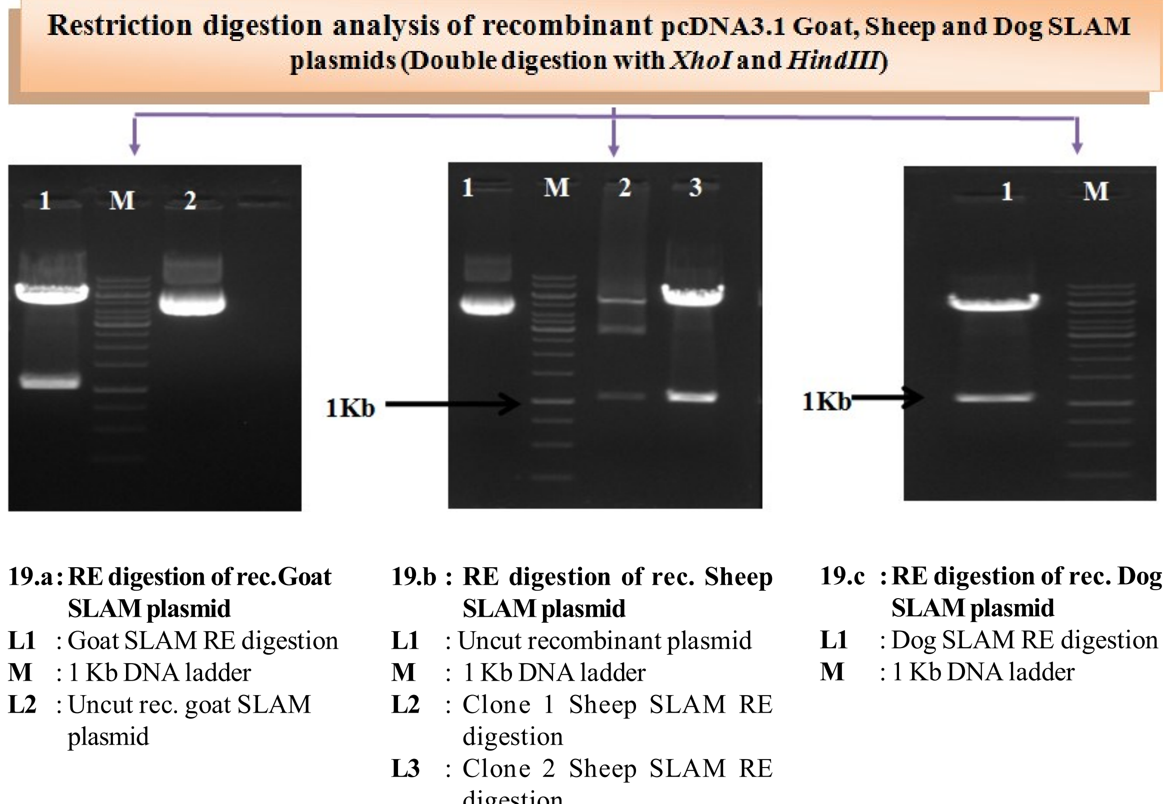


Fig. 19: RE digestion analysis of recombinant pcDNA3.1 Goat (19.a), Sheep (19.b) and Dog SLAM (19.c) plasmids with *XhoI* and *HindIII* enzymes.

Sequence detail of Goat SLAM (insert) in pcDNA3.1 (+)Vector

a. Nucleotide Sequence:

```
TTAAGCTTGTGATGGATCACAAAGGGCTCCTCTCCTCGAATGTCCTGCTGTTTTTCTCTCTGATCATTGAGCTGAGCTGCAGAACAGGTGAGGGCT
TGACCAGTTCCACAAAGACGATTCGTGGGCAGCTGGGAAGCAGCGTGCTGTTGCCCCTGGCATCTGAGGAGATAAGTAGGAGCATGAATAAGAGCA
TCCACATCCTTGTCACAATGGCAGAATCACCCAGAGACACTGTCAAGAAGAAAATAGTGTCCCTAGATCTGCGGAAAGGTGACTCTCCACGTCTGG
AGGATGGCTATGAGTTTCATCTGGAAAACCTGAGCCTGAGGATCCTGAAGAGCAGGAAGGAGGATGAAGGCTGGTACTTTATAAGCCTGGAGGAAA
ATGTTTCAGTCCAGCACTTTAGCCTGCAGCTGAAGCTCTATGAGCAGGTCTCCACTCCGCAAATTAAGGTGTTGAACTCCACCCAGGAAGATGGGA
ACTGCAGTCTCATGCTGGCCTGCGTGGTGGAGAAAGGGGACCACGTGACTTACAACTGGAGTGAGGAAGCAGGTGCCCCCCTGCTGAGTCCCACCA
ATAGCTCCCACCTCTTGTATCTCACTCTTGGCCCTCAGCATGCCAACAACGTCTACATCTGCATTGCGAGCAACCCCATCAGCAACAGCTCTCAGA
CCTTCATCCCTTGGTCCAGATGCAGCTCCAGGCCCCCAGAATCAAGACAATGGGGACTATATACTGGGCTCTTCTTAGGGGGCATCGTTGGCGTTA
TCATGATTCTCCAAGTGGTGATACTACTGTTGAGAAGAAGAGGTAAAACAGACAATTACCAGCCAACAATGGAAGCAAAAAGCCTTACTATCTATG
CCCAAGTCCAGAAATCAGGTTCCATTCAGAAGAGACCTGACCCCCTGCCAGCACAGGACCCCTGCACCACCATTTATGTCGCTGCCACAGAGCCTG
TCCCAGAGCCCATCCAGGAATCAGGTTCCTTCACAGTCTATGCCAGTGTGACGGTTCCAGAGAGCggaggtGACTACAAAGACGATGACGACAAGT
GACTCGAGTT
```

b. Amino Acid Sequence:

```
MDHKGLLSSNVLLFFSLIIELSCRTGEGLTSSTKTIRGQLGSSVLLPLASEEISRSMNKSIHILVTMAESPRDTVKKKIVSLDLRKGDSPRLEDGY
EFHLENLSLRILKSRKEDEGWYFISLEENVSVQHFSLQLKLYEQVSTPQIKVLNSTQEDGNCSLMLACVVEKGDHVTYNWSEEAGAPLLSPTNSSH
LLYLTLGPQHANNVYICIASNPISNSSQTFIPWSRCSSRPPESRQWGLYTGLFLGGIVGVIMILQVVILLLRRRGKTDNYQPTMEAKSLTIYAQVQ
KSGSIQKRPDPLPAQDPCTTIYVAATEPVPEPIQESGSFTVYASVTVPESGGDYKDDDDK
```

FLAG tag

Fig. 20: Sequence details of goat SLAM in pcDNA3.1(+) vector. Nucleotide (a) and Amino acid (b) sequences in correct frame and orientation. FLAG tag (Signal sequence) with RE sites (underlined sequences) located at 32 end of the insert.

Sequence detail of Sheep SLAM (insert) in pcDNA3.1 (+)Vector

a. Nucleotide Sequence:

```
TTAAGCTTGTGATGGATCACAAAGGGCTCCTCTCCTCGAATGTCCTGCTGTTTTTCTCTCTGATCATTGAGCTGAGCTGCAGAACAGGTGAGGGCT
TGACCAGTTCCACAAAGACGATTCGTGGGCAGCTGGGAAGCAGCGTGCTGTTGCCCCTGGCATCTGAGGAGATAAGTAGGAGCATGAATAAGAGCA
TCCACATCCTTGTCACAATGGCAGAATCACCCAGAGACACTGTCAAGAAGAAAATAGTGTCCCTAGATCTGCGGAAAGGTGACTCTCCACGTCTGG
AGGATGGCTATGAGTTTCATCTGGAAAACCTGAGCCTGAGGATCCTGAAGAGCAGGAAGGAGGATGAAGGCTGGTACTTTATAAGCCTGGAGGAAA
ATGTTTCAGTCCAGCACTTTAGCCTGCAGCTGAAGCTCTATGAGCAGGTCTCCACTCCGCAAATTAAGGTGTTGAACTCCACCCAGGAAGATGGGA
ACTGCAGTCTCATGCTGGCCTGCGTGGTGGAGAAAGGGGACCACGTGACTTACAACTGGAGTGAGGAAGCAGGTGCCCCCCTGCTGAGTCCCACCA
ATAGCTCCCACCTCTTGTATCTCACTCTTGGCCCTCAGCATGCCAACAACGTCTACATCTGCATTGCGAGCAACCCCATCAGCAACAGCTCTCAGA
CCTTCATCCCTTGGTCCAGATGCAGCTCCAGGCCCCCAGAATCAAGACAATGGGGACTATATACTGGGCTCTTCTTAGGGGGCATCGTTGGCGTTA
TCATGATTCTCCAAGTGGTGATACTACTGTTGAGAAGAAGAGGTAAAACAGACAATTACCAGCCAACAATGGAAGCAAAAAGCCTTACTATCTATG
CCCAAGTCCAGAAATCAAGTTCCATTCAGAAGAGACCTGACCCCCTGCCAGCACAGGACCCCTGCACCACCATTTATGTCGCTGCCACAGAGCCTG
TCCCAGAGCCCATCCAGGAATCAGGTTCCTTCACAGTCTATGCCAGTGTGACGGTTCCAGAGAGCggaggtGACTACAAAGACGATGACGACAAGT
GACTCGAGTT
```

b. Amino Acid Sequence:

```
MDHKGLLSSNVLLFFSLIIELSCRTGEGLTSSTKTIRGQLGSSVLLPLASEEISRSMNKSIHILVTMAESPRDTVKKKIVSLDLRKGDSPRLEDGY
EFHLENLSLRILKSRKEDEGWYFISLEENVSVQHFSLQLKLYEQVSTPQIKVLNSTQEDGNCSLMLACVVEKGDHVTYNWSEEAGAPLLSPTNSSH
LLYLTLGPQHANNVYICIASNPISNSSQTFIPWSRCSSRPPESRQWGLYTGLFLGGIVGVIMILQVVILLLRRRGKTDNYQPTMEAKSLTIYAQVQ
KSSSIQKRPDPLPAQDPCTTIYVAATEPVPEPIQESGSFTVYASVTVPESGGDYKDDDDK
```

FLAG tag

Fig. 21: Sequence details of sheep SLAM in pcDNA3.1(+) vector. Nucleotide (a) and Amino acid (b) sequences in correct frame and orientation. FLAG tag (Signal sequence) with RE sites (underlined sequences) located at 32 end of the insert.

Sequence detail of Dog SLAM (insert) in pcDNA3.1 (+)Vector

a. Nucleotide Sequence:

```
TTAAGCTTGTGATGGATTCCAGGGGCTTCCTCTCCCTGCGCTGCCTGCTGGTCCTCGCCCTGGCGTCCAAGCTGAGCTGTGGGACAGGTGAGAGCT
TGATGAATTGCCCAGAAGTCCCTGGGAAGTTGGGAAGCAGTTTGCAGCTGTCCTTGGCATCCGAAGGGATAAGCAAGAGGATGAACAAGAGCATCC
ACATCCTTGTCACAAGGGCGGAATCACCGGGAAACAGTATCAAGAAGAAAATAGTGTCTCTGGATCTGCCAGAAGGGGGGTCTCCACGCTACCTGG
AAAATGGCTATAAATTTCATCTGGAAAACCTGACCCTGAGGATCCTGGAAAGCAGGAGGGAAAATGAAGGCTGGTACTTCATGACCCTGGAGGAGA
ACTTTTCAGTTCAACACTTTTGTCTGCAGCTGAAGCTCTATGAGCAGGTCTCCACTCCAGAAATTAAGGTGTTGAACTGGACCCAGGAGAATGGGA
ACTGCAGCATGATGCTGGCCTGCGAAGTGGAAAAGGGGGACAATGTGGTTTACAGCTGGAGTGAGAAACTGGGGATTGACCCACTGATCCCAGCCA
ACAGTTCTCACCTCTTGCACCTCAGCCTCGGCCCTCAGCATGTCAACAACGTCTATGTCTGCACCGTGAGCAACCCCGTCAGCAACCGCTCATGGT
CCTTCAACCCATGGTCCAAGTGCAGGCCAGAGTCTTCGGTGCCAAGACAATGGAGACTGTATGCTGGGCTCTTCTTAGGGGGTATCGTTGGTGTCA
TCTTGATTTTCGAAGTGGTATTACTGCTGTTGAGAAGAAGAGGTAAAACAAATCATTACAAGCCAACAAAGGAAGAAAAAAGCCTTACCATCTATG
CCCAAGTCCAGAAATCAGGTTCTACTCAGAAGAAACCTGATCCCTTGCCAGCTGAGGACCCCTGCACCACCATTTATGTTGCTGCCACAGAACCTG
TCCCAGAACCTGCCCCAGAACCTGTGCAGGAACCACATTCTATCACGGTCTATGCCAGCGTGACGTTCCCAGAGAGCggaggtGACTACAAAGACG
ATGACGACAAGTGACTCGAGTT
```

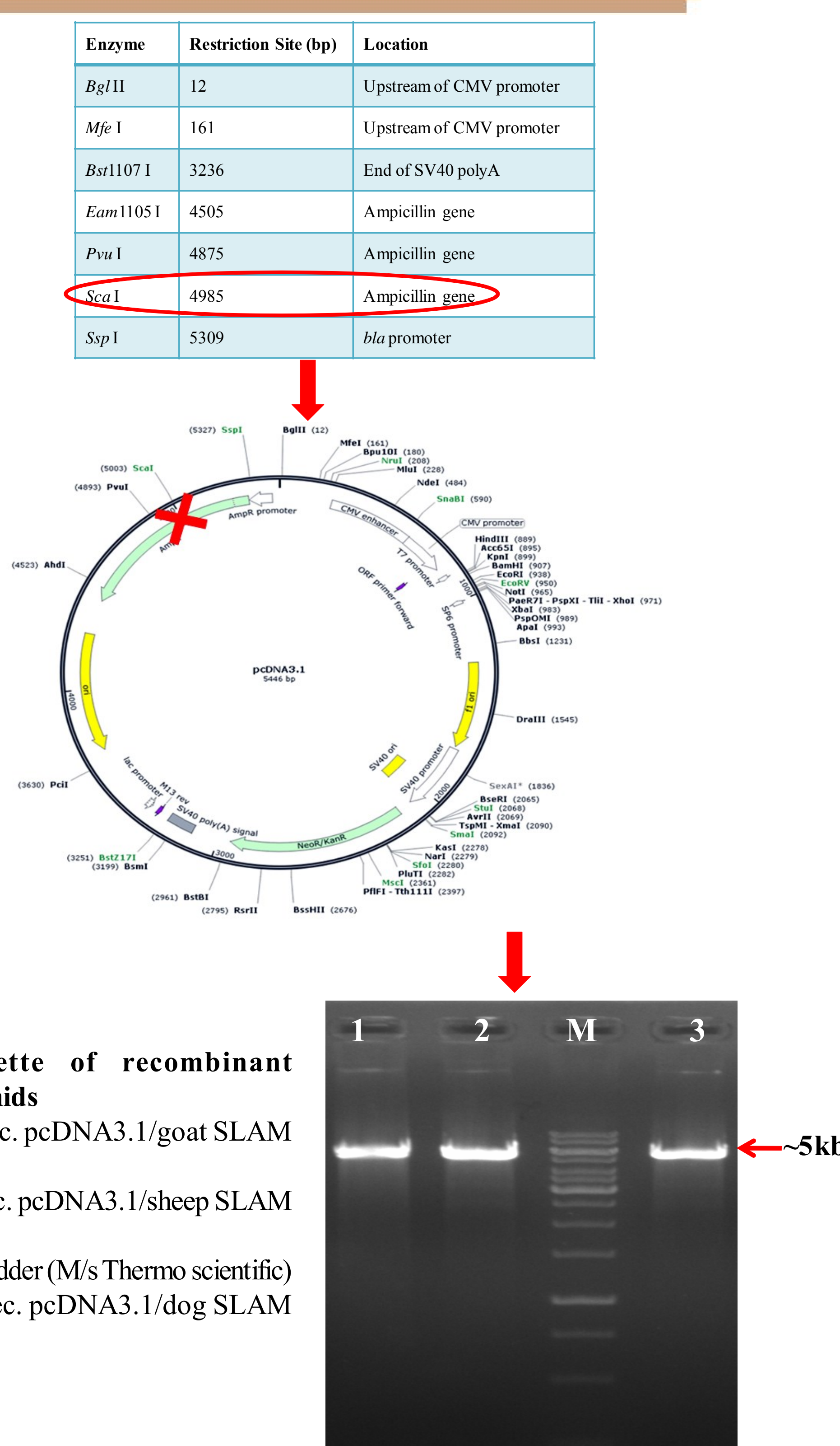
b. Amino Acid Sequence:

```
MDSRGFLSLRCLLVLALASKLSCGTGESLMNCPEVPGKLGSSLQLSLASEGISKRMNKSIHILVTRAESPGNSIKKKIVSLDLPEGGSPRYLENGY
KFHLENLTLRILESRRENEGWYFMTLEENFSVQHFCLQLKLYEQVSTPEIKVLNWTQENGNCSMMLACEVEKGDNVVYSWSEKLGIDPLIPANSSH
LLHLSLGPQHVNNVYVCTVSNPVSNRSWSFNPWSKCRPESSVPRQWRLYAGLFLGGIVGVILIFEVVLLLLRRRGKTNHYKPTKEEKSLTIYAQVQ
KSGSTQKKPDPLPAEDPCTTIYVAATEPVPEPAPEPVQEPHSITVYASVTFPESGGDYKDDDDK
```

FLAG tag

Fig. 22: Sequence details of dog SLAM in pcDNA3.1(+) vector. Nucleotide (a) and Amino acid (b) sequence in correct frame and orientation. FLAG tag (Signal sequence) with RE sites (underlined sequences) located at 32 end of the insert.

Linearization of Expression Cassette



Enzyme	Restriction Site (bp)	Location
Bgl II	12	Upstream of CMV promoter
Mfe I	161	Upstream of CMV promoter
*Bst*1107 I	3236	End of SV40 polyA
*Eam*1105 I	4505	Ampicillin gene
Pvu I	4875	Ampicillin gene
Sca I	4985	Ampicillin gene
Ssp I	5309	*bla* promoter

Linearize cassette of recombinant pcDNA3.1 plasmids

L1 : Single cut rec. pcDNA3.1/goat SLAM plasmid

L2 : Single cut rec. pcDNA3.1/sheep SLAM plasmid

M : 1 Kb DNA ladder (M/s Thermo scientific)

L3 : Single cut rec. pcDNA3.1/dog SLAM plasmid

Fig. 23: Linearization of recombinant goat, sheep and dog SLAM plasmids with *ScaI* RE Enzyme showing product size of around 5kb when resolved on agarose gel electrophoresis.

Kill curve analysis (G418 antibiotic) in Vero cells

Conducted 3 times

800µg/ml

700µg/ml

600µg/ml

21st Day post selection

500µg/ml

400µg/ml

Healthy Vero cells

Fig. 24: Kill curve analysis using G418 antibiotic in Vero cells. Vero cells kept under G418 pressure (800µg/ml-100µg/ml) for 21 days for assessing the optimal concentration which kill all the healthy cells. 600µg/mL(Red circle) was found to be the lowest optimum dose of antibiotic which killed the healthy Vero cells within two week of selection.

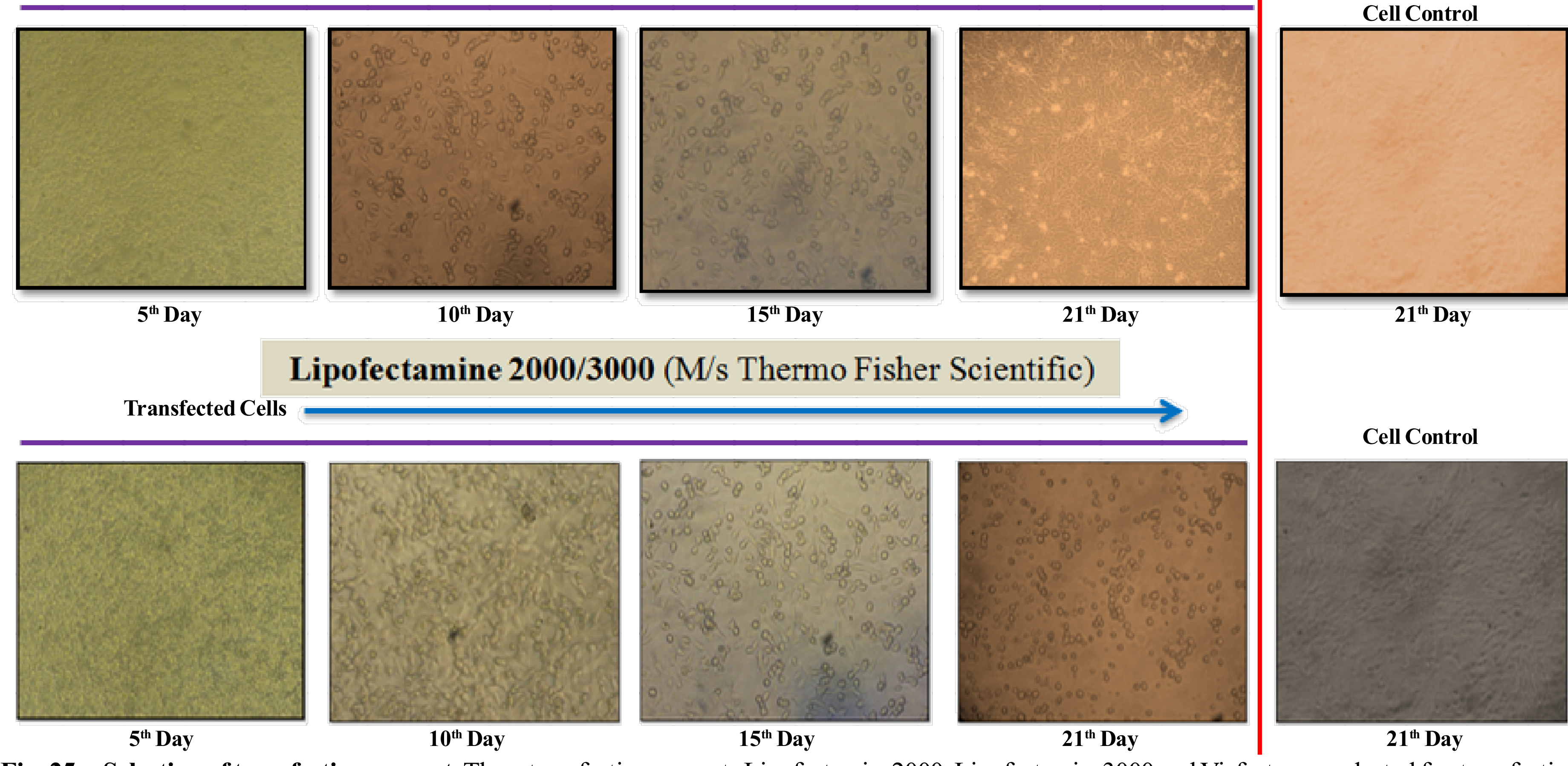


Fig. 25: Selection of transfection reagent. Three transfection reagents Lipofactamine2000, Lipofectamine3000 and Viafect were selected for transfection. Transfection was done in Vero cells with G418 resistant recombinant plasmid in a four well plate as per the manufacturer's protocol. Both Lipofectamine 2000 and 3000 worked well as evidenced by the survival of cells under antibiotic pressure (Upper lane of transfected cells). In case of Viafect all the cells died after 15 days alike healthy Vero cells (Lower lane of transfected cells).

selection both in transfected as well as control wells. The non-transfected Vero cells get detached from the plate surface floating in the media and complete death was observed after 21day of antibiotic pressure. The transfected positive clones survived under neomycin pressure and resistant clones started growing after 15 days of transfection (attached in patches to the plate surface) along with majority of dead cells floating in the media. These resistant clones become almost confluent monolayer after 21-24 days of transfection whereas no live cells were observed in the native Vero cells kept under antibiotic selection. The Vero/goat/SLAM (VGS) cells (**Fig:26**), Vero/sheep/SLAM (VSS) cells (**Fig:27**) and Vero/dog/SLAM (VDS) cells (**Fig:28**) were generated using above protocol. The supernatant along with the dead cells from the transfected wells were discarded and the live cells were transferred successfully to 25cm^2 flask under neomycin pressure after a brief trypsinization.

4.13 Characterization of stable Vero cell line constitutively expressing goat, sheep and dog SLAM

The stable cells were characterized by studying integration of the SLAM gene into the genome of Vero cells, by checking RNA transcript and determining expression of SLAM protein on the Vero cells.

(i) Characterization at transcript level

Total mRNA was isolated from both native Vero and stable cells (VGS, VSS and VDS) to study the expression of SLAM gene. The mRNAs were subsequently used as template in cDNA synthesis and PCR was carried out for full length SLAM transcript amplification using gene specific goat, sheep and dog SLAM expression primers. Vero cell mRNA-specific cDNAs were included as negative control in this experiment. Subsequent electrophoretic migration of the amplicons in 1.0% agarose gel revealed successful amplification of SLAM gene from VGS, VSS and VDS using SLAM expression primers of goat (1017bp), sheep (1017bp) and dog (1029bp) **(Fig:29.a & b).** There was no amplification in negative control confirming expression of SLAM transcript in engineered cells.

(ii) Characterization for checking genomic integration

Genomic integration of SLAM gene was determined by PCR amplification of the gene from Vero/goat/SLAM (VGS), Vero/sheep/SLAM (VSS) and Vero/dog/SLAM (VDS)

genomic DNA using SLAM expression primers of goat, sheep and dog respectively. Genomic DNA of non-transfected Vero cells was included in this study as negative control. Subsequent electrophoretic migration of PCR amplicons in 1.0% agarose gel revealed a DNA fragment of approximate 1kb size from VGS, VSS and VDS corresponding to the amplicons of respective species specifying amplification of 1017 bp length of goat & sheep SLAM and 1029 bp of dog gene at different passage level (**Fig:30 a & c)** including passage-15 and passage-23 (**Fig:30 b**) . The absence of the amplicons in negative control confirms the integration of SLAM gene into the genome of the transfected Vero cells.

(iii) Characterization of SLAM at protein level by Immunofluorescence/ Immunocytochemistry

Expression of SLAM protein on the cell membrane was checked by immunofluorescence test. Transfected cells i.e. VGS, VSS, VDS (at P-4 level) and native Vero cells were stained with M2-anti-FLAG monoclonal antibodies (1:500) and anti-mouse FITC (Fluorescein isothiocyanate) conjugate (1:100) and fluorescence was visualized under fluorescent microscope. Native Vero cells used as negative control in this experiment. All the three engineered cells (VGS, VSS and VDS) were showing expression of SLAM protein on their membrane under fluorescence condition as indicated by apple green fluorescence (**Fig:32, 33 & 34**), whereas no fluorescence was observed in native Vero cells. Thus the expression of SLAM protein was confirmed on the cell membrane of transfected Vero cells.

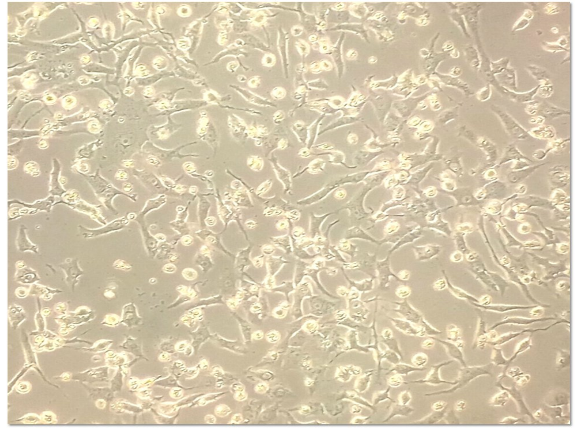
Once the stable cells were confirmed for presence of SLAM both at genomic and protein level, the cells were propagated further into 75 cm^2 flask and preserved in liquid nitrogen [LN_2(-196ºC)] at different passages.

The preserved VGS, VSS and VDS cells were revived successfully after three months of preservation (**Fig: 31a, b & c**) and SLAM transcript was checked in revived cells by RT-PCR **(Fig: 31.d).**

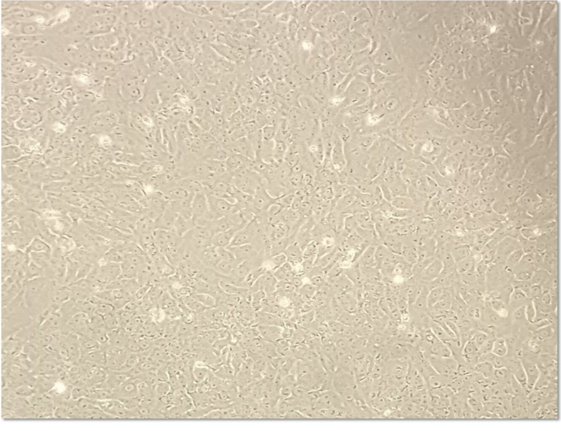
(VI) Relative Expression of SLAM gene in different cell lines by RT-qPCR.

In order to check the relative expression of SLAM gene in different cell lines, real time PCR was done from the cDNA of different cell lines at different passage level. The relative

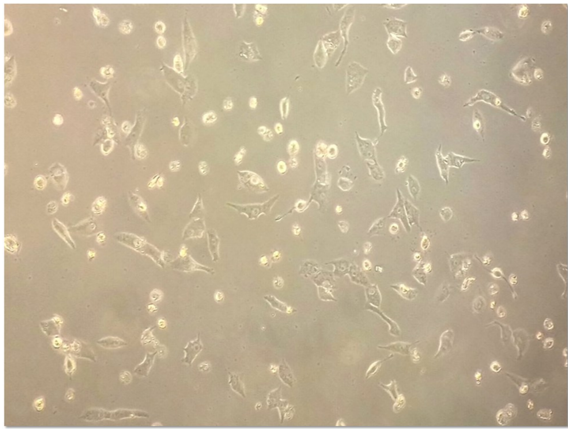
Generation of stable Vero/goat/SLAM (VGS) cell line



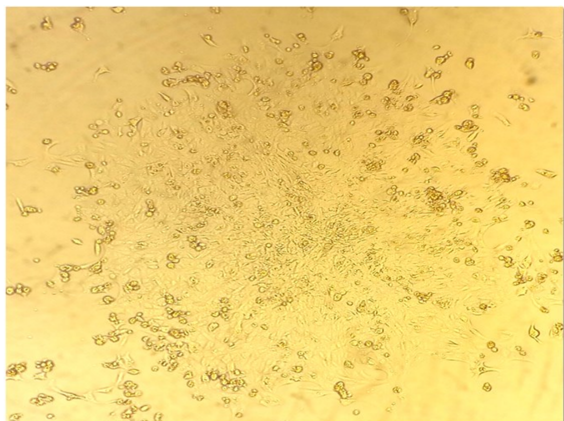
26a: Vero/goat/SLAM cells after 10th day of antibiotic selection



26b: Non-transfected Vero cells without antibiotic selection



26c: Vero cells after 10th day of antibiotic selection

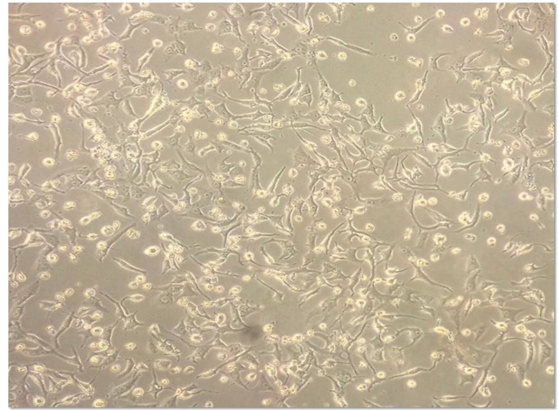


26d: Transfected Vero/goat/SLAM cells after 21 days of antibiotic selection

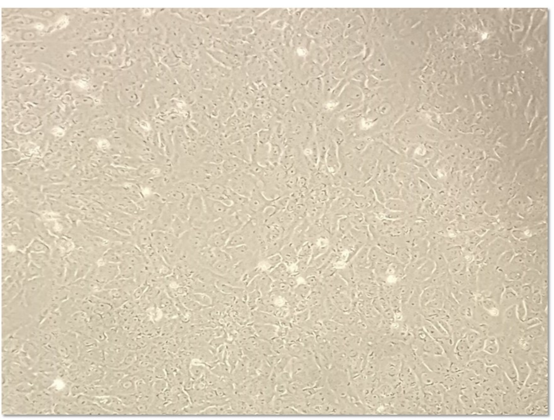
26e: Non-transfected Vero cells after 21 days of antibiotic selection

Fig. 26: Generation of stable Vero/goat/SLAM (VGS) cell line. Vero/goat/SLAM and Vero cells after 10th day (26.a-c) and 21st day of antibiotic selection (26.d-e). More death (round glistening cells) was observed in Vero cells kept under antibiotic selection (26.c) in comparison to Vero/goat/SLAM cells (26.a). After 21st days of antibiotic selection, the transfected cell grown in discrete bunches (resistant clone). There was no live cells in the non-transfected Vero cells kept under antibiotic after 21st day of selection (26.e).

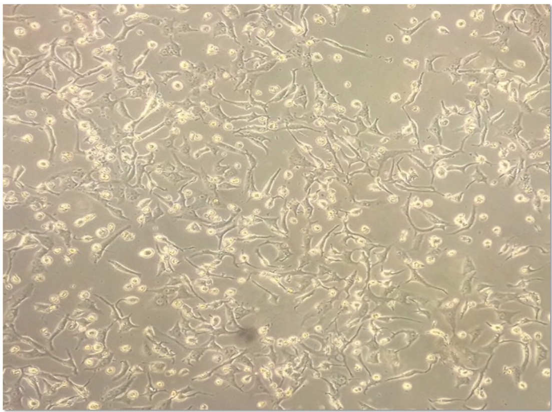
Generation of stable Vero/sheep/SLAM (VSS) cell line



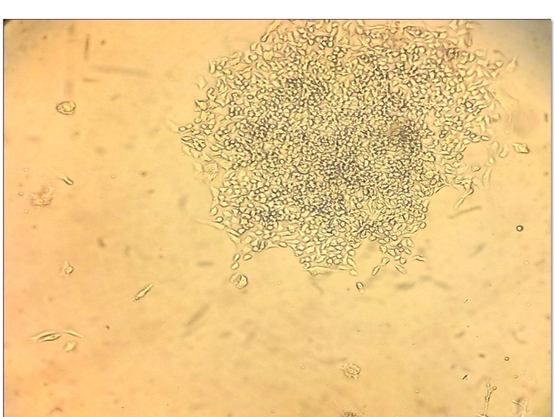
27a: Vero/sheep/SLAM cells after 10th day of antibiotic selection



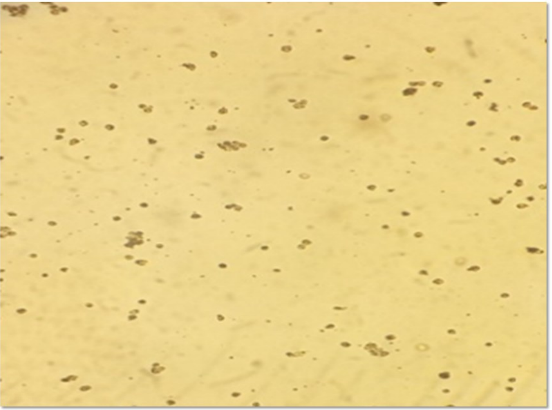
27b: Non-transfected Vero cells without antibiotic selection



27c: Vero cells after 10th day of antibiotic selection



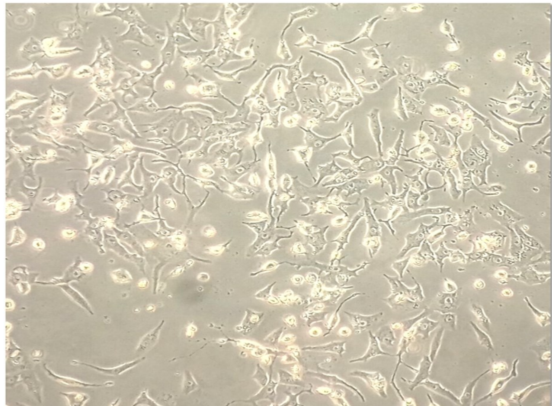
27d: Transfected Vero/sheep/SLAM cells after 21 days of antibiotic selection



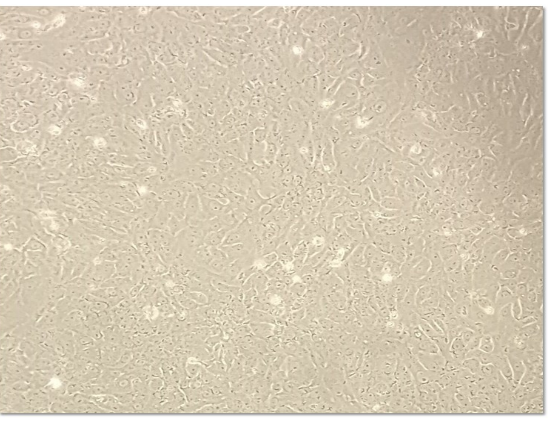
27e: Non-transfected Vero cells after 21 days of antibiotic selection

Fig. 27: Generation of stable Vero/sheep/SLAM (VSS) cell line. Vero/sheep/SLAM and Vero cells after 10th day (27.a-c) and 21st day of antibiotic selection (27.d-e). More death (round glistening cells) was observed in Vero cells kept under antibiotic selection (27.c) in comparison to Vero/goat/SLAM cells (27.a). After 21st days of antibiotic selection, the transfected cell grown in discrete bunches (resistant clone). There was no live cells in the non-transfected Vero cells kept under antibiotic after 21st day of selection (27.e).

Generation of stable Vero/dog/SLAM (VDS) cell line

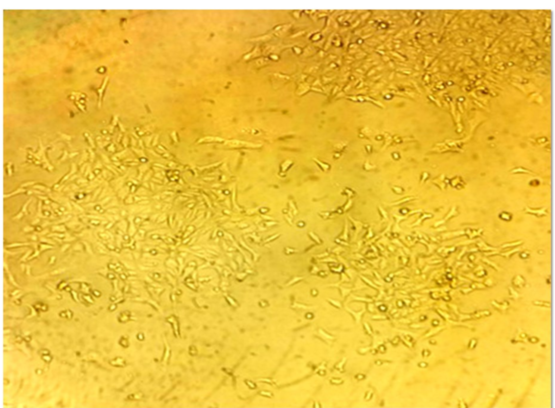


28a: Vero/dog/SLAM cells after 10th day of antibiotic selection

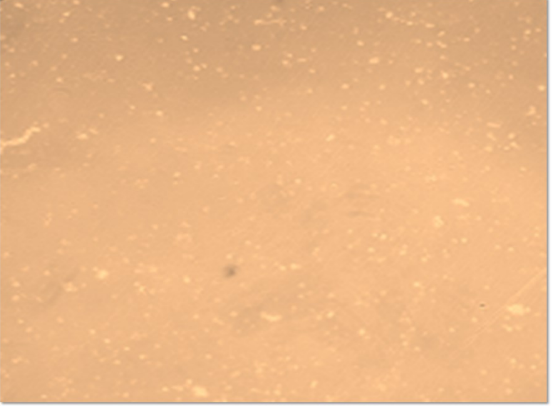


28b: Non-transfected Vero cells without antibiotic selection

28c: Vero cells after 10th day of antibiotic selection



28d: Transfected Vero/dog/SLAM cells after 21 days of antibiotic selection

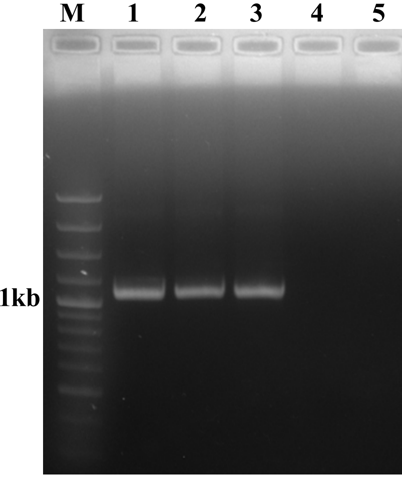


28e: Non-transfected Vero cells after 21 days of antibiotic selection

Fig. 28: Generation of stable Vero/dog/SLAM (VDS) cell line. Vero/dog/SLAM and Vero cells after 10th day (28.a-c) and 21st day of antibiotic selection (28.d-e). More death (round glistening cells) was observed in Vero cells kept under antibiotic selection (28.c) in comparison to Vero/goat/SLAM cells (28.a). After 21st days of antibiotic selection, the transfected cell grown in discrete bunches (resistant clone). There was no live cells in the non-transfected Vero cells kept under antibiotic after 21st day of selection (28.e).

Characterization of stable Vero cells at transcript level

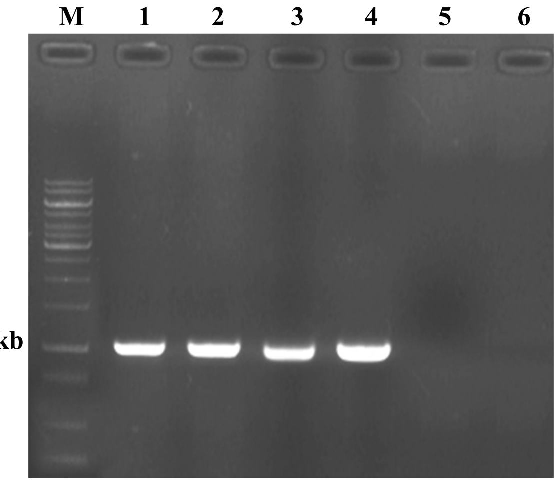
Full length RNA transcript of Vero/goat/SLAM cells



29a: RT-PCR of SLAM gene from Vero/ Goat/ SLAM cells at different passage

M: 100bp plus DNA ladder (M/s Thermo Scientific)
L1 : Vero/goat/SLAM at Passage-4
L2 : Vero/goat/SLAM at Passage-5
L3 : Vero/goat/SLAM at Passage-6
L4 : Native Vero Cell
L5 : Non-template control

Full length RNA transcript of Vero/dog/SLAM & Vero/sheep/SLAM cells

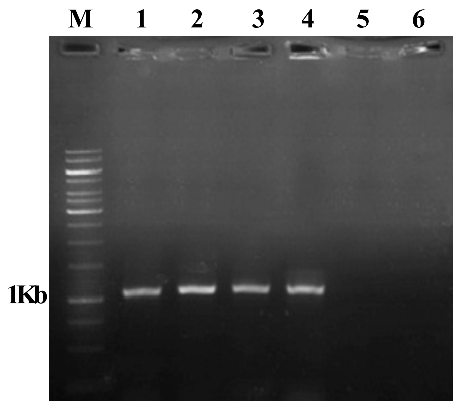


29b: RT-PCR of SLAM gene from Vero /Dog /SLAM and Vero /Sheep/SLAM cells at different passage

M : 1 kb DNA ladder (M/s Thermo Scientific)
L1 : Vero/Dog /SLAM at Passage-4
L2 : Vero/Dog /SLAM at Passage-7
L3 : Vero/Sheep /SLAM at Passage-4
L4 : Vero/Sheep /SLAM at Passage-6
L5 : Native Vero cell
L6 : Non-template control

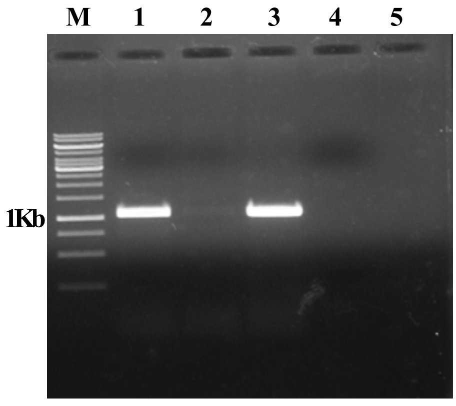
Fig. 29: Amplification of full length SLAM transcript of Vero/goat/SLAM, Vero/sheep/SLAM and Vero/dog/SLAM at different passage level. No transcript was detected from native Vero cells.

Characterization of stable cells at genomic level



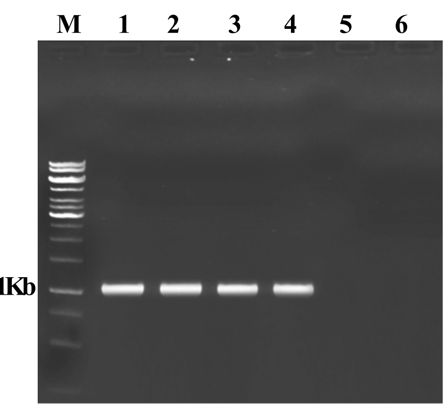
30a: Integration of goat and sheep SLAM gene into genome of Vero cells

M : 1Kb DNA ladder (M/s Thermo scientific)
L1 : Goat SLAM gene amplification from DNA of Vero/goat/SLAM cells **(P-4)**
L2 : Goat SLAM gene amplification from DNA of Vero/goat/SLAM cells **(P-7)**
L3 : Sheep SLAM gene amplification from DNA of Vero/sheep/SLAM cells **(P-4)**
L4 : Sheep SLAM gene amplification from DNA of Vero/sheep/SLAM cells **(P-7)**
L5 : Native Vero cells
L6 : Non template control



30b: Integration of goat and sheep SLAM gene into genome of Vero cells at passage 15 and 23

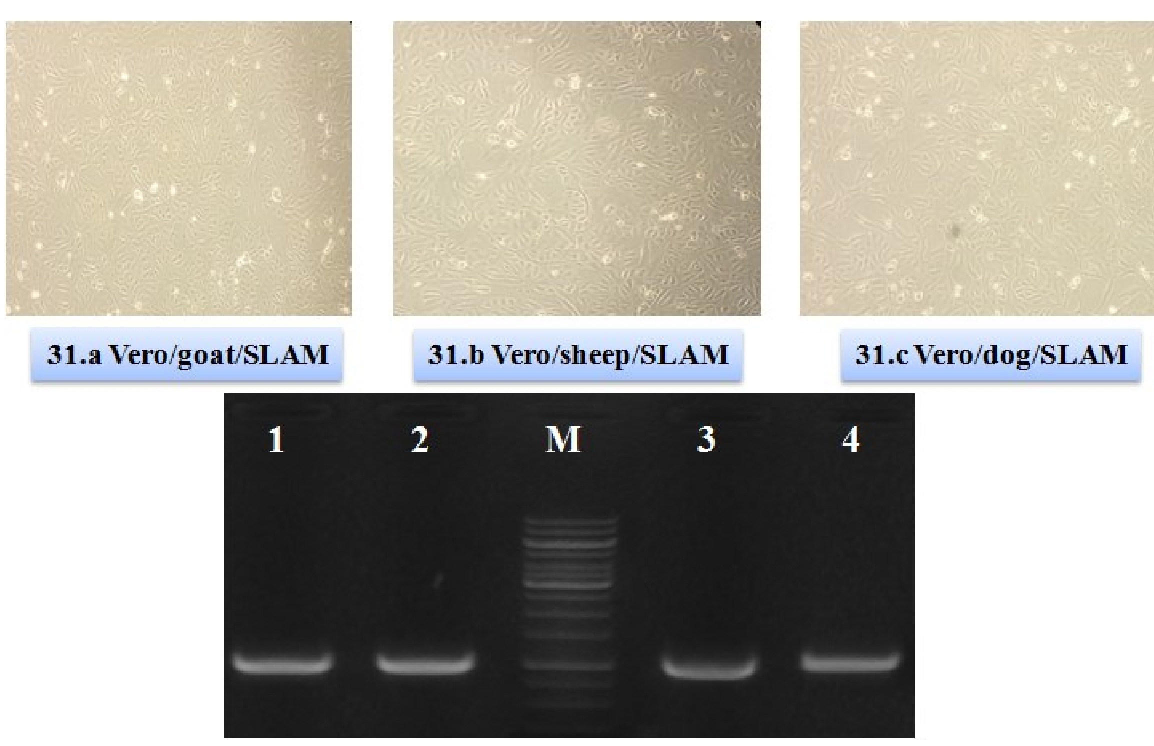
M : 1Kb DNA ladder (M/s Thermo scientific)
L1 : Goat SLAM gene amplification from DNA of Vero/goat/SLAM cells **(P-15)**
L2 : Goat SLAM clone (no integration; clone was lost)
L3 : Sheep SLAM gene amplification from DNA of Vero/sheep/SLAM cells **(P-23)**
L4 : Native Vero cells
L5 : Non-template control



30c: Integration of dog SLAM gene into genome of Vero cells

M : 1Kb DNA ladder (M/s Thermo scientific)
L1 : Dog SLAM gene amplification from DNA of Vero/dog/SLAM cells **(P-3)**
L2 : Dog SLAM gene amplification from DNA of Vero/dog/SLAM cells **(P-4)**
L3 : Dog SLAM gene amplification from DNA of Vero/dog/SLAM cells **(P-5)**
L4 : Dog SLAM gene amplification from DNA of Vero/dog/SLAM cells **(P-7)**
L5 : Native Vero cells
L6 : Non-template control (NTC)

Fig. 30: Genomic integration detection of goat (30.a&b), sheep (30.a&b) and dog (30.c) SLAM gene into DNA of Vero cells at different passage level. There was no integration was detected in the DNA of native Vero cells.

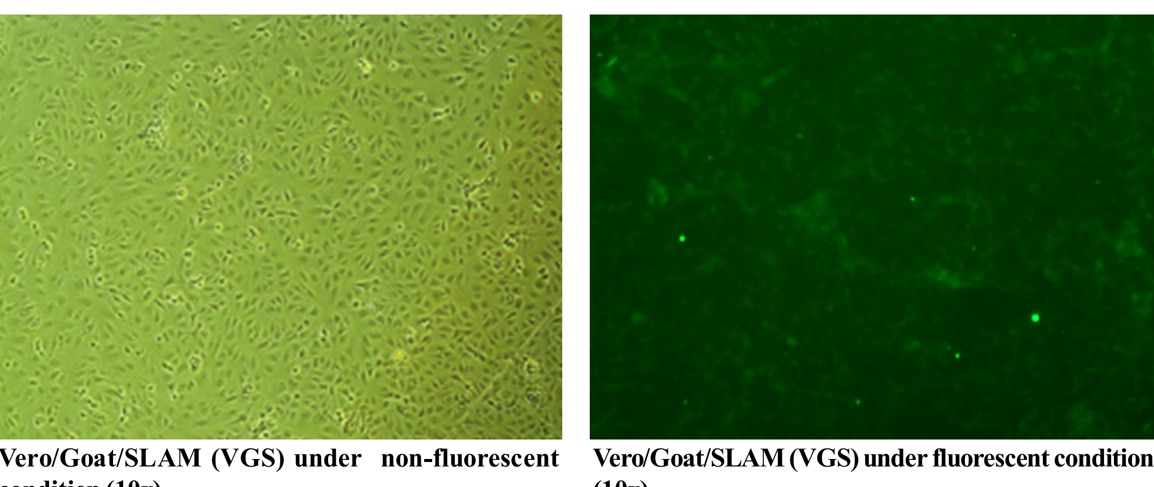


31.(d) RT-PCR of SLAM gene from Vero /goat /SLAM cells, Vero/ sheep/ SLAM cells and Vero/dog/SLAM cells after preservation of three months

L1 : Goat SLAM gene **(P-5)**
L2 : Goat SLAM gene **(P-8)**
M : 1 kb DNA ladder
L3 : Sheep SLAM **(P-5)**
L4 : Dog SLAM **(P-6)**

Fig. 31: Successful revival of Vero/goat/SLAM, Vero/sheep/SLAM and Vero/dog/SLAM cells after three months of preservation (31 a-c) and checking of full length SLAM transcript in all the three cell lines at different passage level (31d).

Characterization of stable cells at protein level: Vero/Goat/SLAM (VGS)



Vero/Goat/SLAM (VGS) under non-fluorescent condition (10x)

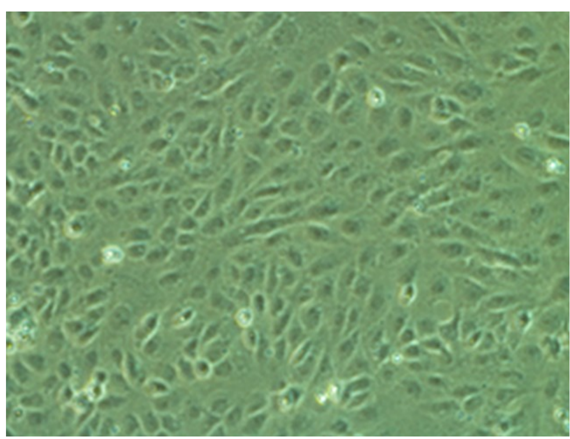
Vero/Goat/SLAM (VGS) under fluorescent condition (10x)

Native Vero cells under non-fluorescent condition (10x)

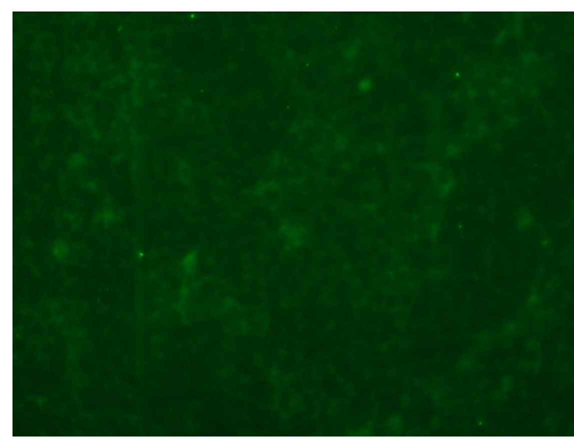
Native Vero cells under fluorescent condition

Fig. 32: Staining of **Vero/goat/SLAM** cells expressing FLAG tagged goat SLAM protein with anti-FLAG monoclonal antibodies (Sigma-Aldrich) and anti-mouse FITC conjugate (Sigma-Aldrich)

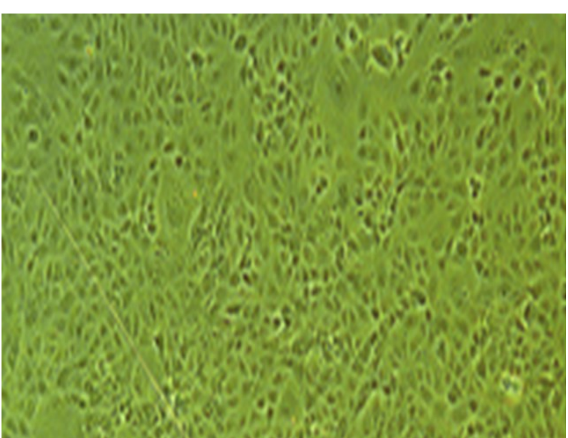
Characterization of stable cells at protein level: Vero/sheep/SLAM (VSS)



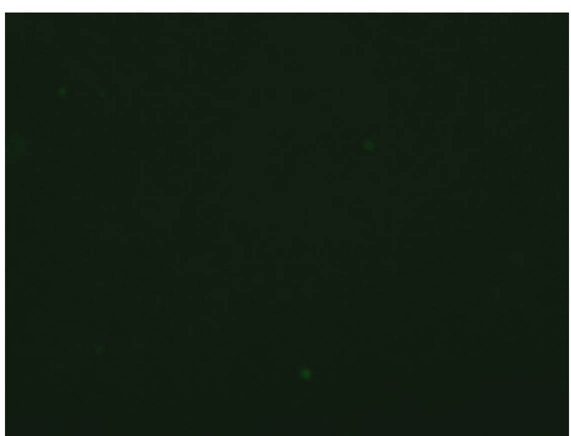
Vero/sheep/SLAM (VSS) under non-fluorescent condition (10x)



Vero/sheep/SLAM (VSS) under fluorescent condition (10x)



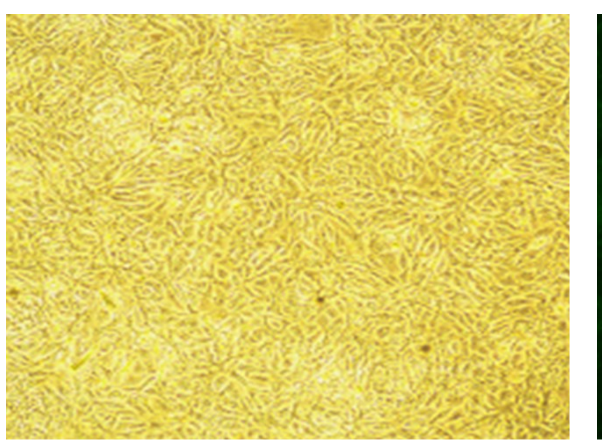
Native Vero cells under non-fluorescent condition (10x)



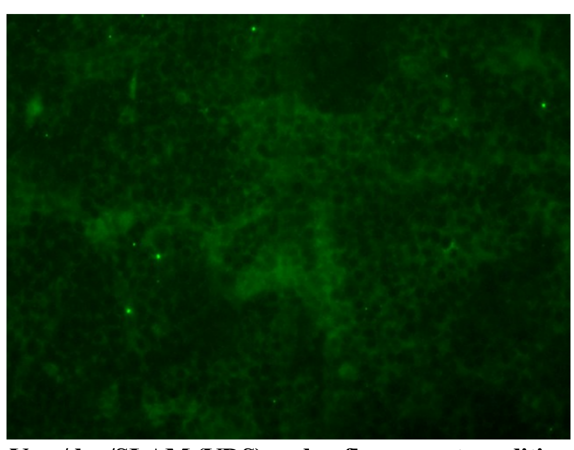
Native Vero cells under fluorescent condition

Fig. 33: Staining of **Vero/sheep/SLAM** cells expressing FLAG tagged sheep SLAM protein with anti-FLAG monoclonal antibodies (Sigma-Aldrich) and anti-mouse FITC conjugate (Sigma-Aldrich)

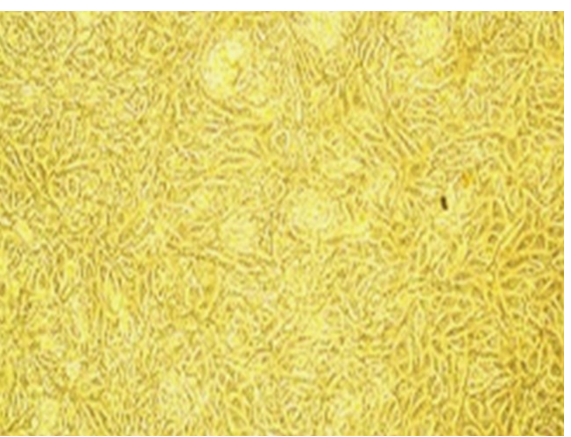
Characterization of stable cells at protein level: Vero/dog/SLAM (VDS)



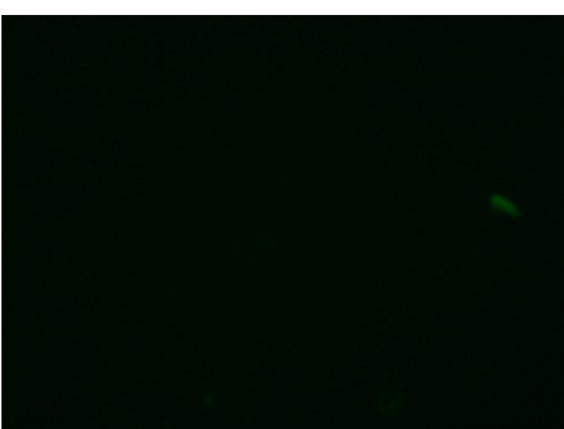
Vero/dog/SLAM (VDS) under non-fluorescent condition (10x)



Vero/dog/SLAM (VDS) under fluorescent condition (10x)



Native Vero cells under non-fluorescent condition (10x)



Native Vero cells under fluorescent condition

Fig. 34: Staining of **Vero/dog/SLAM** cells expressing FLAG tagged dog SLAM protein with anti-FLAG monoclonal antibodies (Sigma-Aldrich) and anti-mouse FITC conjugate (Sigma-Aldrich)

expression of SLAM at P-3 level in VGS was 3 fold, in VSS 2.65 fold and in VDS 3.26 fold in comparison to their respective calibrator (cDNA synthesized from goat, sheep and dog PBMCs 48hrs post stimulation). It was observed that as the passage number increases the relative expression of SLAM gene in engineered cells decreased. The maximum expression of SLAM gene was noticed in VDS (3.26 fold change log_2) among all the three cell lines. **(Fig: 35).**

4.14 Studies on comparative sensitivity of native and stable cell lines to PPRV and CDV

4.14.1 Relative susceptibility study of native Vero and Vero/SLAM for PPR vaccine virus

(i) One-step growth curve of PPRV vaccine virus in different cell lines.

One-step growth curve of "PPRV Sungri/96" was performed in different cell line viz., Vero, VGS, VSS and VDS at 1.0 m.o.i. The supernatant (cell free virus) and cell lysate (cell associated virus) was collected at 0h, 6h, 12h, 18h, 24h, 48h, 72h, 96h, 120h and 144h. Titration of both the cell free and cell associated virus was performed in Vero cells. Growth kinetics of PPRV in all the three engineered cell lines (VGS, VSS and VDS) was more or less similar to the native Vero cells. In supernatant samples, PPRV titre showed two peaks, one at 18h and second at 96h **(Fig:36.a)**. However, the overall titre was found to be more in engineered cells as compared to native Vero cell (**Table. 4a).** In case of cell associated-virus, also two peaks one at 18h and second at 72h post infection were noticed **(Fig:36.b).** Like supernatants, the overall titre of PPR virus was found to be marginally more in engineered cells than the native ones. On comparison of cell free and cell associated PPR virus titre in all these cell lines, it was observed that, the titre was little more in supernantant than the cell lysate **(Table 4a & 4b)**.

(ii) Checking of antigenic load of PPRV in different cell lines by s-ELISA

The one-step growth curve samples, both supernatant and lysate of all the four cell lines (Vero, VGS, VSS and VDS) were subjected to monoclonal antibody based sandwich ELISA to check the antigenic load of PPRV. In case of cell-free virus the antigenic mass in the VGS, VSS and VDS were higher in comparison to native Vero. Highest antigenic load was

found in VDS as compared to other cell lines. In case of cell-associated virus the antigenic load in VGS, VSS and VDS was found to be almost similar to native Vero cells. There was no significant difference was found in the antigenic load of PPRV in all the four cell lines **(Fig: 37).**

(iii) Studies on comparative replication efficacy of PPRV in native Vero and stable cell lines (VGS, VSS and VDS) using different multiplicity of infection (m.o.i).

PPR vaccine virus was infected to confluent monolayer with different moi (1.0, 0.1, 0.01 and 0.001 moi) along with cell control to the all four cell lines in 24-well plate. Media was changed every third day with EMEM containing 2% fetal bovine serum (FBS).

After 6th day post infection, when CPE reached 80-90%, plates were harvested. Then after three cycles of freezing and thawing, the viruses were subjected to infectivity assay. At higher m.o.i. (m.o.i. 1.0) all the three cell lines (VGS, VSS and VDS) exhibited lower (VGS and VSS) or similar (VDS) titre as compared to native Vero cells. But as the m.o.i. decreases (0.1, 0.01, 0.001) titre of PPR virus also decreased in VGS, VSS and VDS in comparison to native Vero cells. Overall, virus titre was found to be highest in VDS among all three engineered cell lines, yet lower than the native Vero cell (**Fig: 38)**

4.14.2. Studies on relative susceptibility of native Vero and stable Vero/SLAM cell lines to canine distemper virus

(i) One-Step Growth Curve of canine distemper virus in different cell lines

One-step growth curve of CDV was performed in different cell lines i.e. VGS, VSS, VDS and native Vero similar to the PPR virus. The supernatant (cell free virus) and cell lysate (cell associated virus) was collected at 0h, 6h, 12h, 18h, 24h, 48h, 72h, 96h, 120h and 144h. Titration of both the cell free and cell associated virus was performed in Vero cells. In cell free virus first peak of titre was noticed at 18h post infection in all the cells except in native Vero where it was found around 24h post infection. The second peak in titre was observed at 48h post infection. After 48 h post infection virus titre start decreasing continuously and again start rising after 120h post infection (**Fig:39.a**). In case of cell- associated virus at 0h virus titre was around 3.5-4.5 $TCID_{50}$/ml and then the virus titre peak was found at 18h and 48h post infection. After 48h virus titre started decreasing upto 120h and then started rising upto 144h post infection of study period of the experiment (**Fig:39.b**). The overall titre in both cell supernatant and cell lysate was found highest in VDS as compared to rest of the cell including native Vero (**Table 5a & 5b).**

Relative Expression of SLAM gene by RT-qPCR

ΔΔCt Method: Comparative Ct method (Livak and Schmittgen, 2001)

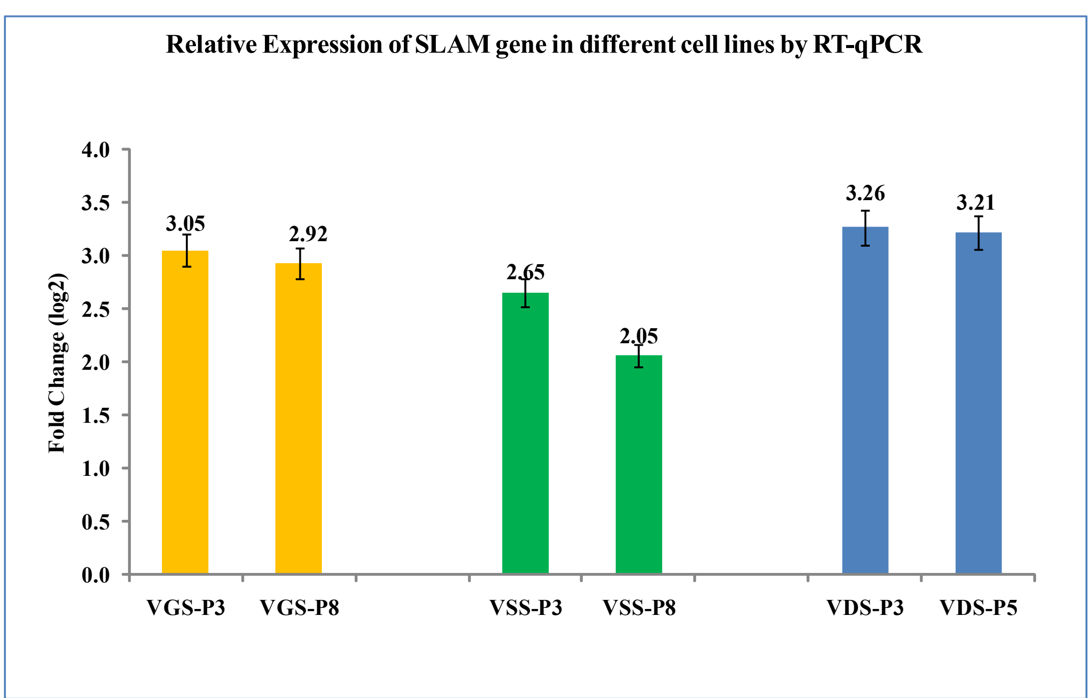
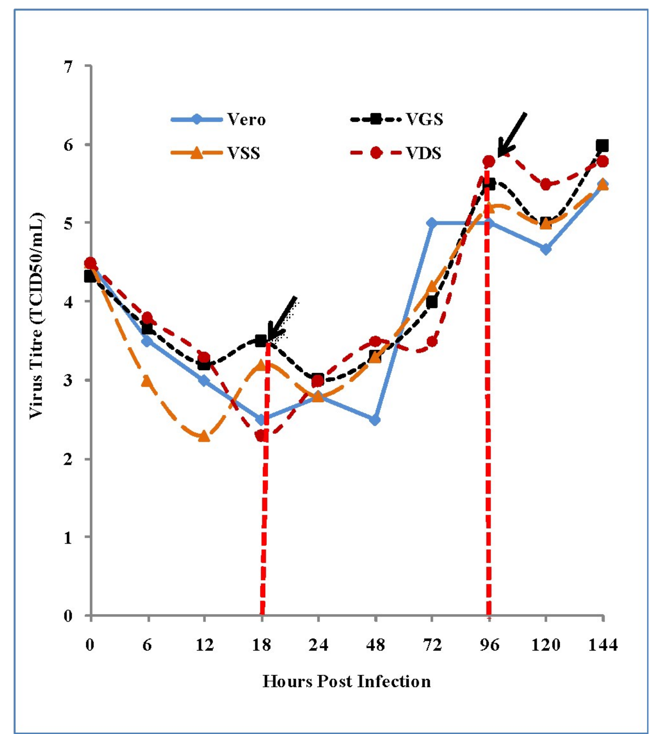
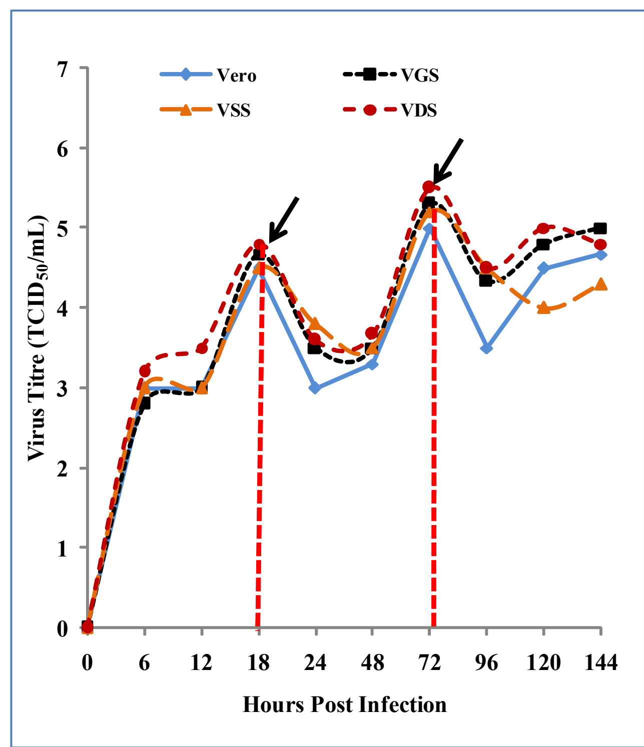


Fig. 35: Relative expression of SLAM gene in Vero/goat/SLAM (VGS), Vero/sheep/SLAM (VSS) and Vero/dog/SLAM (VDS) cell lines at different passage level by real time PCR. Relative expression of SLAM gene was quantified by ΔΔCt Method or Comparative Ct method using PBMCs of respective species as calibrator. VDS showed highest expression as compared to VGS and VSS.

One-Step Growth Curve (1.0 moi) : PPRV in Different Cell Lines



36.a PPR Virus Titre in Different Cell Lines (Supernantant)



36.b PPR Virus Titre in Different Cell Lines (Cell-Lysate)

Fig. 36: One-step growth curve of *peste-des-petits ruminants-virus in* native Vero (Blue continuous line), VGS (Black dotted line), VSS (orange dotted line) and VDS (red dotted line) cell lines. Two peak of viral titre was found in supernatant one at 18h and second at 96h post infection (36.a) while in cell lysate it was at 18h and 72h post infection (36.b).

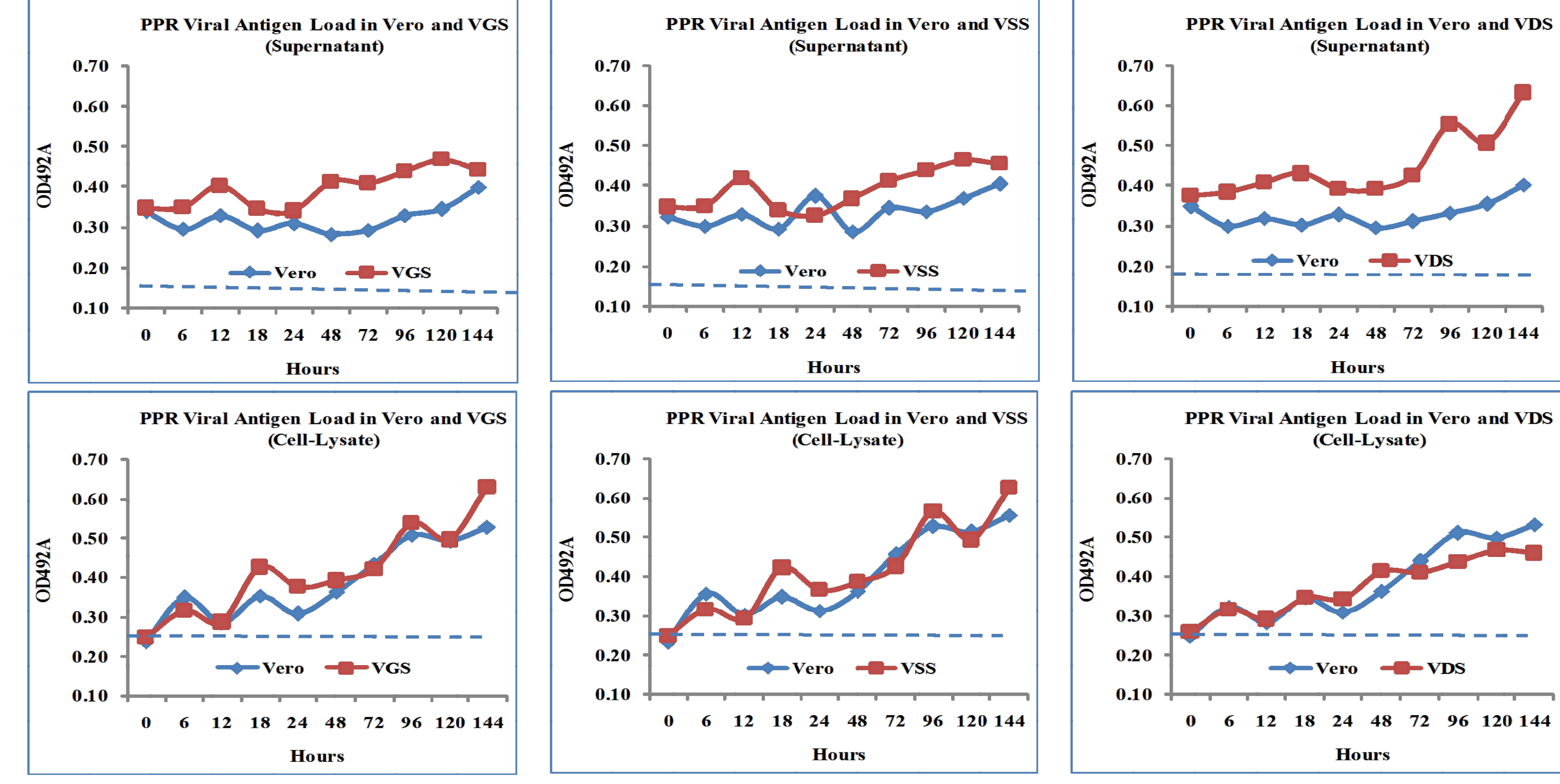
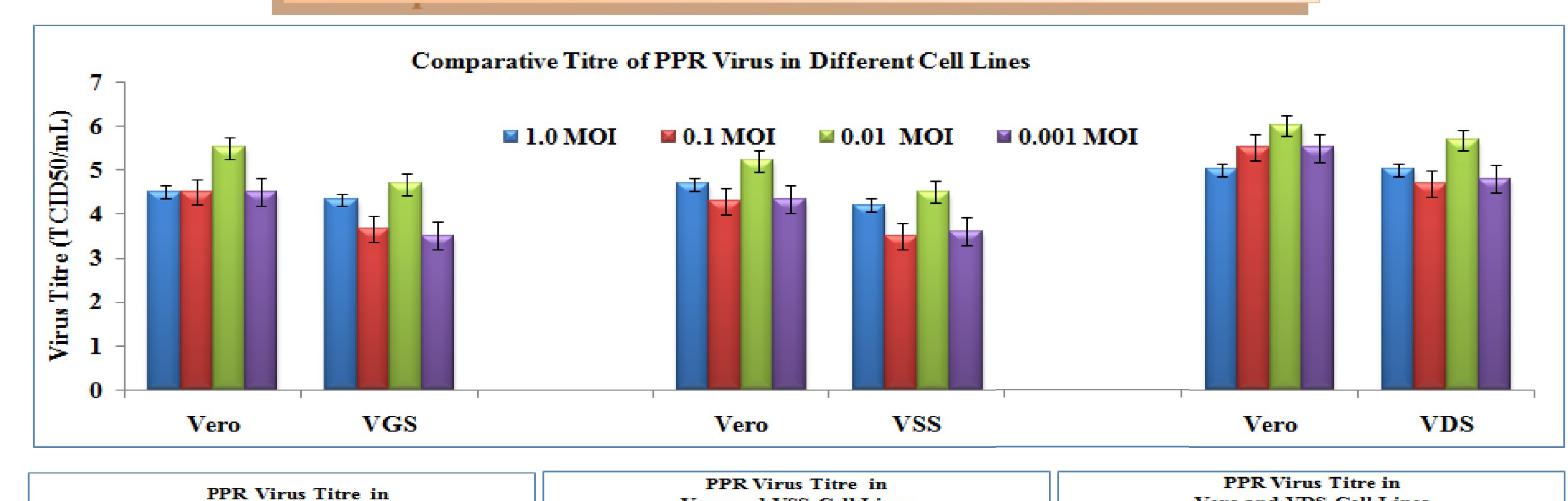


Fig. 37: Antigenic mass of one-step growth curve samples (both supernatant and lysate) determined by monoclonal antibody based PPR sandwich ELISA. PPR viral load found to be more in supernatant of stable cells than native vero cells. Antigenic load in cell lysate was more or less similar in all the cell lines.

Comparative Titre of PPR Virus in Different Cell Lines



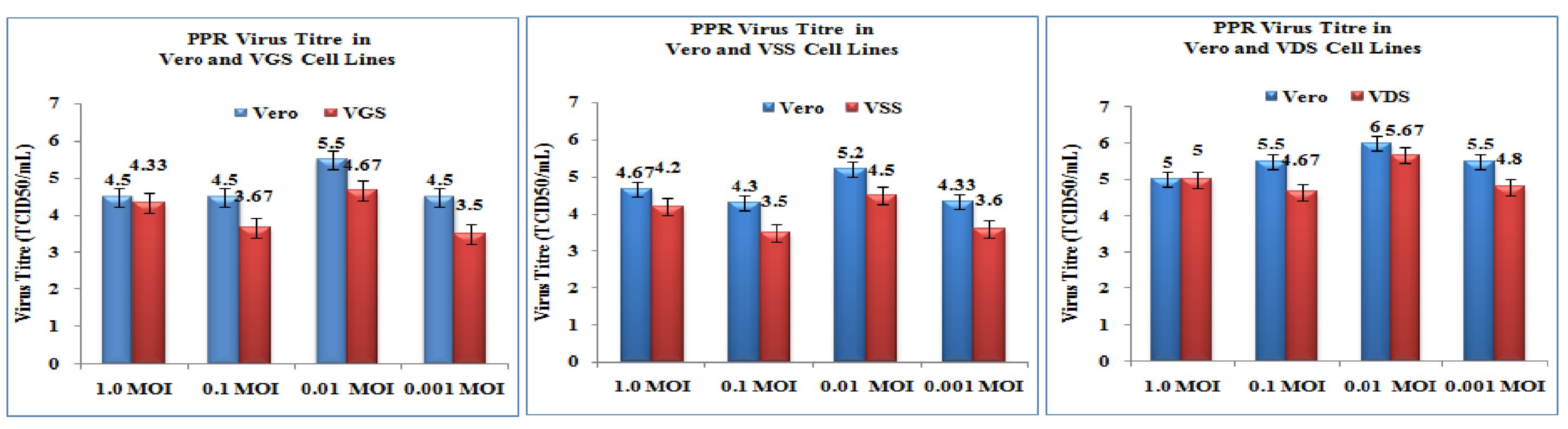
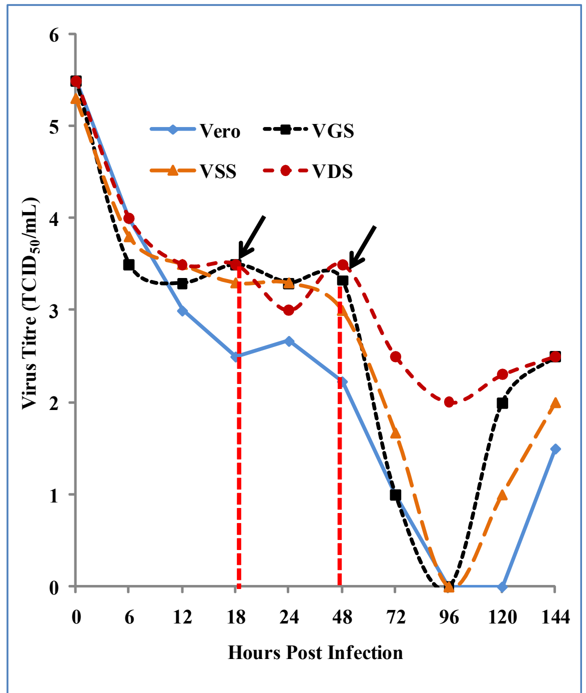
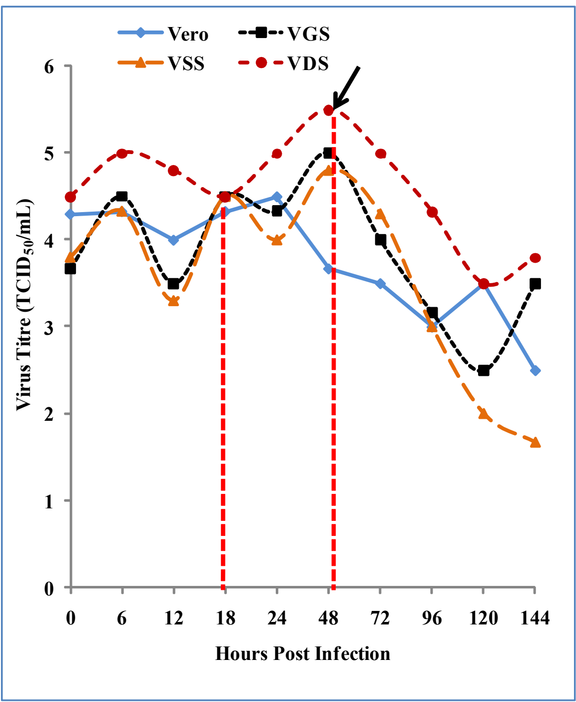


Fig. 38: Comparative titre of PPR virus in Vero, VGS, VSS and VDS cell lines at different moi. All the three cell lines (VGS, VSS and VDS) exhibited lower titre as compared to native Vero cells irrespective of moi used for infection.

One-Step Growth Curve (1.0 moi) : CDV in Different Cell Lines



39a: Canine Distemper Virus Titre in Different Cell Lines (Supernatant)



39b: Canine Distemper Virus Titre in Different Cell Lines (Cell-Lysate)

Fig. 39: One-step growth curve of *canine distemper virus* in native Vero (Blue continuous line), VGS (Black dotted line), VSS (orange dotted line) and VDS (red dotted line) cell lines. Two peak of viral titre was found at 18h and 48h post infection both in supernatant and cell lysate. The highest titre was observed in VDS cells as compared to other cells.

Table 4a: Comparative Titre ($TCID_{50}$/mL) of PPRV in supernatant of one-step Growth Curve in different cell lines Hours

Hours	PPRV-Vero	PPRV-VGS	Difference	PPRV-Vero	PPRV-VSS	Difference	PPRV -Vero	PPRV -VDS	Difference
0	4.5	4.33	-0.17	4.5	4.5	0	4.5	4.5	0
6	3.5	3.67	0.17	3.5	3	-0.5	3.5	3.8	0.3
12	3	3.2	0.2	3	2.3	-0.7	3	3.3	0.3
18	2.5	3.5	1	2.5	3.2	0.7	2.5	2.3	-0.2
24	2.8	3	0.2	2.8	2.8	0	2.8	3	0.2
48	2.5	3.3	0.8	2.5	3.3	0.8	2.5	3.5	1
72	5	4	-1	5	4.2	-0.8	5	3.5	-1.5
96	5	5.5	0.5	5	5.2	0.2	5	5.8	0.8
120	4.67	5	0.33	4.67	5	0.33	4.67	5.5	0.83
144	5.5	6	0.5	5.5	5.5	0	5.5	5.8	0.3
Mean	**3.897**	**4.15**	**0.253**	**3.897**	**3.9**	**0.003**	**3.897**	**4.1**	**0.203**

Table 4b: Comparative Titre ($TCID_{50}$/mL) of PPRV in cell-lysate of one-step Growth Curve in different cell lines

Hours	PPRV-Vero	PPRV-VGS	Difference	PPRV-Vero	PPRV-VSS	Difference	PPRV -Vero	PPRV -VDS	Difference
0	0	0	0	0	0	0	0	0	0
6	3	2.8	-0.2	3	3	0	3	3.2	0.2
12	3	3	0	3	3	0	3	3.5	0.5
18	4.5	4.67	0.17	4.5	4.5	0	4.5	4.8	0.3
24	3	3.5	0.5	3	3.8	0.8	3	3.6	0.6
48	3.3	3.5	0.2	3.3	3.5	0.2	3.3	3.67	0.37
72	5	5.3	0.3	5	5.2	0.2	5	5.5	0.5
96	3.5	4.33	0.83	3.5	4.5	1	3.5	4.5	1
120	4.5	4.8	0.3	4.5	4	-0.5	4.5	5	0.5
144	4.67	5	0.33	4.67	4.3	-0.37	4.67	4.8	0.13
Mean	**3.447**	**3.69**	**0.243**	**3.447**	**3.58**	**0.133**	**3.447**	**3.857**	**0.41**

Table 5a: Comparative Titre ($TCID_{50}$/mL) of CDV in supernatant of one-step Growth Curve in different cell lines

Hours	PPRV-Vero	PPRV-VGS	Difference	PPRV-Vero	PPRV-VSS	Difference	PPRV -Vero	PPRV -VDS	Difference
0	5.5	5.5	0	5.5	5.3	-0.2	5.5	5.5	0
6	4	3.5	-0.5	4	3.8	-0.2	4	4	0
12	3	3.3	0.3	3	3.5	0.5	3	3.5	0.5
18	2.5	3.5	1	2.5	3.3	0.8	2.5	3.5	1
24	2.67	3.3	0.63	2.67	3.3	0.63	2.67	3	0.33
48	2.23	3.33	1.1	2.23	3	0.77	2.23	3.5	1.27
72	1	1	0	1	1.67	0.67	1	2.5	1.5
96	0	0	0	0	0	0	0	2	2
120	0	2	2	0	1	1	0	2.3	2.3
144	1.5	2.5	1	1.5	2	0.5	1.5	2.5	1
Mean	**2.24**	**2.79**	**0.55**	**2.24**	**2.69**	**0.45**	**2.24**	**3.23**	**0.99**

Table 5b: Comparative Titre ($TCID_{50}$/mL) of CDV in cell-lysate of one-step Growth Curve in different cell lines

Hours	PPRV-Vero	PPRV-VGS	Difference	PPRV-Vero	PPRV-VSS	Difference	PPRV -Vero	PPRV -VDS	Difference
0	4.3	3.67	-0.63	4.3	3.8	-0.5	4.3	4.5	0.2
6	4.33	4.5	0.17	4.33	4.33	0	4.33	5	0.67
12	4	3.5	-0.5	4	3.3	-0.7	4	4.8	0.8
18	4.33	4.5	0.17	4.33	4.5	0.17	4.33	4.5	0.17
24	4.5	4.33	-0.17	4.5	4	-0.5	4.5	5	0.5
48	3.67	5	1.33	3.67	4.8	1.13	3.67	5.5	1.83
72	3.5	4	0.5	3.5	4.3	0.8	3.5	5	1.5
96	3	3.17	0.17	3	3	0	3	4.33	1.33
120	3.5	2.5	-1	3.5	2	-1.5	3.5	3.5	0
144	2.5	3.5	1	2.5	1.67	-0.83	2.5	3.8	1.3
Mean	**3.763**	**3.867**	**0.104**	**3.763**	**3.57**	**-0.193**	**3.763**	**4.593**	**0.83**

(ii) Studies on comparative replication efficacy of CDV in native Vero and stable Vero cell lines (VGS, VSS and VDS) using different multiplicity of infection (moi).

The pre-titrated canine distemper vaccine virus was infected to confluent monolayer at different moi (1.0, 0.1, 0.01 and 0.001 moi) in all the four cells (3 engineered + native Vero cells) along with cell control in a 24-well plate. Media was changed every third day with EMEM containing 2% serum. After 5th day of post infection when CPE reached up to 80-90%, plates were harvested and after three cycles of freeze-thaw, the viruses were subjected to infectivity assay. At m.o.i. 1.0, equal titre of CDV was observed in native Vero, VGS and VSS But as the moi decreases, titre also decreased in VSS when compared to native Vero. In VGS, equal titre was observed irrespective of moi (barring 0.01moi) as compared to native Vero cell. The CDV grown in VDS cell showed higher titre than the native Vero cells at all moi (**Fig: 40**) and at 0.001 moi titre was higher by 1.0 log than native Vero cells.

4.15 Comparative mean titre of PPRV and CDV in different cell lines as compared to native Vero

The comparative mean titre of PPRV and CDV was analyzed from one-step growth kinetics experiment (in both supernatant and cell-lysates) in comparison to native Vero cells. In case of supernatant, PPR virus was having more titre in VGS and VDS than the native Vero, but in VSS titre was equal to native Vero cells. In supernatant the CDV was having higher titre in all the three cells (VGS, VSS and VDS) in comparison to native Vero cells. It was observed that the mean titre of CDV in VDS cells was 0.99 log higher than native Vero cells **(Fig: 41 a).**

The mean titre in cell lysates revealed that the PPR virus was having more titre in VGS, VSS and VDS in comparison to native Vero cells. While the CDV showed higher titre in VGS and VDS cells. Titre of CDV in VSS cells was lower in comparison to native Vero cells. The mean titre of CDV in VDS cells was 0.83 times higher in comparison to native Vero cells **(Fig: 41 b).** The overall comparative mean titre of both viruses showed that the CDV replication efficacy was much higher in all the engineered cell lines and highest in VDS cell.

4.16 Isolation of virulent PPRV from field sample in stable Vero cells expressing the SLAM receptor

Isolation of PPR 'wild type' virus was attempted both in VGS cell and native Vero cells. The lymph node, lung and spleen samples tested positive by s-ELISA and RT-PCR were used for isolation. The inoculum was added to the cell monolayer and checked upto third blind passage (BP-3) by RT-PCR. There was no positive indication in both the cell lines was observed. So isolation in either of these cell lines could not be successful **(Fig: 42).**

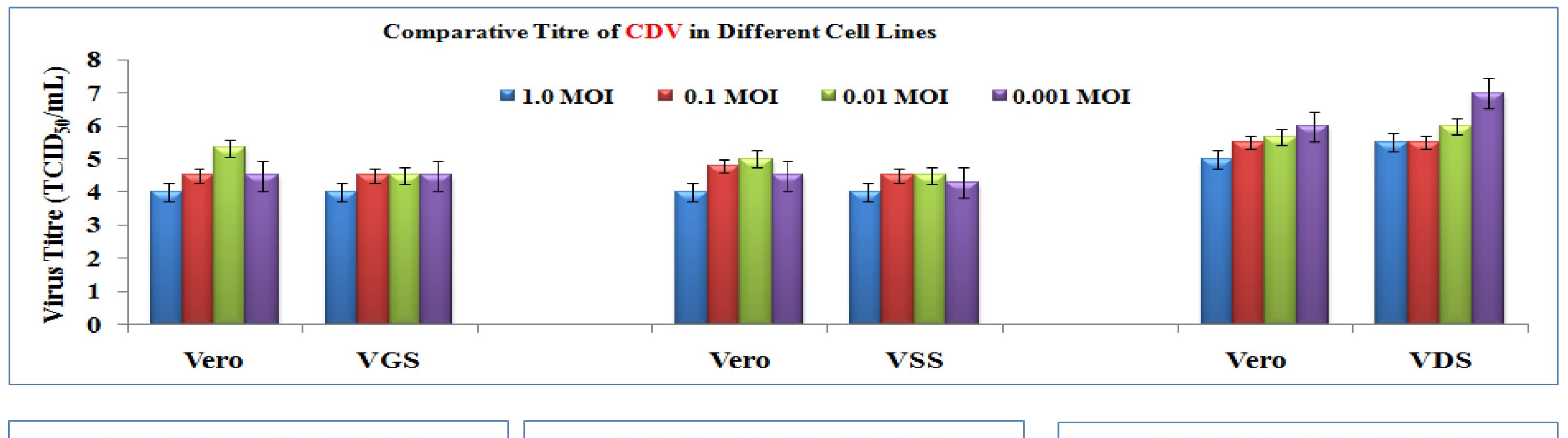
Same goat tissue samples were also used in Vero/dog/SLAM (VDS) cells for isolation. The cell culture harvest of passage-1 was checked in Lateral Flow Device (LFD) and by RT-PCR using NP3/NP4 primer. The samples have given some positive indication in LFD as shown by test line (T-line) and 351bp amplicon size in 1% agarose gel (**Fig: 44**).

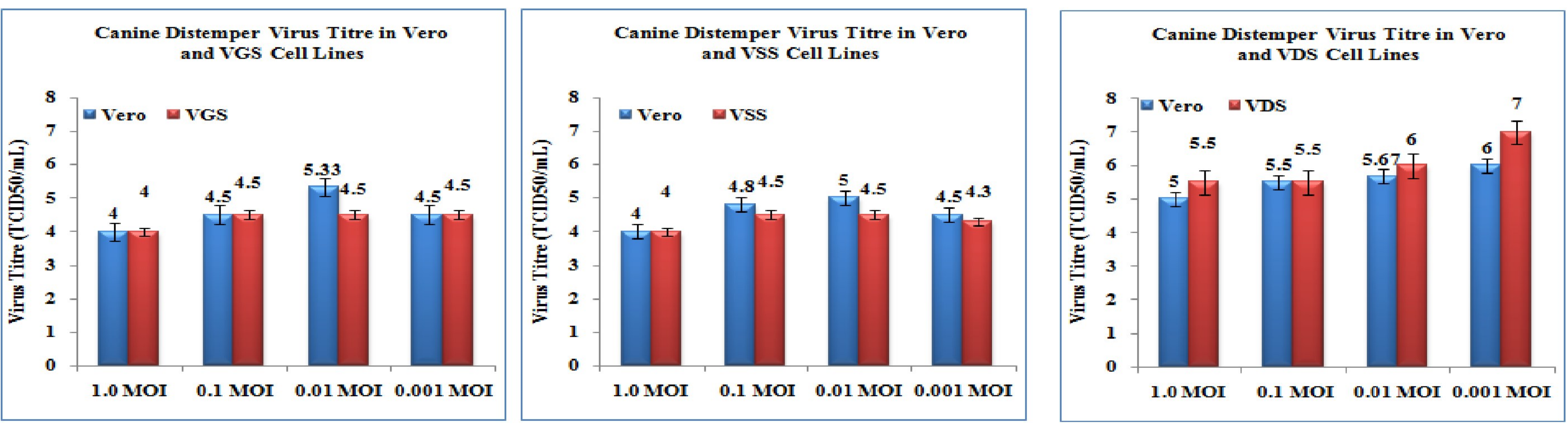
4.17 Isolation of Canine distemper virus from dog

Attempt was made to isolate canine distemper virus from the blood of clinically affected dog. The blood was inoculated (1:1 dilution with EMEM) to the cell monolayer of Vero and VGS cells. We checked the cell culture harvest upto third blind passage (BP-3) by RT-PCR. There was also no positive indication in both the cell lines. Here also we could not succeed in isolation the wild type canine distemper virus **(Fig: 43).**

✍ ✍ ✍

Comparative Titre of CDV in Different Cell Lines





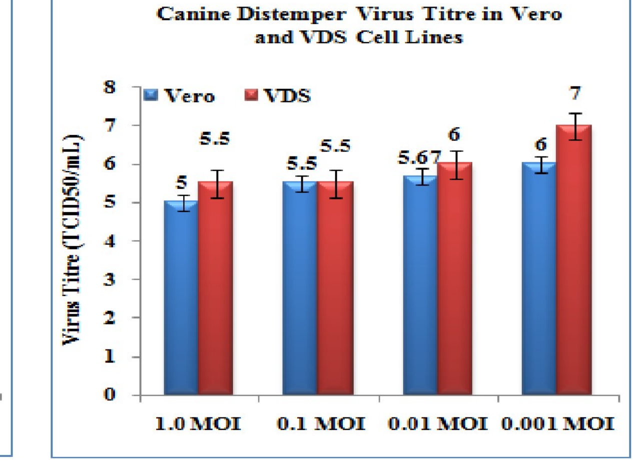


Fig. 40: Comparative titre of canine distemper virus in Vero, VGS, VSS and VDS cell lines at different moi. At 1.0 moi the titre of CDV in all the three cell lines was almost equal to native Vero cell. Upon decreasing the moi, the CDV titre also deceased in VGS and VSS. VDS exhibited higher titre (up to 1.0 log at 0.001 moi) than the native Vero cell irrespective of moi.

Comparative Mean Titre of PPRV and CDV in Different Cell Lines as Compared to Vero

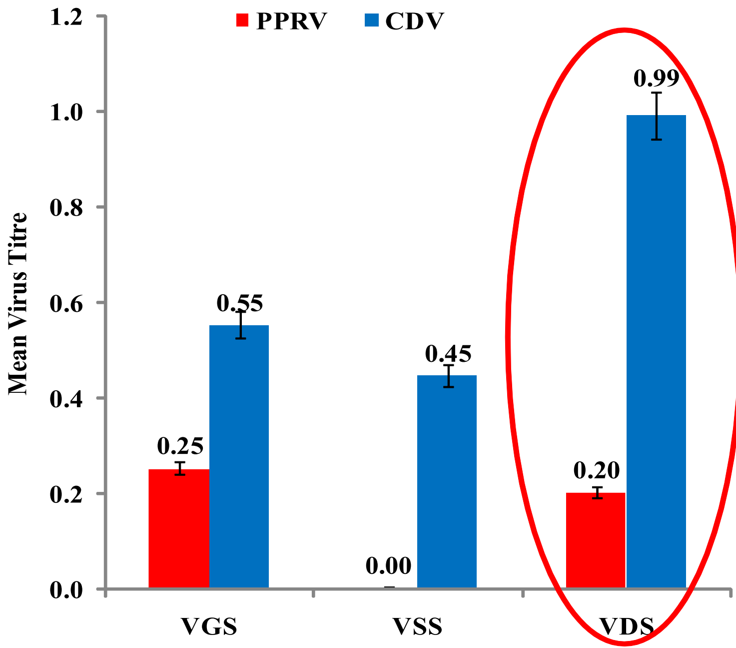


Fig. 41a: **Mean titre difference between PPRV and CDV Supernatant in different cell lines as compared to Vero**

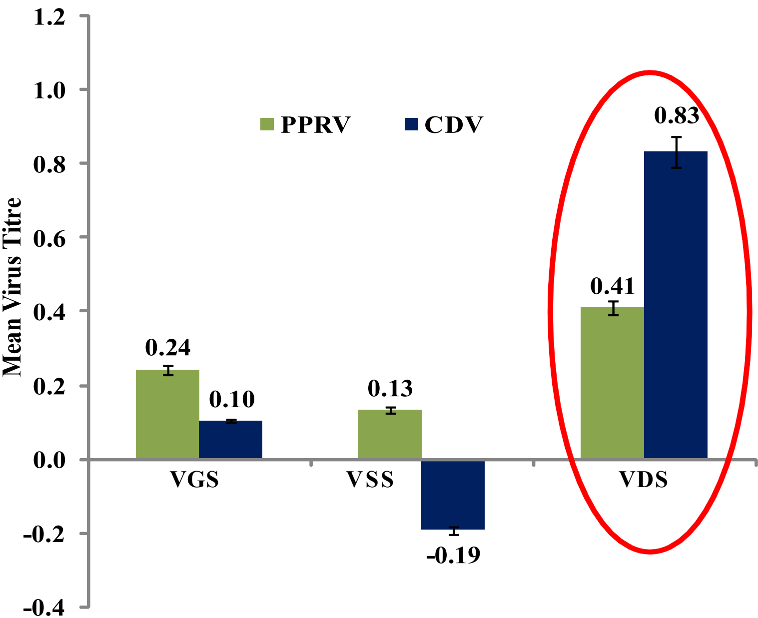
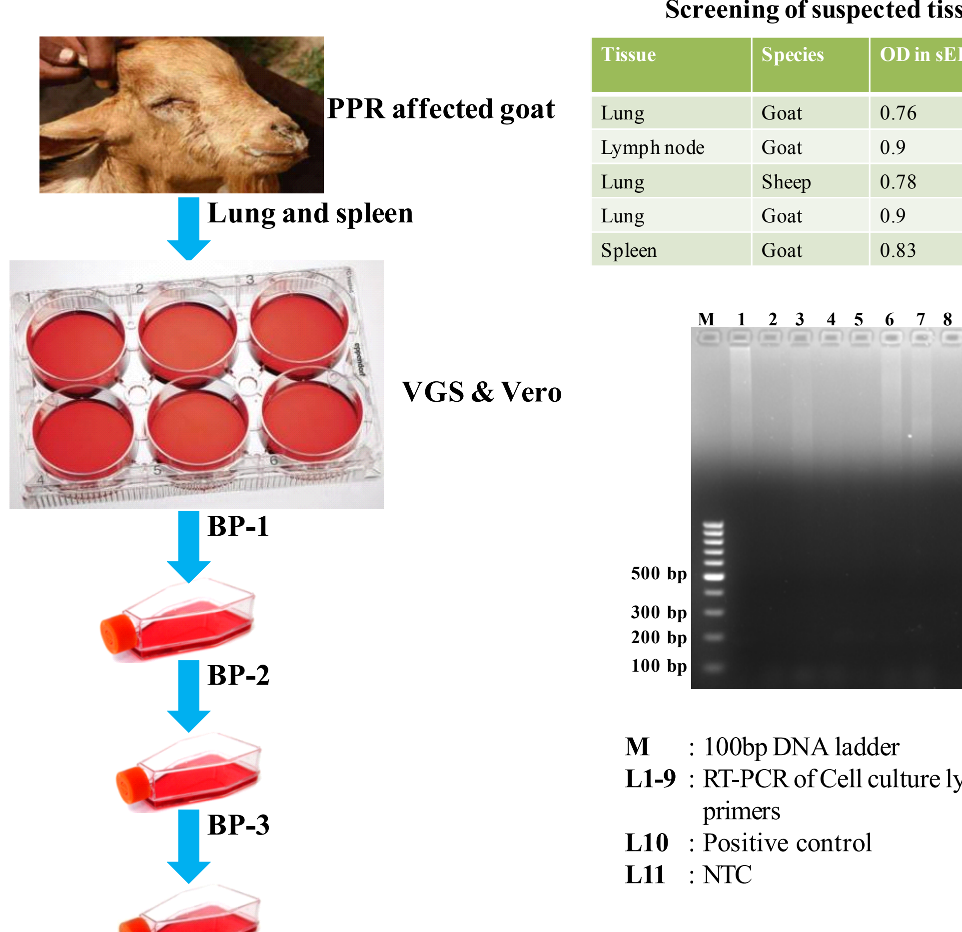


Fig. 41b: **Mean titre difference between PPRV and CDV cell lysate in different cell lines as compared to Vero**

Fig. 41: **Comparative mean titre of PPRV and CDV in VGS, VSS and VDS cell lines as compared to native Vero**. Both PPRV and CDV was found to have higher titre in supernatant as well as cell lysate in all the cell lines (except in VSS). The overall mean titre of CDV was found to be higher in comparison to PPRV. In VDS cells, the mean titre of CDV was found be higher by 0.99log in supernatant (41.a) and 0.83log in cell lysate (41.b).

PPR Virus isolation from tissue samples in Vero/goat/SLAM (VGS)



Screening of suspected tissue samples

Tissue	Species	OD in sELISA	RT-PCR by NP3/NP4
Lung	Goat	0.76	+
Lymph node	Goat	0.9	+
Lung	Sheep	0.78	+
Lung	Goat	0.9	+
Spleen	Goat	0.83	+

M : 100bp DNA ladder
L1-9 : RT-PCR of Cell culture lysate with NP3/NP4 primers
L10 : Positive control
L11 : NTC

Fig. 42: Details of PPRV isolation from pathological samples in Vero/goat/SLAM and native Vero cells. Isolation was tried up to third passage in both the cell lines, no virus could be isolated in any cell lines.

CDV isolation from tissue samples in VGS

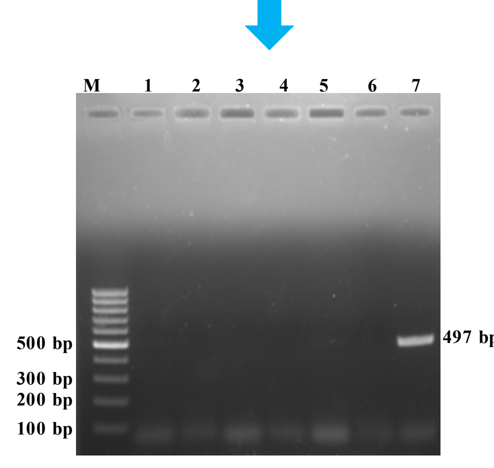
Screening of suspected tissue samples

Tissue	Species	RT-PCR
Blood	Stray dog	+
Blood	Dog 549	+

RNA isolation from cell culture harvest

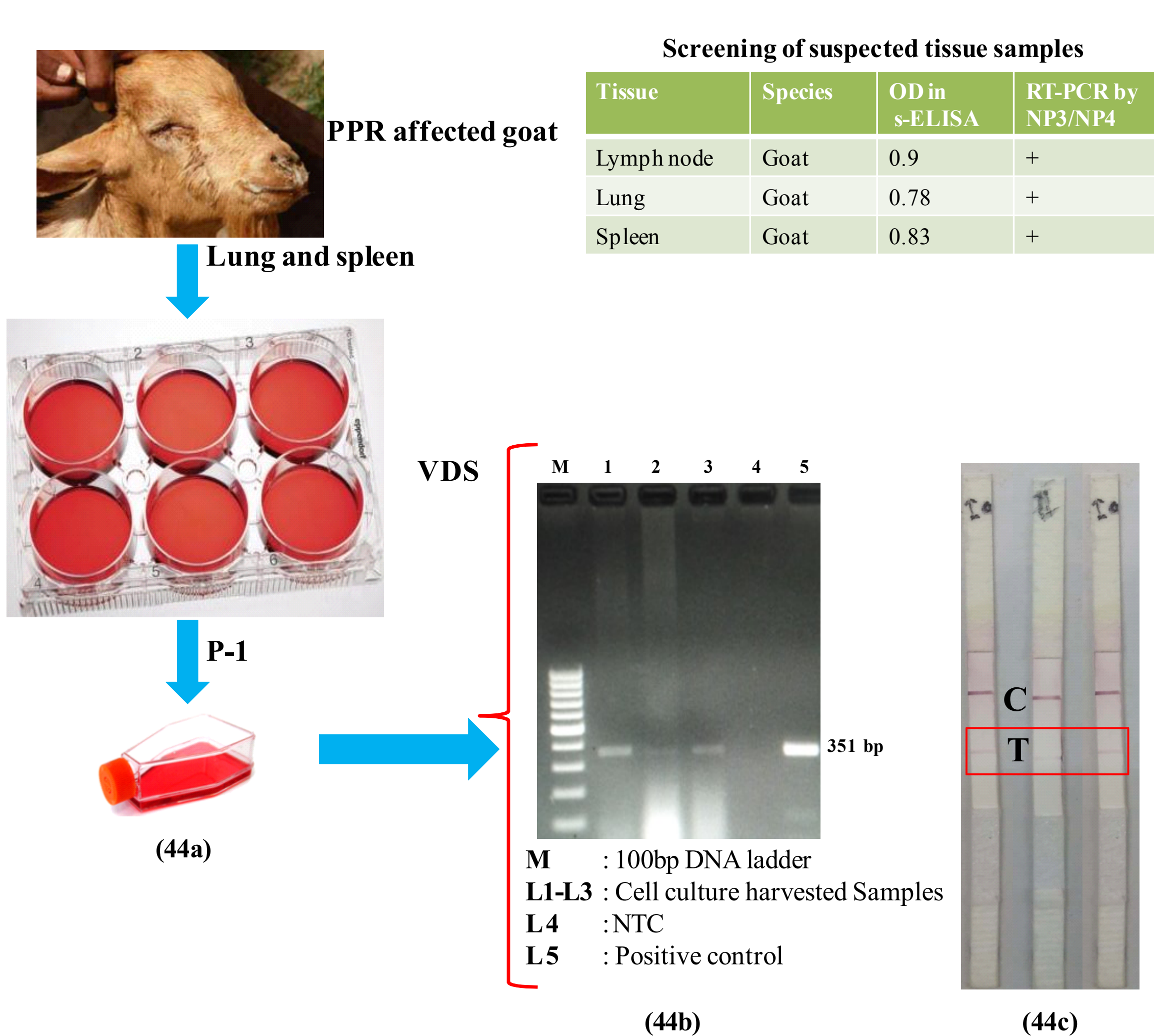
cDNA synthesis

RT-PCR by in-house designed N-gene specific primer (497bp)



M : 100bp DNA ladder
L1-5 : Cell culture harvest from BP1-BP3
L6 : NTC
L7 : Positive control

Fig. 43: Details of CDV isolation from blood samples in Vero/goat/SLAM and native Vero cells. Isolation was tried up to third passage in both the cell lines, no virus could be isolated in any cell lines.



Screening of suspected tissue samples

Tissue	Species	OD in s-ELISA	RT-PCR by NP3/NP4
Lymph node	Goat	0.9	+
Lung	Goat	0.78	+
Spleen	Goat	0.83	+

Fig. 44: Details of PPRV isolation from pathological samples in Vero/dog/SLAM cells. Indication of isolation was noticed at P-1 as evidenced by RT-PCR (44b) and Lateral Flow device (LFD) (44c).

Discussion

5 DISCUSSION

India harbours a large population of dogs, sheep and goat. These animals (sheep and goats) not only provide food security in terms of animal protein but also play crucial role in sustainable development of the farmers or animal raisers. Although, India harbours a good number of sheep, goat and dog population, health management have been major challenge in enhancing the productivity. *Peste-des-petits-ruminants* (PPR) is considered the main constraint to increased sheep and goat production in endemic areas (Diallo, 2006). Approximately more than one billion (63%) sheep and goats are at risk of transmission of PPR virus infection. Due to its huge impact on small-ruminant production, PPR has emerged as an essential global animal health concern. Therefore, FAO proposed that PPR would be the next animal disease to be eradicated after Rinderpest (FAO, 2013). Like PPR, canine distemper (CD), a severe systemic disease affecting carnivores worldwide. CD initially diagnosed as a life-threatening disease in domestic dogs (*Canis familiaris*), it has subsequently been recognized in a wide range of hosts including some non-human primates, posing a conservation risk to several free-ranging and captive non-domestic carnivores (Beineke *et al.,* 2015).

Morbilliviruses are classified under the subfamily Paramyxovirinae, the family *Paramyxoviridae* and the order *Mononegavirales* (Gibbs *et al*., 1979). There are seven members of the genus *morbillivirus*: *measlesvirus* (MV), is the prototype *morbillivirus*, and causes disease in primates, *rinderpest virus* (RPV) is closely related to MV and used to cause severe disease in cattle, *peste-des-petits-ruminants* virus (PPRV) which causes disease in goats, sheep and wild ruminants, *canine distemper virus* (CDV), which causes distemper

in dogs and a large number of other carnivore species, *phocine distemper virus* (PDV), which leads to distemper in several seal species *cetacean morbillivirus* (CeM) which cause disease in dolphins and whales, and recently, *feline morbillivirus* (FmoPV) first described in 2012 as a morbillivirus of cats, involved in chronic kidney disease (CKD) (Kumar *et al*., 2014; de Vries *et al*., 2015). Morbillivirus infection in humans and animals causes profound immunosuppression (de Vries *et al*., 2015); however, the individuals that survive infection usually develop lifelong immunity (Kerdiles *et al*., 2006). A cross-protection is believed to occur among various prototypes of morbilliviruses (Kumar *et al*., 2014). Members of morbilliviruses are enveloped, non-segmented, negative, single-stranded RNA genome that encodes a single envelope-associated matrix protein (M), two glycoproteins (hemagglutinin H and fusion protein F), two RNA-polymerase associated proteins (phosphoprotein P and large protein L), and a nucleocapsid protein (N) that encapsulates the viral RNA. The H gene encodes a key protein for morbillivirus and its animal hosts: the virus uses this protein to attach to cell receptors during the first step of infection (Griffin, 2007). The exact length of various morbillivirus genomes vary due to the variable size of the junction between the matrix (M) and the fusion (F) protein genes (Radecke *et al*., 1995).

Peste-des-petits-ruminants (PPR) is an acute and highly contagious viral disease of domestic and wild small ruminants (Libaeu *et al*., 2014). Clinically, PPR resembles rinderpest in cattle and is characterized by pyrexia (106-107^{0}F), ocular and nasal discharges, erosive stomatitis and diarrhea (Couacy-Hymann *et al.,* 2007; Banyard *et al.,* 2010). Like rinderpest, the causative agent of PPR is a virus belongs to the *Morbillivirus* genus in the family *Paramyxoviridae*. The disease was first identified in Ivory Coast of West Africa in 1942 by Gargadennec and Lalanne, who gave the name “peste-des-petits-ruminants” which means “plague of small ruminants”. In India, the disease was first reported from Arasur village of Villupuram district in Tamilnadu (Shaila *et al.,* 1989). Work on seroprevalence indicates that the disease has steadily progressed over time across the country and now endemic in nature (Singh *et al*., 2004, Balamurugan *et al*., 2011). The disease is caused by PPR virus (PPRV), which has been shown to be the largest member of genus Morbillivirus under family Paramyxoviridae with genome size of 15948 bp (Muthuchelvan, 2004, Siddappa *et al*., 2014).

The PPRV genome encodes six structural the nucleocapsid (N) protein, the matrix (M) protein, the polymerase or large (L) protein, the phosphoprotein (P), two envelope proteins-haemagglutinin (H) and fusion (F) protein] and two nonstructural proteins (V, C) in the order of 3' -N-P/C/V-M-F-HN-L-5' (Munir *et al.,* 2013). The hemagglutinin (H) protein of the PPRV also exhibits neuraminidase activity and, hence, is named the hemagglutinin-neuraminidase (HN) protein. F-gene based molecular epidemiology of virus from all over the world has defined the presence of four different lineages of virus (Banyard *et al.,* 2010; Muniraju *et al.,* 2013). Lineage I in West Africa, Lineage II in Nigeria and Cameroon, lineage III in East Africa and lineage IV in Asia. Of late, analysis based on F and N-gene of PPRV field samples from different parts of India revealed the circulation of lineage IV virus and no other lineage has been reported in last decade (Ahmad*,* 2014).

Akin to PPRV, *canine distemper virus* (CDV) also belongs to genus *Morbillivirus*, family *Paramyxoviridae* and is the causative agent of a severe infectious disease affecting a broad range of wild and domestic carnivores. The diameter of the virus is about 150 to 300 nm (Murphy *et al.,* 1999). The genome of CDV (approximately 15.7 Kb) encodes one non-structural protein (C) and six structural proteins: the nucleocapsid (N) protein, two transcriptase-associated proteins (phosphoprotein, P and large protein, L), the envelope stabilizing matrix (M) protein, and two immunologically important transmembrane glycoproteins (hemagglutinin (H) and fusion (F) proteins) embedded in the viral envelope (Lamb and Kolakofsky, 2001). The organization of the major genes in the CDV genome is 3'-N-P-M-F-H-L-5', each separated by untranslated regions (UTRs) (Sidhu *et al.,* 1993). CDV is also known as a highly prevalent viral infectious disease of carnivores, posing a conservation threat to endangered species around the world (McCarthy *et al*., 2007, Beineke *et al*., 2009, Martella *et al*., 2010, Gilbert *et al*., 2014). It was first isolated by Carré in 1905. Even recently, it was proposed to rename the virus "Carnivore Distemper Virus" due to its constant threat for different carnivorous species and to commonly documented outbreaks in non-dog carnivores (Terio *et al*., 2013). CDV has also been found in naturally infected non-canine hosts (Kapil *et al*., 2011) and recent dramatic outbreaks in breeding colonies of rhesus macaques (*Macaca mulatta*) and cynomolgus macaques (*Macaca fascicularis*), has drawn attention of a possible zoonosis in the post-

measles eradication times (de Vries *et al*., 2014). Of all the morbilliviruses CDV is the most promiscuous, infecting many different carnivore species. Generally, CDV exhibits lympho-, neuro- and epitheliotropism resulting in systemic infection of almost all organ systems including respiratory, digestive, urinary, lymphatic, endocrine, cutaneous, skeletal and central nervous system (CNS) (Von Messling *et al*., 2004, Lempp *et al*., 2014). The disease course and pathogenesis in canine distemper resemble those of human measles virus infection including, fever, rash, respiratory signs, lymphopenia, and profound immunosuppression with generalized depletion of lymphoid organs during the acute disease phase (Von Messling *et al*., 2006). In addition, CDV infection shows a high incidence of neurological complications (Lempp *et al*., 2014).

There is a correlation between the distribution of receptors and virus tropism. It is one of the major determinants, which determine the host range and play a crucial role in pathogenesis. Identification of cellular receptors has provided fundamental new insights into the pathogenesis of morbilliviruses and their interactions with the host immune system (de Vries *et al*., 2015). Investigations aimed at identifying the receptors for morbillivirus started with CD46 for vaccine strains of *measles virus* (MV) (Naniche *et al*., 1993), followed by the lymphoid cell receptor, SLAM (Tatsuo *et al*, 2000), and the epithelial cell receptor, nectin-4, for wild-type viruses (Noyce *et al*., 2011). CD46 or membrane cofactor protein (CD46/MCP) is used by MeV to adapt and grow in cell culture (Dorig *et al*., 1993, Naniche *et al*., 1993). However CD46/MCP is not a natural receptor involved in wild-type MeV pathogenesis and is not used by other morbillivirus species. Specific interactions between cellular receptors and the viral hemagglutinin protein (H) facilitate virus entry into host cells (Muhlebach *et al*., 2011, Noyce *et al*., 2011, Pratakpiriya *et al*., 2012, Birch *et al*., 2013) by inducing virus-cell and cell-cell membrane fusion in cooperation with the fusion protein (F) (Baker *et al*., 1999, Takimoto *et al*., 2002). Importantly, morbilliviruses are highly lymphotropic viruses that use the signaling lymphocyte activation molecule (SLAM/CD150) as an immune cell entry receptor that is expressed on the surface of activated T- and B-lymphocytes, macrophages and dendritic cells (Tatsuo *et al*., 2001, Hsu *et al*., 2001, Von Messling *et al*., 2004, Muhlebach *et al*., 2011). SLAM is also well-established receptor for *canine distemper virus* (CDV) (Von

Messling *et al*., 2006; Wenzlow *et al*., 2007), PPRV (Sarkar *et al*., 2007; Pawar *et al*., 2008; Adombi *et al*., 2011; Meng *et al*., 2011) *Rinderpest virus* (RPV) (Baron *et al*., ý2005) and *Phocid distemper virus* (PDV) (Melia *et al*., 2014).

Recently, epithelial cell receptor, nectin-4 is also identified in MeV (Muhlebach *et al*., 2011; Zhang *et al*., 2013), CDV (Pratakpiriyaa *et al*., 2012; Noyce *et al*., 2013), PPRV (Birch *et al*., 2013) and *Phocid distemper virus* (Melia *et al*., 2014). The nectin-4 gene is evolutionary more conserved in comparison to SLAM gene among the mammalian species (Birch *et al*., 2013). It is recently identified as exit receptor for morbilliviruses i.e. MeV, CDV and PPRV (Noyce *et al*., 2011 & Birch *et al*, 2013). The nectin-4 receptor is not responsible for the species specificity in morbillivirus infection; it is the SLAM which is responsible for species susceptibility for the morbillivirus infection (Ohishi *et al*., 2010).

After the establishment of both SLAM and Nectin-4 as cellular receptors for PPRV and CDV, people have used these receptors for various purposes. One of the key use is the isolation of pathogen (CDV or PPRV) (Seki *et al*., 2003, Adombi *et al.,* 2011) and increased production of antigen (Nizamani *et al*., 2014, Fakri *et al.,* 2015). In both, the case, development of stable cell line expressing either SLAM or nectin-4 receptor have been used (Tatsuo *et al*.,2000, Seki *et al*.,2003, Sarkar, 2007, Adobmi *et al*., 2011, Noyce *et al*., 2013, Fakri *et al.*, 2015).

Based on the above backgrounds, attempts were then made, in the present study to develop a stable Vero cell line (which normally does not express SLAM) constitutively expressing caprine, ovine and canine SLAM or Nectin-4 and to study if this cell line has better susceptibility to PPR virus (both virulent and vaccine virus) and canine distemper virus in comparison to native Vero cell line. In order to develop such cell lines, primers were designed for both the genes (SLAM and Nectin-4) using available sequences in the GenBank. cDNAs were obtained by reverse transcription of total mRNAs isolated from ConA-stimulated PBMCs of sheep, goat, and dog. Subsequent PCR amplification of these cDNAs using SLAM specific primer pairs, generated amplicons of more than 1000bp (approximate 1017bp) size specifying amplification of SLAM genes of goat and sheep and 1029bp for the dog. The amplified

SLAM genes of goat, sheep, and dog were then checked for identity and specificity by cloning and sequencing. The SLAM gene sequences of these species were aligned with human and dog SLAM gene sequences which showed the highest homology between goat and sheep both at the nucleotide level (99.8%) and protein level (99.4%). These findings revealed that the species of sheep and goat are phylogenetically closer with regard to SLAM homology, than other species *viz*. cattle, buffalo, dog, and human. The dog SLAM showed the highest homology with Redfox and raccoon both at nucleotide (98.5% and 98.9%) and protein level (99.1% and 99.4%). When this gene was compared with Asiatic lion SLAM, homology of 84.0% and 74.3% was observed at nucleotide and amino acids level, respectively. These findings suggested that dog SALM is closer to Redfox and raccoon SLAM gene and possibly evolved from SLAM of wild life during evolution (Hara *et al*., 2013). This could be the one of the possible reason for cross-species jumping of CDV to the wild life population like lion, leopard, and tigers (Appel *et al*., 1994; Guiserix *et al*., 2007; terio *et al*., 2013; Gilbert *et al*., 2015). The caprine SLAM distantly related to dog SLAM as it showed 80.2% homology at the nucleotide and 69.8% at amino acids level. Similarly, the caprine SLAM is also distantly related to Human SLAM (78.9% homology at the nucleotide and 63.9% at amino acids level). This could be again one of the reasons of non-reactivity of the commercially available monoclonal antibody against human SLAM with goat SLAM in western blot (Adombi *et al*., 2011).

The full-length goat nectin-4 was amplified for the first time and upon sequencing, it was found to be 1533bp in length, while in NCBI data base the predicted goat nectin-4 sequences are 1530bp long. The variation in full-length amplicon by three nucleotides may be due to the transcript variant of the goat nectin-4. Upon sequence analysis, the goat nectin-4 was found to be more closely related to the predicted sheep nectin-4 (Accession no.XM_015092338) and showed 94.4% homology at the nucleotide level and 95.4% at amino acids level. Homology with human nectin-4 showed 89.3% and 94.2% at nucleotide and amino acids level, respectively. The goat nectin-4 when compared with a predicted sequence of cattle nectin-4 (Accession no: NM_001024494), showed 97.3% homology at the nucleotide level and 97.4% an amino acids level. So these data support that nectin-4 is evolutionarily

conserved among the mammalian species and in agreement with the earlier finding of Bieringer *et al*., 2013. Phylogenetic analysis of goat nectin-4 showed that it forms monophylectic clade with other ruminant's species like cattle, buffalo and sheep. These findings suggested that goat nectin-4 evolved from nectin sequences from these ruminants. The sheep and dog nectin-4 genes were also amplified and cloned, but upon sequencing, both the clones failed at several occasions. Most of the time either they did not match with any significant sequence in the database or no read came in the chromatogram. Failure in amplification and then sequencing of sheep and dog specific nectin-4 genes could be due to non-availability of true sequences in the GenBank. The primers were designed from predicted sequences which might not have worked in sheep and dog. Since nectin-4 genes could not be amplified (except goat) hence, SLAM gene of sheep, goat and dog was taken further for stable cell preparation.

The full-length SLAM gene from goat, sheep and dog were cloned in an expression vector [pcDNA3.1(+) plasmid vector] and sequenced. After getting the specific sequence, the recombinant pcDNA3.1(+) plasmid vector containing caprine, ovine and canine SLAM gene was made endotoxin-free. This is a crucial step before transfection, as the presence of bacterial endotoxin along with the recombinant plasmid vector may cause the death of the transfected cells due to toxicity. The transfected cells were subjected to G418/neomycin sulphate selection pressure for 21-28 days to develop a stable cell line constitutively expressing caprine, ovine and canine SLAM. G418 is a neomycin sulfate analogue which inhibits protein synthesis of eukaryotic cells and subsequently leads to the death of the cells (www.invitrogen.com). However, pcDNA3.1(+) vector contains an in built bacterial gene aminoglycoside (e.g. neomycin) phosphotransferase which inhibits the action of G418 thus allowing the stable transformants to survive under G418 selection pressure (Southern and Berg, 1982). The resistant clones were selected in 6 well plates with 1×10^5 cells/well up to 21-24 days. The selected clones survived after 21 days grown in small discrete patches. These clones were transferred to another 6 well plate and put under antibiotic selection. Each time the clones were propagated under antibiotic pressure. This led to selecting only those clones that were stable transformants. The chance of mixed population of non-transfected cells (~1-2%) is possible because single cell cloning is not done. Once the transfected cells of Vero/SLAM (VGS, VSS and VDS),

started growing in a monolayer, the cells were characterized for SLAM gene at genomic, transcript and protein levels.

It was felt that there could be a possibility of the recombinant SLAM containing plasmid to remain in episomal form. In such cases of the insert remaining as an extrachromosomal entity, it would be possible for the selected clone to survive under G418 selection pressure, albeit only transiently. Hence repeated passaging of the stably transformed cell line was done and integration of the SLAM gene into the genome of Vero/SLAM was confirmed by PCR amplification of the 1017bp (goat and sheep) and 1029bp (dog) SLAM gene from VGS, VSS and VDS cells genomic DNA at different passage level, whereas no integration of SLAM gene was detected in DNA of native Vero cells. This confirmed the integration of SLAM gene into the DNA of transfected Vero cells. Moreover, the recombinant plasmid DNA integrates randomly into the genome of the transfected cells and, therefore, it does not always ensure the expression of the desired gene (clone into the vector plasmid), because the same transgene integrated at different locations in the genome may show different levels of expression due to "position effect" (Henikoff *et al*., 1992; Grigliatti *et al*., 2001; Zhang *et al*., 2016). This effect is presumed to arise due to: (i) the regulatory elements present in the regions flanking the transgene, (ii) the general availability of the region for transcription, (iii) the presence of *cis*- or trans-acting regulatory elements conferring tissue-specific expression.

The integration of SLAM gene into the genome of the Vero/SLAM cells was noticed up to passage level 23 by PCR. This is an inadvertent finding because according to some workers (Keniscope *et al.,* 2008, Feng *et al*., 2016) stable cell lines should not used beyond 5-10 passages because after that the integrated gene may lost, resulting in halt in protein expression. But Centre for Disease Control and Prevention (WHO measles and Rubella Laboratory Network) is using Vero-hSLAM (Vero-human-SLAM) cells up to 15 passage for routine virus propagation and isolation after that they are discarding the cells. We have checked genomic integration in one of the clone of Vero/goat/SLAM cells and the genomic integration from this clone was lost at passage-15. Expression of SLAM in the transfected cells was studied by isolating total mRNA from VGS, VSS and VDS cells at different passage level by RT-PCR. Presence of PCR amplicons of desired size *i.e.* 1017bp for SLAM expression primers

of goat and sheep and 1029bp of dog confirms the unequivocal expression of the cloned gene in these cell lines. The findings are in concurrence to the previous findings of different workers (Sarkar *et al.,* 2007, Adombi et. al., 2011) who also confirmed the RNA transcript from transfected cell stably expressing SLAM genes by RT-PCR.

Detection of SLAM protein expression was done by immunofluorescence assay using monoclonal antibody (mAb) against FLAG tag. Monoclonal antibody against sheep, goat or dog SLAM is not available commercially, though, it is available for human SLAM [clone A 12, eBiosciences, San Diego, CA, USA (Cat #16-1509-81)]. But, mAb of human SLAM is non-reactive with sheep, goat or dog SLAM stably expressed on Vero cells (Adombi et. al., 2011). To overcome such problem, SLAM gene was tagged with FLAG tag (DYKDDDDK) at their C-terminus. While protein synthesis, whole transcript was supposed to be translated (N'$\rightarrow$C i.e. SLAM'$\rightarrow$FLAG terminal) and FLAG Tag could be detected using antibody against FLAG. Several other workers have used the same strategy to check the SLAM/ Nectin-4 protein expression on the Vero cell membrane using different tag (Seki *et al*., 2003, Feng *et al.*, in 2016). To check the SLAM protein expression by immunofluorescence, FLAG-tagged SLAM protein on the surface of Vero/goat/SLAM, Vero/sheep/SLAM and Vero/dog/SLAM cells reacted with M-2 anti-FLAG monoclonal antibodies (M/s Sigma Aldrich) and FITC (M/s Sigma Aldrich) and upon exposure of UV light under Fluorescence microscope it emited green fluorescence signal whereas no such fluorescence was detected in the native Vero cells. Seki and co-workers (2003) developed Vero.DogSLAMtag cells wherein, haemagglutinin (HA) gene of *Influenza virus* was used as tagging element for tracing the SLAM gene expression by flow cytometry. Feng *et al*., in 2016 also used *Influenza virus* HA epitope as tag for detection of SLAM expression. They examined the SLAM expression by flow cytometry using an HA tag specific antibody.

The expression of SLAM in VGS, VSS and VDS was also quantified by real-time PCR. The relative expression of SLAM in different cell lines at different passage level was quantified by $\Delta\Delta$Ct method or comparative Ct method as described by Livak and Schmittgen, 2001. The PBMCs of respective species was taken as calibrator to calculate the relative expression of the SLAM in different cell lines. Maximum expression of SLAM was found in

VDS cells in comparison to VGS and VSS cells. It was observed that expression of SLAM gene decreased upon increase in the passage number. The exact reason for decreased expression on passaging of cells could not be ascertained however, it may be attributed to lose of integrated SLAM gene, it may be possible to use lower passage of Vero/SLAM cell line for early isolation of virus. Researchers have suggested that stable cell lines should not use beyond 5-10 passages for virus isolation otherwise it will lose the SLAM expression property (Keniscope *et al*., 2008, Feng *et al*., 2016).

Susceptibility of cells expressing signaling lymphocytic activation molecule (SLAM) of homologous host for members of the genus *Morbillivirus* has been studied extensively for MV, CDV and RPV (Tatsuo *et al.*, 2001; Seki *et al.*, 2003), and for PPRV (Sarkar *et al*., 2007). In the present investigation, the novel Vero cell line expressing caprine, ovine and canine SLAM on its surface was infected with vaccine viruses of PPR and *canine distemper virus* to find out its susceptibility for the viruses. To check the replication efficacy of both PPRV and CDV in the developed cell lines one-step growth curve was performed in native Vero, VGS, VSS and VDS. The supernatant and cell lysate were harvested at 0h, 6h, 12h, 18h, 24h, 48h, 72h, 96h, 120h and 144h, and titration of both supernatant and cell lysate of above mentioned time points was determined.

In case of PPRV, in the supernatant, the first peak was found around 18h and the second peak was found around 96h however, in the cell lysate, the first peak was found around 18h and the second peak was found around 72 post infection. The eclipse period of PPRV in all the cell lines including native Vero cells was around 18h with maximum titer at 72h post-infection. After 72h the viral titer started decreasing. These findings are in the consistent to the previous findings by Singh *et al*., 2008, though they have not used the Vero/SLAM; instead native Vero cell was used. Although, the comparative mean titer of PPRV was higher in all the three cell lines (VGS, VSS and VDS) as compared to native Vero cells, titer difference was insignificant (Less than half log). This finding is in contrary to Fakri *et al*., 2016, who observed 0.9log and 1.7log $TCID_{50}$ higher in VeroNectin-4 cells as compared to Vero and VeroDogSLAM cells, respectively. In another work, Sarkar *et al*., 2007, found that Vero-goat SLAM cells showed 1.2 log more titer in comparison to native Vero cells using PPRV

vaccine (PPRV Sungri/96) virus and Nizamani *et al*., 2014, noticed that 0.8 log10 higher virus titer in Vero.DogSLAMtag cells than the Vero cell for both “wild-type” and “vaccine strains” of PPRV Nigeria75/1 strain. The mean log titer difference was 0.24, 0.13 and 0.41 log more in VGS, VSS and VDS cells, respectively as compared to native Vero. The reason(s) for non-significant titer in the cells stably expressing SLAM gene could not be conclusively ascertained. However, use of high titer virus and effect of pH on virus replication cannot be ignored. In this study, the stock virus (PPRV Sungri/96) titer was around 4.5-5.0 $TCID_{50}$/mL. Further, no media was changed during one-step growth curve experiment (till 144 hrs), which left the plate environment to acidic pH. It is well established that PPRV is quite stable between pH 5.8–10.0 (Coetzer and Tustin, 2004) and titer is drastically affected by acidic pH. Since both cell-free and total virus was accounted in this study, changing the media was not done to keep the cell-free virus unaltered. Replication efficacy of PPR vaccine virus was also assessed by different moi (1.0, 0.1, 0.01 and 0.001 moi) in all four cell lines viz; native Vero, VGS, VSS and VDS. At higher m.o.i. (1.0) all the three cell lines (VGS, VSS and VDS) exhibited lower (VGS and VSS) or similar (VDS) titre as compared to native Vero cells. But as the m.o.i. decreases (0.1, 0.01, 0.001) titre of PPR virus also decreased. Overall, virus titer was found to be highest in VDS among all three engineered cell line, yet lower than the native Vero cell.

The one-step growth curve of CDV was also performed in Vero, VGS, VSS and VDS with Onderstepoort vaccine virus. Here supernatants and cell lysates of above mentioned time point (like PPRV) were titrated. The CDV Onderstepoort strain showed first peak at 18h and second peak at 48h post-infection. The eclipse period of CDV was found to be 18h and maximum titre obtained at 48h post-infection which then gradually started decling. The mean titre difference with native Vero cell in supernatant was 0.55, 0.45 and 0.99log more in VGS, VSS and VDS, respectively, whereas cell-associated virus showed 0.83log more titre in VDS as compare to native Vero cells. CDV was also infected with different moi from 0.001, 0.01, 0.1 and 1.0 in Vero, VGS, VSS and VDS cells. At higher moi the titre of CDV in all the three cell lines was almost equal to the titre in Vero. Upon decreasing the moi the CDV titre also deceased in VGS and VSS. There was 1.0 log higher titre observed in VDS than native Vero at moi 0.001.

Findings of this study are in agreement with the Feng *et al*. 2016 who also studied the replication of CDV (Monkey-BJ01-DV) on Vero cells expressing the SLAM receptor from various animal species (Vero/DogSLAM, Vero/MonkeySLAM, and Vero/HumanSLAM cells). At 0.01 m.o.i. Monkey-BJ01-DV replicated to the highest titers on Vero/DogSLAM ($10^{5.2\pm0.2}$ $TCID_{50}$/mL) and Vero/MonkeySLAM ($10^{5.4\pm0.1}$ $TCID_{50}$/ml) at 48 h post-infection whereas replication on the Vero/HumanSLAM cells was reduced approximately 100-fold ($10^{3.3\pm0.3}$ $TCID_{50}$/ml. Bieringer *et al* 2013, got 2-3log higher titre of CDV-A75/17 isolate in a VeroDogSLAM cell than the Vero-hSLAM. However, the titre reached to the normal level (similar to VeroDogSLAM) after three successive passages in Vero-hSLAM.

The comparative mean titre of PPRV and CDV was analyzed by one-step growth kinetics experiment in comparison to native Vero cells. The overall comparative mean titre of both viruses showed that the CDV replication efficacy was much higher in all the cell lines and highest in VDS cell (0.99 log higher in supernatant and 0.83 log higher in cell lysate) than the PPRV.

The proper explanations on the lower titre of PPRV in stably expressing SALM cells remain inconclusive and need further investigation. PPR virus as any other morbillivirus has the propensity to utilize three receptors viz. CD46, CD150 and Nectin-4. Vero cells have CD46 on their surface and this is the receptor utilized by the alternated strain of PPR virus (Galbraith *et al*., 1998; Sannat *et al*., 2014). Transfected Vero cells have caprine, ovine and canine SLAM *i.e.* CD150 besides CD46 on their surface. Hence the PPR vaccine virus is adapted to grow at conditions that permit the exclusive use of CD46 in erstwhile Vero cells. However, in a transfected cell population, there are two receptors available for virus entry viz. CD46 and CD150. So the virus that ordinarily uses the CD46 receptor for entry is drawn by the CD150 receptor. However due to the limited availability of CD150; entry of virus takes a longer duration. It may also be stated that a proportion of virus could exist that may have an affinity for CD46. Entry by CD150 is not simultaneous and presumably, entry by CD150 is not inhibitory for a subsequent entry by CD46. Hence, there is increased presence of virus detected in the supernatant in Vero/SLAM when compared to Vero cells. Further, when the amount of total viral antigen load was compared by a mAb-based (N-protein) sandwich-ELISA; the

relative O.D. values were higher in the supernatant and almost equal in cell lysate of VGS, VSS and VDS cells when compared to normal Vero cells. There was no significant difference found in case of cell lysate. This may be due to the presence of dual receptors and or virus is getting entered in the transfected cells and after completion of short replication cycle, coming out of the cell and released into the supernatant. This needs further study and scientific validation.

In this study, pcDNA3.1 (+) vector (circular plasmid vector) was used for transfection of Vero cells. This is a traditional method for transfection where in the integration of target gene is random and not much efficient as it is seen in site-directed integration methods (Flip-In system or CRISPR-Cas or Lentivirus based system). There could be low transfection rate which lead to low percentage of SLAM rich cells. It is to worth mentioning that the transfected cell was not subjected to single cell cloning. Doing so might have helped in the enrichment of SLAM rich population resulting in to development of much sensitive cell.

Further, low titre in receptor expressing cell lines may be because of receptor inactivation on cell membrane but before establishing the fact we have to check the replication efficacy of PPRV with other isolates and with different batches of the vaccine virus. Again, sequence of SLAM expressed on the cell surface of the Vero cells was not checked. The sequence analysis of SLAM from the transfected cells have to compare with native SLAM sequence used for transfection for change, if any. It may be possible that during random integration in the Vero cells, chance of recombination in SLAM sequence or some deletion in the part of SLAM gene occurred, which may lead to receptor inactivation even after successful expression of protein on cell surface. The amino acids of the V region of the SLAM are responsible for the interacting with PPRV H protein (Ohishi *et al*., 2014). It may be possible that one or two critical amino acids might have changed during integration or during subsequent passage of the cells, resulted in to receptor inactivation. There may be inherent problem in expression vector that some part of the CMV promoter may be lost during integration in Vero cell genome and only the SV40 promoter is surviving which guides only Neomycin resistance gene, so the transfected cell survived in presence of G418 but in actual the gene of interest may be absent in the cell (SLAM receptor gene). After entering into the cell cytoplasm and subsequent integration into Vero cell genome there may be possibilities of conformational changes of SLAM receptor

protein while cell surface expression. The critical residues (52-136 amino acids) of SLAM responsible for PPR H protein interaction may undergo some 3D structure changes that are responsible for virus attachment (Ader-Ebert *et al*., 2015; Ohishi *et al*., 2014). We have sequenced our Goat, Sheep and Dog SLAM there was not a single amino acids change in the binding region but there may be possibilities of conformational changes. The receptor-ligand interaction is possible only when there is 3-dimensional interaction between them without any change in conformation.

Apart from membranous form, SLAM can also be expressed either in a secreted form that lacks the entire 30 amino acid transmembrane domain or as an intra-cytoplasmically expressed isoform that is missing the leader sequence. These isoforms mediate multiple functions by their differential expression in T and B lymphocytes at distinct ages of differentiation/ maturation (Cocks *et al.*, 1995; Punonen *et al.*, 1997; Aversa *et al.*, 1997a). Secretory form of SLAM may inhibit the virus infectivity by interacting with viral attachment protein before it reaches cell surface. Similarly, intracellularly expressed isoform of SLAM may also reduce the possibility of virus-host interaction. Thus, expression of SLAM in any of these isoforms may result in reduced viral infectivity as contrary to the prevalent notion.

Attempts were made to isolate field derived virulent PPRV and CDV in a panel of cells *viz*. Vero/goat/SLAM, Vero/dog/SLAM and Vero cells with an objective to determine if there are any tangible differences between these cell lines with regard to their susceptibility to virulent viruses. Ten percent (w/v) tissue suspensions (n=03) prepared from field tissue (Frozen) samples in PBS were subsequently tested for PPRV antigen by sandwich-ELISA. The VGS cell line did not produce any CPE after 10 days of infection with the virulent virus. Moreover, after 3 blind passages of the virus in respective cell lines, neither CPE was found nor viral antigen was detected in any of these infected cells by RT-PCR. The same tissue sample was inoculated to VDS and after two blind passage, some indications of virus isolation was found by RT-PCR (amplification of 351bp amplicon size) and Lateral Flow Device (by the appearance of T-line). The blood (n=03) from a clinically affected dog with canine distemper was inoculated in VGS and Vero cell lines. There was no appearance of CPE in both the cell lines after 8-10 days of infection. Moreover, after 3 blind passages of the virus in respective cell lines, neither

CPE was found nor viral antigens were detected in any of these infected cells by RT-PCR. Current findings are in partial agreement with Fakri *et al* (2016) who did successful isolation using VeroNectin-4 cells. Only one out of 10 frozen isolated virus samples (10% efficiency) yielded viral replication with an observed CPE, following three passages in VeroNectin-4 cells. No virus isolation was observed using Vero or VeroDogSLAM cells. They concluded that Vero or VeroDogSLAM cell lines are less sensitive to infection than VeroNectin-4 cells. Virus isolation was successful from 13 fresh samples using VeroNectin-4 cells (42% efficiency) and from seven fresh samples using VeroDogSLAM cells (22% efficiency). In the present study, frozen tissue samples were used for isolation of PPRV and relatively old (not fresh) blood samples were subjected for CDV isolation. Non-isolation of either PPRV (though some indication of positivity in RT-PCR) or CDV in any engineered cell line might be due to nature of samples used. A total number of samples may be another factor hence use of fresh sample with more sample number may give some positive result. Amino acid sequence analysis of the 'H' protein of several vaccines and wild type strains of PPRV, RPV, MV and CDV showed that the critical residues identified on the MV 'H' protein for interaction with SLAM (CD150) receptor are conserved in all morbilliviruses (Abraham *et al.*, 2006). Further, the amino acid residues at position 60 (isoleucine) and 61 (Histidine) have been proved to be critical for human SLAM to act as a permissive MV receptor. These amino acids are conserved in human, marmoset and dog SLAM protein which have already been proved to be receptors of *Morbilliviruses*. The sequences obtained in the present investigation revealed the same amino acids at position 61(isoleucine) and 62 (histidine) in sheep and goat SLAM protein, reinforcing the critical interactive role of these with the 'H' protein making it plausible to assume that these two amino acids should contribute to the receptor function for SLAM in these species. While in dog SLAM amino acids position at 60 (Isoleucine), 61 (histidine) and 63 (leucine) are critical for the function of SLAM as a receptor for CDV. However, in the present study, caprine and ovine SLAM does not seem to be critical for entry of the virulent virus into the host. From these observations, it appears that viable viruses from the tissue suspension entered the cells through hitherto unknown receptors/co-receptors, but the numbers of the intracellular virus were not sufficient enough to produce any morphological change of the infected cells. However, it seems that further blind passages are needed to get a perceptible

viral effect in form of a CPE after adaptation of the virus in these cells (Diallo *et al.*, 1989). The possible role of co-receptors should also be investigated further.

The findings in respect to susceptibility of PPRV and CDV revealed that Vero/SLAM is a better host for CDV (at least at the level of entry) yielding more amount of virus than native Vero cells. However, susceptibility of all the four cell lines for propagation of PPR (wild-type and vaccine virus) and CDV (Wild-type) could not be conclusively ascertained. With this limited data, following points emerged from present study:

- Stable Vero cells constitutively expressing goat, sheep and dog SLAM (VGS, VSS and VDS) were generated successfully using pcDNA3.1(+) vector under G418 selection pressure and characterized.
- VDS cell showed relatively high expression of SLAM genes as compared to VSS and VGS cells by RT-qPCR and found to be most sensitive cell line for CDV replication, even at 0.001 moi (1.0 log higher titre).
- The Mean titre difference of CDV in VDS was found to be higher by 0.83 log in Cell-Lysate and 0.99 log in the supernatant as compared to native Vero cell.
- PPRV replication in different cells even at different m.o.i. was found to be at a lower level in comparison to native Vero. Reason(s) for lower replication efficacy of PPRV could not be conclusively ascertained.
- Isolation of PPRV and CDV from field samples in VGS cell could not be succeeded. However, an indication of PPRV isolation in VDS at P-1 level was noticed.

Suggested future research focus

The present study can be used in the areas of virus-receptor interaction as well as molecular pathogenesis. The suggested research focus for future investigation could be:

- Single-cell cloning of the stable cells generated in this study for SLAM receptor enrichment.
- Studies on comparative replication efficacy of PPRV and CDV using already developed similar cell lines (VeroDogSLAM or VeroDogNectin)

- Generation of even more sensitive cell line (nectin-4/SLAM) cell by site-specific integration strategy (CRISPR/Cas, Flip-in system, Lentiviral system, ϕC31 integrase system and transposon).
- Development of cell lines harbouring both SLAM and Nectin-4 receptor and studies on replication efficacy of Morbilliviruses.
- Multistep growth curve using low moi (0.01) with 2 hourly time interval can give more accurate picture of replication efficacy (at least at the level of entry).
- Attempt for isolation on more no. of samples may give some idea of sensitivity of cell line to wild type virus.
- Development of monoclonal antibody/CRISPR-CAS system/SiRNA against goat, sheep and dog SLAM and nectin-4 and determining their role in blocking PPRV and CDV infection.

Summary and Conclusions

6 SUMMARY AND CONCLUSIONS

Morbilliviruses are highly contagious pathogens that cause devastating diseases with high morbidity and mortality in humans and animals across the world. The genus, *Morbillivirus*, belongs to the family *Paramyxoviridae* under the order, *Mononegavirales* (Gibbs *et al*., 1979). Members of the genus include *Measles virus* (MV) of primates, *Rinderpest virus* (RPV) of cattle, *Peste-des-petits-ruminants virus* (PPRV) of small ruminants, *Canine distemper virus* (CDV) of carnivores, *Phocine distemper virus* (PDV) of several seal species, *Cetacean morbillivirus* (CeM) of dolphins and whales, and recently, *Feline morbillivirus* (FmoPV) of cats (de Vries *et al*., 2015).

Peste des petits ruminants (PPR) is an economically important viral disease of small ruminants, characterized by "3Ds" i.e. 'discharge', 'diarrhea' and 'death', with additional fourth major component that is 'bronchopneumonia' (Wohlsein, *et al*., 2006). PPR causes high morbidity and mortality in its natural host and therefore it is considered as one of the economically important diseases of small ruminants. *Canine Distemper* is also known as highly prevalent viral infectious disease of carnivores (dog, lion, leopard etc) posing a conservation threat to endangered species around the world (McCarthy *et al*., 2007, Beineke *et al*., 2009, Martella *et al*., 2010, Gilbert *et al*., 2014). CDV exhibits lympho-, neuro- and epitheliotropism resulting in multisystemic infection (Von Messling *et al*., 2004, Lempp *et al*., 2014). The disease course and pathogenesis in canine distemper resemble those of human measles virus infection including, fever, rash, respiratory signs, lymphopenia, and profound immunosuppression with generalized depletion of lymphoid organs during the acute disease phase (VonMessling *et al*., 2006). In addition, CDV infection shows a high incidence of neurological complications (Lempp *et al*., 2014).

Cellular receptors are one of the major determinants of the host range and tissue tropism of a virus, which helps in understanding the pathogenesis (de Vries *et al*., 2015). It has already been established that *Morbilliviruses* uses SLAMs and Nectin-4 as cellular receptors (Tatsuo *et al*, 2000; Noyce *et al*., 2011). Same as other morbilliviruses, SLAM/CD150 and Nectin-4/ PVRL4 receptors are also utilize by PPRV and CDV (Adobmi *et al*., 2011; Meng *et al.,* 2011; Noyce *et al*., 2013; Birch *et al.,* 2013). The SLAM receptor presents mainly in lymphoid tissues and Nectin-4 in epithelial tissues. Both PPRV and CDV are lymphotropic and epitheliotropic in nature (Coucy-Hymann *et al.,* 2007; Hammouchi *et al.,* 2012, Noyce *et al*., 2012). Lesions and viral replication are mostly confined to lymphoid and epithelial tissues. Relation of virus infectivity and expression of cellular receptors has been elucidated at several occasions (Sarkar *et al.,* 2007; Pawar *et al.,* 2008; Adombi *et al.,* 2010; Meng *et al.,* 2011, Birch *et al.,* 2013, and Manu, 2015)

Virus isolation is a "gold standard" method but less sensitive, especially in heterologous cell lines. Primary cultures of bovine kidney, goat kidney, sheep kidney and lung cells have been utilized for isolation and maintenance for PPRV (EI Hag Ali and Taylor, 1984). CDV isolation is a common practice in mitogen-stimulated dog, ferret lymphocytes and canine kidney cells (Appel *et al*., 1992). Naïve Vero cell line is found to be less sensitive for both attenuated and wild-type PPRV strains (Sreenivasa *et al*., 2006, Nizameni *et al*., 2014). Likelihood of isolating virus is very low and even if successful it often requires multiple, sequential blind passages and many weeks in culture (Abu Elzein *et al*., 1990). Therefore the present study was designed to develop a more sensitive cell line having SLAM or Nectin-4 receptor for propagation and isolation of both PPRV and CDV.

SLAM genes of goat, sheep and dog were PCR amplified from cDNA (synthesised from RNA of mitogen stimulated PBMCs) using self-designed SLAM gene-species specific primers. The amplicons (1017bp size) thus obtained from goat, sheep, and dog (1029bp) were cloned into a mammalian expression vector pcDNA3.1(+) and sequenced commercially. The sequence analysis showed caprine SLAM gene had highest sequence homology with sheep SLAM gene (99.8%) followed by cattle, Buffalo (96.3%), dog (80.2%) and human (78.9%). The dog SLAM showed the highest homology with Redfox and raccoon both at

nucleotide (98.5% and 98.9%) and protein level (99.1% and 99.4%). When this gene was compared with Asiatic lion SLAM, homology of 84.0% and 74.3% was observed at nucleotide and amino acids level, respectively. The caprine SLAM distantly related to dog SLAM as it showed 80.2% homology at the nucleotide and 69.8% at amino acids level. Similar to SLAM gene, Nectin-4 was also amplified cloned and sequenced from goat species. The sheep and dog nectin-4 genes could not be amplified for one or other reasons. Upon sequence analysis, the goat nectin-4 (1533bp) was found to be more closely related to the predicted sheep nectin-4 (Accession no.XM_015092338) and showed 94.4% homology at the nucleotide level and 95.4% at amino acids level. So these data support that nectin-4 is evolutionarily conserved among the mammalian species. Since nectin-4 genes could not be amplified (except goat) hence, SLAM gene of sheep, goat and dog was processed downstream for stable cell preparation.

Vero cells were transfected with the recombinant pcDNA3.1(+) vector containing caprine, ovine and canine SLAM gene using Lipofectamine3000 transfection reagent. The transfected cells were cultured with the G418 antibiotic after 48h of transfection keeping native Vero cells as control. Being a neomycin sulfate analogue, G418 killed all the cells in control wells after 15 days of selection by inhibiting synthesis of cellular proteins. However, in experimental wells, there were few live cells attached to the surface of the cell culture plate in the form of discrete bunches. The tranfected cells then characterized at genomic, transcript and protein level; positive clones were subsequently preserved in LN_2. To check the genomic integration of SLAM gene, genomic DNA was subjected to PCR assay using SLAM-Expression primers of the respective species keeping normal Vero cell as negative control. SLAM gene of all the three species were successfully amplified from Vero/SLAM (Vero/goat/SLAM, Vero/sheep/SLAM, Vero/dog/SLAM) which confirmed the integration of the gene into the cellular genome. Since genomic integration does not ensure expression of the desired gene (cloned into the vector plasmid), so expression of gene was checked at transcript level. Total mRNA was isolated from Vero/SLAM and native Vero cells, cDNA synthesized and subjected to PCR. The SLAM gene was successfully PCR amplified using SLAM-Expression primers of the respective species. The protein expression on Vero cell surface was checked by staining

with M-2 anti-FLAG monoclonal antibodies and anti-mouse FITC conjugate in the form of green fluorescence whereas no such fluorescence was detected in native Vero cells, confirming the expression of SLAM on the Vero cell membrane. The relative expression of SLAM transcript in all three species was also checked by RT-qPCR employing SYBR green chemistry and found highest expression in Vero/dog/SLAM (VDS) cells.

In order to check the replication efficacy of both PPRV (PPRV/Sungri/96) and CDV (Onderstepoort) vaccine viruses in the developed cell lines, one-step growth curve was performed. Vero/goat/SLAM (VGS), Vero/sheep/SLAM (VSS), Vero/dog/SLAM (VDS) and Vero cells were infected with pre-titrated PPRV as well as CDV vaccine virus at 1.0 m.o.i. Viruses were then harvested as supernatant as well as cell-associated forms at various time-intervals *viz*. 0h, 6h, 12h, 18h, 24h, 48h, 72h, 96h, 120h and 144h following infection. The one-step growth curve was made after titration of supernatant and cell lysate for both the viruses. It was found that virus growth kinetics follow the similar pattern in all the four cell lines, however, difference in titer was noticed among the cells. The maximum titer of PPRV was obtained after 72h post-infection and in case of CDV, it was at 48h. The comparative mean titer of PPRV and CDV was more in Vero/SLAM in comparison to native Vero both in supernatants and cell lysates. Though, the comparative mean titer of PPRV was higher in Vero/SLAM cell (VGS, VSS and VDS) as compared to native Vero but, titer difference was not significant. Growth of PPR vaccine virus was also assessed at different moi (1.0, 0.1, 0.01 and 0.001) in all four cell lines. It was observed that titre of PPRV was lower than the native Vero irrespective of m.o.i. The viral antigen load was also assessed by PPR sandwich ELISA. In this assay, supernatant showed higher viral antigen in VGS, VSS, and VDS than Vero. The cell lysates of VGS, VSS, and VDS showed almost similar viral load to native Vero. The reason(s) for non-significant titer/antigen load of PPRV in the cells stably expressing SLAM gene could not be conclusively ascertained. However, before establishing the fact the replication efficacy of PPRV with other isolates and with different batches of the vaccine virus may be verified.

The one-step growth curve of CDV was also performed using Onderstepoort vaccine virus. Here supernatants and cell lysates of above mentioned time point (like PPRV) were

titrated. The maximum titre was obtained at 48h post-infection then gradually started decline. The mean titre difference with native Vero cell in supernatant was 0.55, 0.45 and 0.99 log more in VGS, VSS and VDS, respectively. Whereas cell-associated virus showed 0.83 log more titre in VDS as compare to native Vero cells. Like PPRV, CDV was also infected with different moi from 1.0, 0.1, 0.01 and 0.001 in Vero, VGS, VSS and VDS cells. At higher moi the titre of CDV in all the three cell lines was almost equal to the titre in Vero. Upon decreasing the moi the CDV titre also deceased in VGS and VSS. But in VDS, it was always higher that the native cell, even there was 1.0 log higher titre was observed in VDS than native Vero at moi 0.001. The overall comparative mean titre of both the viruses showed that CDV replication efficacy was much higher in all the cell lines and highest in VDS cell (0.99log higher in supernatant and 0.83slog higher in cell lysate) than the PPRV.

Isolation of virulent PPRV and CDV from frozen pathological samples (n=03) and blood (n=03) was tried in Vero, VGS, and VDS. Even after 3 blind passages of the virus in respective cell lines, neither CPE nor viral antigen was detected by RT-PCR. The same tissue sample was inoculated to VDS and after two blind passage, some indications of PPR virus isolation was found by RT-PCR (amplification of 351bp amplicon size) and Lateral Flow Device (LFD). No CDV could be isolate in any of the cell lines.

Current study with limited data revealed that Vero/SLAM (especially VDS) is a better host for CDV (at least at the level of entry) yielding more amount of virus than native Vero cells. Better susceptibility of VDS for CDV vaccine virus may be due to the higher affinity of the virus to SLAM molecule of homologous host species than any other receptor. On the other hand, non-susceptibility of Vero/SLAM to PPR (both vaccine and virulent) and CDV virulent virus appeared to be contrary to the previous reports. Though, the present data is not supported with fresh (for isolation) and more number of samples, which warrants further investigation in this direction. Generation of more sensitive cell line using both SLAM and nectin-4 receptor by site-specific integration strategy may increase the susceptibility of both vaccine and wild type PPR and Canine distemper viruses many fold.

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Mini Abstract

7 MINI ABSTRACT

Peste-de-petits-ruminants (PPR) and *canine distemper* are an acute, febrile, viral disease of small ruminants and carnivores, respectively. The PPR is caused by PPR virus (PPRV) and characterized by high morbidity and mortality causing great loss in small ruminants production. Canine distemper is caused by canine distemper virus (CDV) and produces multisystemic diseases with respiratory and neurological complications. Cellular receptors like SLAM/CD150 and Nectin-4 play pivotal role in viral pathogenesis. Both SLAM/CD150 and nectin-4 is identified as receptor for PPRV and CDV of their respective host species. Susceptibility of cells expressing these receptors for virus isolation and increased antigen production has been studied in Morbilliviruses. The present work was designed to develop the stable cell lines constitutively expressing SLAM and Nectin-4 of goat, sheep and dog and their susceptibility to PPRV and CDV. Full-length SLAM receptor genes were amplified from mitogen stimulated goat, sheep and dog PBMCs. The amplicons were cloned into a eukaryotic expression vector (pcDNA3.1) and sequenced. Vero cells were transfected with the recombinant vector and three stable cells Vero/goat/SLAM (VGS), Vero/sheep/SLAM (VSS) and Vero/dog/SLAM (VDS) were generated by G418 antibiotic selection pressure. Characterization of transfected cells for SLAM gene was done at genomic, transcript and protein level. The genomic integration and RNA transcript of SLAM gene in different stable cell lines (VGS, VSS and VDS) was confirmed by PCR and RT-PCR, respectively. Similarly, expression of SLAM protein was confirmed by immunofluorescence using anti-FLAG monoclonal antibody and FITC conjugate. Relative expression of SLAM in different cell lines were quantified by RT-qPCR and maximum expression was found in Vero/dog/SLAM (VDS) cells. Comparative susceptibility of Vero and Vero/SLAM cells were tested with vaccine strain of CDV and PPRV. The CDV titre was found to be 1.0 $\log_{10}$ higher in VDS than native Vero cells. The mean titre difference of CDV in VDS cell was found to be higher by 0.83 log in cell-lysate and 0.99 log in supernatant as compared to native Vero cell. The PPRV titre found to be less in all three engineered cells than native Vero cells. Isolation of PPRV and CDV was attempted in engineered cell lines with limited samples. Isolation of PPRV and CDV in VGS and VSS cell could not be succeeded. However, indication of PPRV isolation in VDS at P-1 level was noticed. In conclusion, susceptibility of PPRV and CDV revealed that Vero/SLAM is a better host for CDV (at least at the level of entry) yielding more amount of virus than native Vero cells. However, susceptibility of all the three engineered cell lines for propagation of PPR (wild-type and vaccine virus) and CDV (Wild type) could not be conclusively ascertained.

लघु सारांश

8 लघु सारांश

पेस्ट–डेस पेटीट्स रूमिनेंट्स (पी.पी.आर.) और कैनाइन डिस्टेम्पर क्रमशः छोटे रोमान्थियों तथा मांसाहारीयों की तीव्र, ज्वर, विषाणु जनित रोग है। पी.पी.आर. रोग पी.पी.आर. विषाणु के कारण होता है, जिससे उच्च विकृति और मृत्युदर के कारण छोटे रोमान्थियों के उत्पादन में भारी नुकसान होता है। कैनाइन डिस्टेम्पर रोग कैनाइन डिस्टेम्पर विषाणु के कारण होता है जो श्वसन और तंत्रिका संबंधी जटिलता के साथ बहुआयामी बिमारियों का कारक है। कोशिकाओं के रिसेप्टर जैसेकि सलैम (एस.एल.ए.एम.)। सीडी / 150 और नेक्टीन–4 विषाणु रोगजन्य में मुख्य भूमिका निभाते हैं। स्लैम / सीडी150 और नेक्टीन–4 दोनो को पी.पी.आर.वी. एवं सीडीवी के रिसेप्टर के रूप में जाना जाता है। इन दोनों रिसेप्टर का प्रयोग विषाणु के पृथकीकरण एवं कोशिकाओं की संवेदनशीलता और एंटीजन उत्पादन के वृद्धि में किया जाता है। वर्तमान कार्य को भेड़, बकरी, श्वान के सलैम (एस.एल.ए.एम.) और नेक्टीन–4 रिसेप्टर को स्थिर रूप से विरो कोशिकाओं में व्यक्त करने तथा पी.पी.आर.वी. और सी.डी.वी. की संवेदनशीलता को व्यक्त करने के लिए निर्धारित किया गया था। संपूर्ण लम्बाई स्लैम (एस.एल.ए.एम.) रिसेप्टर जीन को माइटोजन उतेजित बकरी, भेड़ व श्वान के पी.बी.एम.सी. से (एम्पलीफाइड) बढ़ाया गया था। एम्पिलीकान को यूकैरियोटिक अभिव्यक्ति वेक्टर (पी.सी.डी.एन.ए. 3.1) में क्लोन तथा अनुक्रमित किया गया था। वेरो कोशिकाओं को पुनः संयोजक वेक्टर और तीन स्थिर (स्टेबल) कोशिकाओं वेरो / बकरी / स्लैम (वीजीएस), तथा वेरो भेड़ स्लैम (वी.एस.एस.) वेरो श्वान स्लैम (वी.डी.एस.) के साथ जी 418 एन्टीबायोटिक चयन व दबाव द्वारा उत्पन्न किया गया था। स्लैम जीन (एस.एल.ए.एम.) के ट्रांसफेक्टेड कोशिकाओं का निर्धारण जीनोमिक, ट्रांसक्रिप्ट और प्रोटीन स्तर पर किया गया था। विभिन्न स्थिर कोशिका में (वी.जी.एस., वी.एस.एस. व वी.डी.एस.) में स्लैम (एस.एल.ए.एम.) जीन की जीनोमिक एकीकरण और आर.एन.ए. प्रतिलेख क्रमशः पी.सी.आर (पालीमरेज रियेक्सन) तथा आर.टी.–पी.सी.आर. द्वारा पुष्टि की गई थी। इसी प्रकार स्लैम प्रोटीन की अभिव्यक्ति को एन्टी–फ्लैग मोनोक्लोनल एन्टीबाडी और एफ.आई.टी.सी. (फैट) संयुग्मन का उपयोग करके इम्यूनोफ्लोरेसंस द्वारा पुष्टि की गई थी। विभिन्न कोशिकाओं पद्धति में स्लैम की सापेक्ष अभिव्यक्ति आर.टी.–क्यू.पी.सी.आर. द्वारा प्रमाणित किया गया, और अधिकतम अभिव्यक्ति वेरो / श्वान / स्लैम (वी.डी.एस.) कोशिकाओं में मिली थी। वेरो तथा वेरोस्लैम कोशिकाओं की तुलनात्मक संवेदनशीलता का परीक्षण सी.डी.वी. और पी.पी.आर.वी. के टीका विषाणु के साथ किया गया था। साधारण वेरो कोशिकाओं की तुलना में सी.डी.वी. टाईटर वी.डी.एस. में 1.0 $लॉग_{10}$ उच्च पाया गया था। वी.डी.एस. कोशिका में सीडीवी का औसत ट्राईटर कोशिका लाइसेट में 0.83 $लॉग_{10}$ और साधारण वेरो की तुलना में सतह पर (सूपरनैटेन्ट) में 0.99 $लॉग_{10}$ से अधिक पाया गया था। मूल वेरो कोशिकओं की तुलना में पी.पी.आर.वी. टाईटर सभी तीनों इंजीनियर कोशिकाओं में कम पाया गया। सीमित नमूनो के साथ इंजीनियर कोशिकाओं में पी.पी.आर.वी. और सी.डी.वी. का पृथकीकरण का प्रयास किया गया। वी.जी.एस. और वी.एस.एस. कोशिका में पी.पी.आर.वी. तथा सी.डी.वी. का पृथकीकरण सफल नहीं हो सका। हांलाकि पी–1 स्तर पर वी.डी.एस. में पी.पी.आर.वी. पृथकीकरण (आईसोलेशन) का संकेत देखा गया था। निष्कर्ष में पी.पी.आर.वी. और सी.डी.वी. की संवेदनशीलता से पता चला कि वेरा / स्लैम सी.डी.वी. (कम से कम कोशिका में प्रवेश के स्तर द्वार पर) के लिए एक बेहतर मेजबान है जोकि मूल वेरो कोशिकाओं की तुलना में अधिक मात्रा में विषाणु पैदा करता है। हालांकि पीपीआर (क्रूर / प्रचण्ड और टीका विषाणु) और सी.डी.वी. (क्रूर / प्रचण्ड) के उत्पादन के लिए सभी तीनो इंजीनियर कोशिकाओं की संवेदनशीलता को निश्चित रूप से निर्धारित नहीं किया जा सका।

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Appendix

APPENDIX

A. 1X Phosphate Buffer Saline (pH 7.4)

NaCl	4.0 gm
KCL	100 mg
Na_2HPO_4	710 mg
KH_2PO_4	100 mg

Distilled water up to 500 ml

B. DEPC Treated Water

To ensure free from RNAse contamination, water used to dissolve RNA (or prepare buffers required for RNA works) was treated with 0.1% Diethyl pyrocarbonate (DEPC).

DEPC (M/s Sigma)	1.00 ml
Distilled water	1000 ml

The DEPC was mixed vigorously and left overnight at 37°C. To remove DEPC, the water was autoclaved at 120ºC at 15lb pressure for 10 minutes before use.

C. Reagents used in agarose gel electrophoresis

1. Tris-acetate-EDTA (TAE) buffer 50X

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

Distilled water was added to make up to a final volume of 1000 ml. A working solution of 1X was used.

2. Ethidium bromide stock solution (10 mg/ml)

Ethidium bromide	100 mg
Distilled water	10 ml

The solution was mixed and stored at 4°C. A final concentration of 0.5µg/ml was used in preparing agarose gel.

3. Loading dye (6x)

Bromophenol blue	0.25% (w/v)
Xylene cyanol FF	0.25% (w/v)
Sucrose	40% (w/v)

Store at -20°C.

D. Reagents used for bacteriological work

1. LB (Luria-Bertani) broth

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Deionized water	950 ml

Adjust the pH to 7.0 with 5N NaOH. Volume was made up to 1L and sterilized by autoclaving at 121° C and 15 lb pressure for 20 minutes and the solution was stored at 4°C

2. LB agar

3.5% (w/v) LB agar (Himedia, Mumbai, India) in deionized water

Sterilized by autoclaving at 121° C and 15 lb pressure for 20 minutes and the solution was stored at 4°C

3. Ampicillin stock (100 mg/ml)

Ampicillin	500 mg
Distilled water	5 ml

Sterilized by filtration through 0.22 ì filter and stored at -20°C.

4. 100mM $CaCl_2.2H_2O$ (100 mL)

$CaCl_2.2H_2O$	1.47 gm
Milli Q water upto	100 mL

Mix the contents properly and filter sterilizes by 0.22 µm membrane filter and store at 4°C.

5. TSS solution (Store at 4°C)

2X LB medium	20 ml
30% w/v PEG containing 0.6 ml of 2M Mg^{2+} solution	20ml
DMSO	3.0 ml
Sterile double distilled water	17 ml

Sterile through 0.22µ filter

6. SOB medium (per100 ml)

Tryptone	2.0 g
Yeast Extract	5.0 g
NaCl	0.05 g
250 mM KCl	1.0 ml
Double distilled water	90.0 ml

Adjusted pH to 7.0 with 5N NaOH. Volume made upto 100 ml and sterilized by autoclaving for 20 min at 15 psi on liquid cycle and stored at 4°C.

E. Reagents used in cell culture

1. EMEM (1 Liter)

EMEM powder (Sigma)	9.4 gm
$NaHCO_3$ (7.5%)	15 ml
Glutamine (3.0%)	10 ml
Antibiotic Antimycotic Solution (100X)	1 ml

The EMEM powder was dissolved in 1 liter of distilled water (DW) and autoclaved at 121°C at 15 lb pressure for 15 minutes. 10 ml of Glutamine (3%), 15 ml of $NaHCO_3$ (7.5%) and 1 ml of antibiotic and antimycotic solutions (100X) were added per liter of media and incubated at 37°C overnight before use.

2. **Growth media (10% serum)**

EMEM	90 ml
Foetal calf serum	10 ml

3. **Maintenance media (2% serum)**

EMEM	98 ml
Foetal calf serum	2 ml

4. **Trypsin Versene solution (0.25%)**

NaCl	10 gm
KCl	0.250 gm
Na_2HPO_4	1.9 gm
KH_2PO_4	0.250 gm
Trypsin	2.5 gm
Versene (EDTA)	1.4 gm
0.4% Phenol red	1.0 µl

Distilled water was added to make the volume up to 1000 ml.

5. **Sodium bicarbonate stock (Stock D)**

7.5% (w/v) solution was prepared in distilled water and sterilized by filtration through 0.2 µm membrane filter. Kept at 4°C. One liter of media requires up to 15 ml of this solution.

6. **L-Glutamine stock**

3% (w/v) solution was prepared in distilled water and sterilized by filtration through 0.2 µm membrane filter. Aliquots were made and stored at –20°C. Just before use it was warmed it 37°C for few minutes only till a clear solution is visible. Keeping glutamine at 37°C for longer time reduces its activity. One liter of media requires 10 ml of glutamine.

7. **Sodium pyruvate (100mM)**

10mL of Sodium pyruvate solution (100x) in 1liter of EMEM

8. **1 molar HEPES [(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)] 100mL**

23.8 gm dissolved in 100mL of distilled water and autoclaved at 121ºC at 15lb pressure for 15 min.

9. **Neomycin sulphate (G418) (50mL)**

G418	703mg
100mM HEPES	1192mg

Dissolved in 50mL of EMEM

Adjust the pH 7.4-7.5 and filtered through 0.22µ filter

10. **Antibiotic and antimycotic solution (100x)**

10mL of Antibiotic and antimycotic solution (100x) in 1liter of EMEM

11. **Opto-MEM media for transfection**

Opti-MEM (M/s Invitrogen, Cat no#31985062) used for transfection in Vero cells.

F. Reagents for Immunocytochemistry

1. 80% Acetone (10mL)

8mL acetone mix with 2mL of PBS

2. 100mM Glycine (10mL)

75mg glycine dissolved in 10 mL of PBS

3. Permeabilization buffer (20mL)

Bovine serum albumin (0.5%)	100mg
Tween-20 (0.5%)	100µL

Dissolved in 20 mL of PBS

4. Blocking (20mL)

Bovine serum albumin (3%)	600mg
Tween-20	40 µL

Dissolved in 20 mL of PBS

G. Reagents for sandwich ELISA

1. Coating buffer-Phosphate buffer saline (PBS)

137 mM NaCl

2.7 mM KCl

10 mM Na_2HPO_4

2 mM KH_2PO_4

The contents were dissolved in 1litre of distilled water. The pH was adjusted to 7.4, autoclaved and stored at room temperature.

2. Blocking buffer

PPR negative serum (0.5% v/v)	500 µl
Tween-20(0.1%)	100µl
Phosphate buffer saline (pH 7.4)	upto 100 ml

3. Substrate buffer (phosphate-citrate buffer)

0.0347M Citric acid

0.0667M $Na_2HPO_4.12H_2O$

pH was adjusted to 4.0 and stored at 4°C.

4. Substrate Solution for ELISA

1 OPD tablet	30.0mg
(M/s Sigma-Aldrich, St. Louis, USA)	
Citrate buffer	75ml

The OPD solution was distributed in 10 ml aliquots in an amber/brown colored tubes and stored at -20ºC. Half an hour before use, the OPD solution was thawed at room temperature or in water bath and 3% (4il/ml solution) H_2O_2 was added.

5. Stopping Solution (1M H_2SO_4) for ELISA

Distilled water	945ml
Concentrated H_2SO_4	55ml

Concentrated H_2SO_4 was added to distilled water slowly. Stop solution was kept in an amber colored air tight bottle for further use at room temperature.

VITAE

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2. M.V.Sc	ICAR-IVRI, Izatnagar/Mukteswar	2014	87.67%
3. Ph.D.	ICAR-IVRI, Izatnagar	2019	87.31%

Fellowships/Awards:

- ICAR-National Talent Scholarship (NTS) during B.V.Sc. & A.H. programe
- ICAR- JRF during M.V.Sc programe
- ICMR-SRF during PhD programe
- Registered in "Uttar Pradesh Veterinary Council" (UPVC)
- Registered in "Veterinary Council of India" (VCI)
- Life member of "Society for Biosafety".
- Life member of "Indian ASSOCIATION of Veterinary Microbiologists, Immunologists and Specialists in Infectious Diseases" (IAVMI)
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- Life member of "Indian Science Congress".

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