PATHOLOGY OF ENTERIC AND CENTRAL NERVOUS SYSTEM AFFECTIONS IN PIGS CAUSED BY PORCINE ENTERIC PICORNAVIRUSES

Thesis

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

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Roll No. 1583

FOR THE DEGREE OF

Doctor of Philosophy
(Veterinary Pathology)

2016

Dedicated to....

My beloved family

&

Veterinary Fraternity

भारतीय पशु चिकित्सा अनुसंधान संस्थान
(सम विश्वविद्यालय)
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Dated: 9/2/ **2016**

Certificate

This is to be certified that the research work embodied in this thesis entitled "Pathology of enteric and central nervous system affections in pigs caused by porcine enteric picornaviruses" *submitted by* **Dr. Pradeep Kumar Ray, Roll No. 1583,** *for the award of* **Doctor of Philosophy** *Degree in* **Veterinary Pathology** *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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Chairman
Advisory Committee

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We have gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented for the award of **Doctor of Philosophy** *Degree of this Institute.*

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Pradeep Kumar Ray

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(PRADEEP KUMAR RAY)

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ABBREVIATIONS

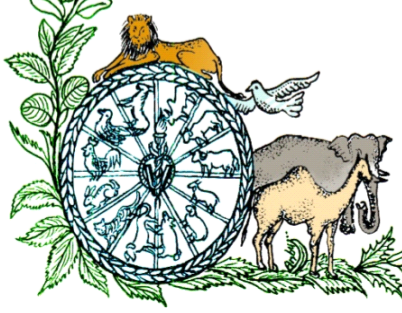
%	:	Per cent
/	:	Per
°C	:	Degree Celsius
3'UTR	:	3' UnTranslated Region
5'UTR	:	5' UnTranslated Region
APTES	:	3'-Aminopropyl Triethoxysilane
BHQ	:	Black Hole Quencher
Bp	:	Base Pair
cDNA	:	Complementary Deoxyribonucleic Acid
CHL	:	Central Histopathology Lab
CNS	:	Central Nervous System
Cp	:	Cytopathogenic
CPE	:	Cytopathic Effects
CS	:	Calf Serum
CSFV	:	Classical Swine Fever Virus
Ct	:	Threshold Cycle
DEPC	:	Diethyl Pyrocarbonate
DIG	:	digoxigenin
DMEM	:	Dulbecco's Minimum Essential Medium
DNA	:	Deoxyribonucleic Acid
dNTP	:	Deoxyribonucleoside triphosphates
DPI	:	Days post infection
DW	:	Distilled water
EVG	:	Enterovirus G
FAM	:	6-carboxyfluorescein
FAT	:	Fluorescent Antibody Technique
FBS	:	Foetal bovine Serum

FISH	:	Fluorescent in sity hybridization
gm	:	Gram
Hrs	:	Hours
IHC	:	Immunohistochemistry
IPTG	:	Isopropyl beta-D -Thiogalactopyranoside
ISH	:	*In Situ* Hybridization
IVRI	:	Indian Veterinary Research Institute
Kb	:	Kilobase pair
KDa	:	kilo Dalton
LB broth	:	Luria - Bertari
LN	:	Lymph node
M	:	Molar
MAb	:	Monoclonal antibody
Mg	:	Milligram
Min	:	Minutes
mL	:	Milliliter
Ml	:	Millilitre
mM	:	Millimolar
MNCs	:	Mononuclear cells
MSC	:	Mesenchymal stem cell
NCBI	:	National Cerntre for Biotechnology Information
ncp	:	non- cytopathogenic
NCSFRL	:	National Classical Swine Fever Referral Laboratory
Ng	:	Nanogram
nm	:	Nanometre
NS	:	Non Structural
NTC	:	No Template Control
ORF	:	Open Reading Frame
PBS	:	Phosphate Buffered Saline

PCR	:	Polymerase Chain Reaction
PK15	:	Porcine Kidney 15
PTV	:	Porcine Teschovirus
PSV	:	Porcine Sapelovirus
RNA	:	Ribo nucleic acid
RPM	:	Rotations per minute
rRT-PCR	:	Real-Time Reverse Transcriptase Polymerase Chain Reaction
RT-PCR	:	Reverse Transcriptase Polymerase Chain Reaction
Sec	:	Seconds
Spp	:	Species
TBE	:	Tris borate EDTA
Temp	:	Temperature
UV	:	Ultra Violet
Mg	:	Microgram
µl	:	Microlitre

CONTENTS

Introduction



1 INTRODUCTION

India has a population of about 11.13 million pigs (India, 2012). Raising pigs is particularly important for the livelihood of poorest of the poor and marginal sections of the society in India. Crop farming alone cannot meet the needs, many rely on livestock–mostly pigs, to meet the demands of nutrition and livelihood. The pig has one of the highest feed conversion efficiency and they are prolific breeders which makes pig rearing a profitable farming practice. However, the profitability of pig farming can be reduced to drastic low levels due to incidence of various diseases caused by various pathogens such as viruses, bacteria and fungi. The wide range of pathogens which cause enteric and neurological disorders of the pig provide a special challenge to the skills of the clinicians and pathologists. Enteric Picornaviruses are one such kind of pathogens which cause diarrhoea and nervous disorders along with many other diseases in swine causing considerable losses to the farmers. Enteroviruses are important pathogens of human beings also and produce illness manifested by nervous symptoms (Kennedy, 2004; Pallansch and Roos, 2006).

The family Picornaviridae is comprised of small, icosahedral, non-enveloped viruses with a single positive strand RNA genome. The family Picornaviridae belongs to the order Picornavirales and currently consists of 50 species grouped into 29 genera.

The porcine enteric picornaviruses, were previously classified within the genus Enterovirus. The original 11 porcine enterovirus (PEV) serotypes, PEV-1 to PEV-11, were placed in three groups – I, II and III – on the basis of cytopathic effect (CPE) in cell culture, serological assays and replication in different cell cultures (Knowles *et al.,* 1979). Now porcine

enterovirus have been reclassified based on their genome sequence data into three genera: porcine teschovirus (previously CPE type I), with 13 distinct serotypes, Enterovirus- G (Enterovirus G1-G11 and formerly classified as CPE type III and Porcine Enterovirus-B) and porcine sapelovirus (formerly classified as CPE type II and Porcine Enterovirus A) (Zell *et al.*, 2001; Krumbholz *et al.,* 2002). The species Porcine sapelovirus consists of a single serotype, PSV (formerly PEV 8).

The first evidence of porcine enterovirus/teschovirus infection to be reported was the occurrence of Teschen disease with high mortality, in Czechoslovakia over 80 years ago (Trefny, 1930). The disease was identified for the first time by Professor Antonin Klobouk as a virosis initially called "*encephalomyelitis enzootica suum non purulenta*" (Kouba, 2009).

In recent times, Enterovirus encephamomyelitis in pigs caused by porcine Teschovirus has been described in Indiana, USA (Pogranichniy *et. al* 2003), Japan (Yamada *et al.*, 2004), Canada (Salles *et. al.,* 2011), Haiti (Deng *et al.,* 2012), Taiwan (Chiu *et al,* 2012). Prevalence of porcine Teschovirus has been confirmed in China (Feng *et al.* 2007, Wang *et al.,* 2010 and Zhang *et al*. 2010) and Czech Republic (Prodelalova *et al,*. 2009). Concurrent Polioencephalomyelitis due to *Porcine Teschovirus* and Postweaning Multisystemic Wasting Syndrome (PMWS) associated with PCV2 in a piglet was recorded in Japan (Takahashi *et. al*., 2008). Antibodies against porcine Teschovirus have been detected in Lithuania (Sereika *et al.*, 2007).

Porcine enteric picornaviruses are common swine pathogens which cause a wide range of illnesses in swine ranging from asymptomatic infection to acute fatal encephalomyelitis (Trefny, 1930; Harding *et al.*, 1957; Yamada et al. 2004), reproductive disorders (Dunne *et al.*, 1965), diarrhea (Izawa, 1962; Honda *et al.,* 1990), pneumonia (Meyer *et al.*, 1966) and dermal lesions (Knowles, 2006). The clinical signs observed in teschovirus encephalomyelitis are anorexia, lassitude, fever up to 41.5°C and locomotor disturbances, hypersensitivity, tremors, clonic spasms of the legs, flaccid paralysis, opisthotonos and nystagmus. Convulsions may be observed in young pigs. In the final clinical stage, paralysis proceeding from the hind part through the loins to the fore part of the body is observed (OIE, 2009).

Cytopathogenic porcine enteroviruses have been isolated from pigs with clinical signs of diarrhea (Kresse *et al.,* 1977). Clinical signs observed in pigs from such herds are diarrhea, debilitation and gauntness. Porcine enteroviruses have been isolated from healthy as well as diarrhoeal domestic pigs in Japan but PEVs with type II cytopathic effect (CPE) have a closer association with enteric pathogenicity (Honda *et. al*, 1990). Porcine Teschovirus 8 has been isolated from samples of organs of pigs showing signs of acute diarrhea, respiratory distress, and death on a swine farm in Jilin Province, northern China (Zhang *et. al,* 2010).

Another enteric picornavirus, the swine vesicular disease (SVD) virus is the causative agent of a highly contagious disease of pigs which was first recorded in Italy in October 1966 (Nardelli *et al*., 1968). Subsequent outbreaks have been reported in Hong Kong, Japan and Europe (Knowles and Sellers, 1994; Brocchi *et al*., 1997). SVD is characterized by vesicles on the coronary bands, heels of the feet and occasionally on the lips, tongue, snout and teats. It is clinically indistinguishable from Foot-and-Mouth Disease, where lies its main importance in livestock farming. SVD is antigenically closely related to the human pathogen coxsackievirus B5 (Graves, 1973; Brown *et al*., 1973) and has been classified along with human enteroviruses as species Enterovirus B (ICTV, 2012). SVD and Coxsackie B5 virus can be differentiated by neutralization and immunodiffusion tests. Using these tests, it has been shown that the Coxsackie-like illnesses may be caused by the swine virus in human beings especially to the personnel engaged on experiments with SVD (Brown *et al.,* 1976).

Enterovirus encephamomyelitis can be diagnosed by virus isolation, histopathology, RT-PCR and by immunohistochemistry. Microscopically, lesions are confined primarily to the brain stem and spinal cord (Pogranichniy *et al.,* 2003, Yamada *et al.*, 2004, Salles *et al.,* 2011). Lesions are characterized by multifocal gliosis and perivascular cuffs composed of lymphocytes and macrophages, plasma cells, and rare eosinophils; satellitosis, chromatolysis, neuronal necrosis, and neuronophagia. In the spinal cord, lesions are bilateral, asymmetric, and most severe in the ventral horns. Infrequently, cuffs and gliotic foci are seen in the white matter along with occasional small foci of axonal degeneration and phagocytic vacuoles (Yamada *et al.*, 2004, Salles *et al.,* 2011). Lesions are more common in the cerebellum than the cerebrum and typically involve the molecular layer. Meninges overlying the lesions may be

infiltrated by macrophages and lymphocytes. Spinal ganglia may show infiltration of macrophages and lymphocytes around neurons.

For identification of Porcine enteric Picornaviruses reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time RT-PCR have been developed (Zell *et al.*, 2000, Palmquist *et al.,* 2002, Jimenez-Clavero *et al.*, 2003, Krumbholz *et al.*, 2003, La Rosa *et al.*, 2006, Zhang *et al.*, 2013) .

The porcine Teschovirus antigen has been demonstrated by immune-histochemical methods in the nerve cells of the brain stem and in the spinal cord and ganglion cells of the spinal ganglion from infected pigs (Yamada *et al.*, 2007, Salles *et al.,* 2011). In the nervous system, PTV antigens have been found in the cytoplasm of neuronal cells and glial cells distributed in the spinal ventral horn and brain stem, and also in the cytoplasm of ganglion cells in the spinal ganglion (Yamada *et al.*, 2008). Porcine Teschovirus antigens has been demonstrated in visceral and lymphoid organs of non-suppurative encephalitic pigs in the endemic field situation indicating long term viremia (Chiu *et al*. 2014). *In-situ* hybridization (ISH) along with quantitative real time PCR has been used for study of pathogenesis of PTV to confirm the fecal-oral mode of pathogenesis but it has been suggested that the intranasal infection and retrograde ascending infection from the tonsils are also important in pathogenesis. The high viral load in regional lymph nodes and the presence of PTV in the dark zone of the germinal center and adjacent Para-cortex of regional lymph nodes suggest that when the immune system is pre-activated by the commonly-observed multi-infection in the field, PTVs may synergize in causing disease.

Enteroviruses are important pathogens for human beings also. The clinical manifestations of Enteroviruses (EVs) in human beings range from conjunctivitis, respiratory tract infection, myocarditis, meningitis, encephalitis, and neonatal sepsis like illness (Cherry, 1998., Pallansch and Roos, 2006). Encephalitis is a rare presentation of EV infection, but many EV serotypes (Coxsackievirus A9, A10, and B5, echovirus 4, 5, 9, 11, 19, and 30, and EV 71, 75, 76 and 89) have been reported in encephalitis cases from different parts of the world including India (Dalwai *et. al,* 2009; Kumar *et al.,* 2011; Lewthwaite *et al.,* 2010; Lin *et al.*, 2003; Sapkal, *et al.*, 2009). Encephalitis is a significant cause of morbidity and mortality in children each year

in Uttar Pradesh. EV was the main etiologic agent (22.1%) in an epidemiological investigation of 204 clinical cases of encephalitis in children in UP, India (Kumar *et al.* 2012). Seasonal outbreaks of acute encephalitis syndrome (AES) occur with striking regularity in India and lead to substantial mortality. Several viruses, endemic in many parts of India, account for AES. Although Japanese encephalitis virus (JEV) is a key aetiological agent for AES in India, many recent studies suggest that enteroviruses also account for outbreaks of AES (Joshi *et al.*, 2012). Muzaffarpur and adjoining districts in Bihar, suffer repeated epidemics of acute encephalopathy in children for the past 16-17 years. An outbreak of this mystery disease, with high case fatality (63.3%) was reported in children from Muzaffarpur district, Bihar, in June 2011 (Sahni, 2012)**.**

Porcine enteric picornaviruses are ubiquitous in distribution in swine species. A wide range of illnesses associated with these viruses such as mild to severe encephalomyelitis, diarrhoea, respiratory distress, reproductive disorders and dermal lesions in swine has been reported all over the world. Diseases with such symptoms are seen frequently in Indian pig population. But there is complete lack of information about Porcine enteric picornaviruses viruses in Indian pig population, their association with diseases and other characteristics. At the same time, enteroviruses including coxsackievirus B5 have been widely reported in various regions of India with their association with AES in children. This viewed with the fact of close antigenic relationship of coxsackievirus B5 and swine vesicular disease virus demands an exploration of enteroviruses in Indian swine population. So a thorough investigation is necessary to study the prevalence of porcine enteric picornaviruses in local pig herds and their association with various illnesses in swine. This study was planned with the following objectives:

Objectives

1. **To study the prevalence of enteric Picornavirus infections in pigs.**
2. **To study the pathology of enteric and central nervous system affections caused by enteric Picornaviruses in pigs.**
3. **Molecular characterization of prevalent enteric Picornaviruses.**

Review of Literature

2 REVIEW OF LITERATURE

2.1 Classification of the Picornaviruses

Picornaviruses are classified within the order *Picornavirales* which also includes families *Dicistroviridae, Marnaviridae, Iflavirus* and *Secoviridae* (Sanfaçon *et al.*, 2011). The order consists of a diverse range of viruses with a single stranded positive sense RNA genome, grouped together on the basis of capsid structure and viral life cycle similarities. *Picornaviridae* is an extremely large and genetically diverse virus family, which contains many human and veterinary pathogens of great clinical importance, including poliovirus (PV) and foot and mouth disease virus (FMDV). The family *Picornaviridae* currently consists of 50 species grouped into 29 genera (Knowles *et al.,* 2012; Adams *et al.*, 2013) which cause clinical disease in a wide range of human and animal hosts including pigs.

2.2 Porcine enteric Picornaviruses

Due to the physicochemical properties of their virions, porcine enteric Picornaviruses were previously classified as enteroviruses. Based on parameters such as (i) cytopathic effect (CPE), (ii) replication properties in various host cell lines, and (iii) serological assays, the Porcine Enterovirusess were divided into three CPE groups: I (serotypes 1 to 7 and 11 to 13), II (serotype 8), and III (serotypes 9 and 10) (Zoletto, 1965; Knowles *et al.*, 1979).

The porcine enteric picornaviruses, previously classified within the genus Enterovirus are now reclassified based on their genomic sequence data into three genera: Teschovirus, with 13 serotypes, porcine sapelovirus (formerly classified with the Enterovirus species PEV

A) and Enterovirus G (Zell *et al.*, 2001; Krumbholz *et al.*, 2002; Oberste *et al.*, 2003, Knowles *et al.,* 2012).

Classification of Family Picornaviridae (Adapted from the Picornaviridae database http://www.picornaviridae.com/ November 2014).

Genus	Species	Serotypes
Avihepatovirus	***Duck hepatitis A virus***	1
Avisivirus	***Avisivirus A***	1
Cosavirus	*Cosavirus A*	1
Dicipivirus	*Cadicivirus A*	1
Enterovirus	*Enterovirus A*	25(sero)types: coxsackievirus A2 (CV-A2), CV-A3, CV-A4, CV-A5, CV-A6, CV-A7, CV-A8, CV-A10, CV-A12, CV-A14, CV-A16, enterovirus A71 (EV-A71), EV-A76, EV-A89, EV-A90, EV-A91, EV-A92, EV-A114, EV-A119, EV-A120, EV-A121 and the simian enteroviruses SV19, SV43, SV46 and baboon enterovirus A13 (BA13).
	Enterovirus B	61 (sero)types: coxsackievirus B1 (CV-B1), CV-B2, CV-B3, CV-B4, **CV-B5 (incl. swine vesicular disease virus [SVDV])**, CV-B6, CV-A9, echovirus 1 (E-1; incl. E-8), E-2, E-3, E-4, E-5, E-6, E-7, E-9 (incl. CV-A23), E-11, E-12, E-13,E-14, E-15, E-16, E-17, E-18, E-19, E-20, E-21, E-24, E-25, E-26, E-27, E-29, E-30, E-31, E-32, E-33, enterovirus B69 (EV-B69), EV-B73, EV-B74, EV-B75, EV-B77, EV-B78, EV-B79, EV-B80, EV-B81, EV-B82,EV-B83, EV-B84, EV-B85, EV-B86, EV-B87, EV-B88, EV-B93, EV-B97, EV-B98, EV-B100, EV-B101, EV-B106, EV-B107, EV-B110, EV-B111 and the simian enterovirus SA5.
	Enterovirus C	23 (sero)types: poliovirus (PV) 1, PV-2, PV-3, CV-A1, CV-A11, CV-A13, CV-A17, CV-A19, CV-A20, CV-A21, CV-A22, CV-A24, EV-C95, EV-C96, EV-C99, EV-C102, EV-C104, EV-C105, EV-C109, EV-C113, EV-C116, EV-C117 and EV-C118.
	Enterovirus D	Five (sero)types, EV-D68, EV-D70, EV-D94, EV-D111 and EV-D120
	Enterovirus E (group A *Bovine enterovirus)*	Four types: EV-E1 to EV-E4
	Enterovirus F (group B bovine enteroviruses)	Six types :EV-F1 to EV-F6.

Genus	Species	Serotypes
	*Enterovirus G (*formerly named Porcine *enterovirus* B).	Eleven (sero)types: EV-G1 to Ev-G11
	Enterovirus H (formerly name *Simian enterovirus A*)	Single serotype EV-H1
	Enterovirus J	Six simian enterovirus types SV6, EV-J103, EV-J108, EV-J112, EV-J115 and EV-J121.
	Rhinovirus A (formerly named Human r hinovirus A)	80 serotypes
	Rhinovirus B (formerly named *Human rhinovirus B*)	32 sero types
	Rhinovirus C Rhinovirus	C (formerly named Human rhinovirus C) 5 4 serotypes
Aquamavirus	*Aquamavirus A*	One type: aquamavirus A1
Cardiovirus	*Theilovirus*	12 types: Theiler's murine encephalomyelitis virus (TMEV), Vilyuisk human encephalomyelitis virus (VHEV), Thera virus (TRV), Saffold virus (SAFV) 1-9
	Encephalomyocarditis virus	1 type: encephalomyocarditis virus (EMCV).
Aphthovirus	*Equine rhinitis A virus*	1 type: equine rhinitis A virus (ERAV)
	Bovine rhinitis A virus	BRAV 1 & 2
	Bovine rhinitis B virus	1 type: bovine rhinitis B virus (BRBV)
	Foot-and-mouth disease virus	7 types: O, A, C, Southern African Territories (SAT) 1, SAT 2, SAT 3 and Asia 1
Hepatovirus	*Hepatitis A virus*	1 type: Hepatitis A virus (HAV)
Parechovirus	*Human parechovirus*	16 types:Human parechovirus (HPeV) 1-16
	Ljungan virus	4 types: Ljungan virus (LV) 1-4
Erbovirus	*Equine rhinitis B virus*	3 types: equine rhinitis B virus (ERBV) 1-3
Kobuvirus	*Aichivirus A (formerly Aichi virus)*	Five viruses (genotypes) Aichi virus 1 (found in humans), canine kobuvirus 1, murine kobuvirus 1, feline kobuvirus 1 and a kobuvirus found in sewage
	Aichivirus B(formerly *Bovine kobuvirus*)	Two types bovine kobuvirus 1 (BKV-1) (Yamashita *et al.*, 2003) and sheep kobuvirus 1 (SKV) (Reuter *et al.*, 2010).
	Aichivirus C (aka porcine kobuvirus).	Porcine kobuvirus 1 (PKV-1)
Passerivirus	*Passerivirus A*	1
Pasivirus	*Pasivirus A.*	1
Rosavirus A	*Rosavirus A*	1
Salivirus A	*Salivirus A*	1

Genus	Species	Serotypes
Gallivirus	*Gallivirus A*	1
Hunnivirus	*Hunnivirus A*	1
Mischivirus	*Mischivirus A.*	1
Megrivirus	*Melegrivirus A.*	1
Mosavirus	*Mosavirus A*	1
Oscivirus	*Oscivirus A*	1
Sapelovirus	*Porcine sapelovirus*	1 type: Porcine sapelovirus (PSV) (formerly PEV-8)
	Simian sapelovirus	3 types: Simian sapleovirus (SSV) 1-3
	Avian sapelovirus	1 type: Avian sapelovirus (ASV)
Senecavirus	*Seneca Valley virus.*	1
Tremovirus	*Avian encephalomyelitis virus*	Three AEV strains
Teschovirus	*Porcine teschovirus*	13 (sero)types, Porcine teschovirus (PTV) 1 to 13

2.3 Picornavirus virions structure

The Picornavirus has a non-enveloped capsid of about 30 nm diameter with icosahedral symmetry. The capsid is composed of 60 copies of four non-identical proteins (VP1, VP2, VP3, and VP4), arranged in sixty repeating protomeric units of an icosahedron. They are non-enveloped and therefore their infectivity is not affected by inorganic solvents. Despite the genetic heterogeneity observed with the capsid coding sequences of picornaviruses, the basic structure of the capsid is conserved throughout the family (Racaniello, 2007).The capsid proteins VP1 to VP3 are each folded into eight-stranded antiparallel β-sheets with a jelly-roll topology. These β-barrels of five copies of VP1 are located around the fivefold axis, while VP2 and VP3 are around the threefold axis. Crystallographic studies have shown that VP4 lies buried in close association with the RNA core, whereas VP1, VP2 and VP3 are exposed at the virion surface (Smith and Baker, 1999). The N terminus of VP2 has been regarded as being in the interior of the mature virion, close to VP4, since VP2 and VP4 are generated from their precursor VP0 by maturation cleavage (VP0 to VP2+VP4). Due to its internal location, this region has seemed to be free from the pressure of neutralizing antibodies and to have evolved independently of the neutralizing type (Kaku *et al.*, 2001).

One of the most striking features of Picornavirus surface is the circular canyon around the fivefold axis, which has been first seen in human rhinovirus 14 (Rossmann *et al.* 1985). The

picornavirus family has a deep depression or canyon‘ (12-15 Å depending of the serotype) on the capsid surface structure, running around each fivefold vertex. In the inner side of the canyon there are conserved amino acid residues involved in the attachment to cell receptors (Rossmann *et al.,* 1985). This site is protected from the immune surveillance by the inability of neutralizing antibodies to penetrate into the canyon (due to their larger size), but can still be accessed by cell surface receptors. The canyon is located roughly between VP1 on the ‘north’ side (the side closer to the fivefold axis), and VP2/VP3 on the ‘`south’ side. This organization leaves five copies of VP1 as a protrusion at the fivefold axis. All known enterovirus and rhinovirus structures contain a hydrophobic pocket at the base of the canyon, covered by loops of VP1 (Rossmann *et al.*, 2002). This space is assumed to be normally filled by a natural pocket factor, a fatty acid with an aliphatic chain of variable length. These pocket factors, including sphingosine (PV1, PV3), palmitate (CVB3), and myristic acid (BEV), are believed to be inserted into the virion upon release from the infected cell. Binding of the receptor into the canyon probably competes with the binding of the pocket factor into the hydrophobic pocket. Release of the pocket factor destabilizes the virus and thereby initiates uncoating. Replacement of the pocket factor by antiviral compounds can inhibit both attachment and uncoating (Salvati *et al.,* 2004).

2.4 Picornavirus genome

Picornaviruses have single-stranded positive sense RNA genomes of approximately 7,000-8,500 nucleotides with similar but not identical organizations across the family (Racaniello, 2007). The 52 end of the genome is linked to a small peptide (VPg), and the 32 end terminates with a poly(A) tract. There is a single open reading frame flanked by untranslated regions (UTRs) at both ends. The 52 UTR is especially long (~600-1,200 nts) and contains a number of important replication and translation control elements, including an internal ribosome entry site that is directly involved in the initiation of protein translation.

The picornavirus genome consists of a single open reading frame translated as a single polyprotein. However, the complete polyprotein is cleaved co-translationally and so is never evident in the cell (Racaniello, 2007). Unlike mammalian RNA, picornavirus RNA lacks a methylated 5’cap structure and initiation of translation at the ribosome is carried out by the

IRES within the 5'UTR. The translated polyprotein is divided into capsid and non-structural regions, known as P1, P2 and P3 (Rueckert and Wimmer, 1984). These regions encode four structural (VP1 to VP4) and seven non-structural proteins (2A to 2C and 3A to 3D), which are cleaved by virus-encoded proteinases in a cascade of proteolytic processing events.

The four structural proteins are assembled to constitute the icosahedral capsid, where the neutralizing epitopes are located. The structural proteins are located within the N terminal one third of the polyprotein, while the remainder includes proteins involved in modifying the cellular environment to optimize virus replication and the proteins directly responsible for replication. In the majority of picornaviruses, the N terminus of the structural precursor protein (P1) is modified by the covalent addition of a myristic acid residue, which is thought to have important roles both in particle assembly and in the cell entry process. P1 is typically about 90 kD and is further proteolytically processed into the mature viral proteins (VP1–4 or P1 A–D) found in the viral capsid. VP1–3 together form the icosohedral shell of the virion, while VP4 is distributed on the inner surface of the particle (Tuthill *et al.,* 2010).

Nonstructural proteins are associated with membranous replication complexes and play an essential role in virus replication (Teterina *et al.,* 2006). Proteins 2A and 3C are proteases, 3D is the RNA-dependent RNA polymerase, 2C is a helicase used during RNA encapsidation. Proteins 2B and 3A have been associated with various functions in the replication of viral RNA. Protein 3B (VPg) is covalently linked to the 5'UTR region of viral genome and acts as a primer for RNA chain initiation and full length RNA synthesis (Pathak *et al.,* 2008).

An important property of RNA viruses' genome is represented by its high frequency mutation and recombination rates. RNA replication is extremely error-prone, due to the lack of proofreading activity of the viral RNA-dependent RNA polymerase, taking the error rate to approximately one per genome replication (Bouslama *et al.,* 2007). Consequently, enteroviruses exist as a dynamic mutant population termed quasispecies, near to a mutational meltdown or error catastrophe (Koonin *et. al.,* 2008). This high variability within RNA virus families acts like an evolutionary force (Domingo *et al.,* 2006). In addition, an intratypic and intertypic form of homologous recombination between quasi-species ensures adaptability in the environment,

contributing to viral pathogenesis and to the continuous emerging of new viruses (Arbiza *et al.,* 2010).

Phylogenetic analysis of RdRp/VP2 and analysis of the predicted RNA secondary structure of the 3-NTR suggested that PEVs should be reclassified genetically into at least three groups, one that should be assigned to PTVs and two PEV subspecies represented by strain PEV-8 V13 and strain PEV-9 UKG410/73 (Kaku *et al.,* 2001). The 3'-NTR was 62 or 63 nucleotide (nt) in the Talfan group, 79 nt in the PEV-8/V13 group and 69 or 72 nt in the PEV-9/UKG410/73 group. The predicted RNA secondary structures varied between groups; a single loop was observed in the Talfan group, four loops in the PEV-8/V13 group and two loops in the PEV-9/UKG410/73 group.

Genome organization of the Porcine Enterovirus group III (i.e., PEV-9 and -10) corresponds to the typical enterovirus or rhinovirus genome, while PEV group II (PEV-8) differs from the enteroviruses by two deviant features: the polyprotein is preceded by a leader protein, and the 2A protein has an unusual length of about 226 amino acids. PEV group III viruses are typical enteroviruses. They differ from other enteroviruses by a prolonged stem-loop D of the 5'cloverleaf structure (Krumbholz *et al.,* 2002).

2.5 Occurrence

2.5.1 Porcine enteric Picornaviruses

Teschen disease (previously also known as Klobouk's disease), actually called *Teschovirus encephalomyelitis*, is a virulent fatal viral disease of swine, characterized by severe neurological disorders of encephalomyelitis. Since 1996 disease was reported to the OIE by many countries such as Belarus (1996, 1999 and 2005), Japan (2002), Latvia (1997 and 2000–2002), Madagascar (1996–2000, 2002 and 2004–2005), Moldavia (2002–2004), Romania (2002), Russia (2004), Uganda (2001) and Ukraine (1996–2005). In many of these cases it is not known whether diagnosis was made only on clinical grounds or with aid of laboratory tests (OIE, 2009).

Teschen disease was initially discovered in the Teschen district of North-Eastern Moravia. During the 1940s and 1950s it caused serious losses to the pig production industry

in Europe, particularly in the former Czechoslovakia. By the year 1952, the reported number of new cases of Teschen disease reached 137,396, i.e., an incidence rate of 2794 per 100,000 pigs, in 14,801 villages with 65,597 affected farms, i.e., 4.43 affected farms per village and 2.10 diseased pigs per affected farm. The average territorial density of new cases was 1.07 per km^2. The last cases were detected in 1973 and from that time Czech and Slovak territories have been free from this dangerous infection (Kouba, 2009).

A porcine enterovirus (PEV) classified to group I was isolated from spinal cords and brains of animals affected with neurologic disorders in a commercial swine herd in Indiana, USA. The herd experienced high death loss of nursery pigs with neurologic disorders for a prolonged period. Polioencephalomyelitis was the consistent histopathological lesion in affected animals (Pogranichniy *et al.*, 2003).

Teschovirus encephalitis was identified in an outbreak where seven of 41 piglets in a herd of 70 sows had developed severe, flaccid paralysis of the hind limbs at 40 days of age and became recumbent (Yamada *et al.*, 2004).

Porcine teschovirus 1 (PTV-1) was isolated from brain tissues of diseased piglets in a farm in Inner Mongolia Province, Peoples Republic of China. It was confirmed by electron microscopy, RT-PCR, and sequencing. Comparison of the sequences of the amino acid and nucleotides and phylogenetic analysis of the polyprotein showed that isolated virus is PTV-1. The isolated virus had closest relationship with Talfan strain. They shared 98.9% and 99.5% homology of amino acids and nucleotides, respectively, in the ORF of polyprotein gene (Feng *et al.,* 2007).

Serological status and epidemiological situation of porcine teschovirus 1 (PTV-1) was studied on swine farms in different regions in Lithuania by using virus neutralization test and virus isolation. Antibodies against PTV-1, detected by using VNT, were found on all investigated farms and in all age groups. Negative serum (2.3%, 39 from 1680) samples were found in 15.5% (16 from 103) of 2-4-month-old pigs and in 4.7% (23 from 494) of 4-6-month-old ones. Epidemiological and clinical examination revealed no signs of porcine enterovirus encepalomyelitis on 32 Lithuanian swine farms. PTV-1 was not isolated and identified in the

PK-15 cell culture. The positive serological and negative epidemiological, clinical and virological results suggest that less virulent or avirulent PTV-1 strains were spread on Lithuanian swine farms (Sereika *et al.,* 2007).

A case of concurrent polioencephalomyelitis due to PTV and postweaning multisystemic wasting syndrome (PMWS) associated with PCV2 was described in a piglet in Japan. The lesions were characterized by polioencephalomyelitis with the predominant distribution in the brain stem, as well as lymphocyte depletion and histiocyte infiltration with cytoplasmic inclusion bodies in the lymphoid tissues throughout the body and interstitial pneumonia. It was suggested that the immunosuppressive condition developing in PMWS may have facilitated the infection of the brain with PTV (Takahashi *et al.,* 2008).

PTV was isolated in cell culture and demonstrated by polymerase chain reaction in samples of brain and/or spinal cord in pigs in Indiana during the 2002–2007 period. Virus was demonstrated in pigs with and without lesions as well as with and without nervous clinical disease. Nucleotide sequence analysis of the 5'-nontranslated region of the viral genome revealed that these isolates had low-level genetic heterogeneity but were homologous to porcine PTV serotype 1 (PTV-1). These findings indicate that low-to-moderate virulence strains of PTV with some homology to PTV-1 are endemic in many swine herds of Indiana and are associated with subclinical and clinical nervous disease in weaned pigs (Bangari *et al.*, 2010).

In an epidemiologic surveillance program for swine diseases carried out in Spain, 206 cytopathic viruses were isolated from 600 porcine fecal samples between 2004 and 2005. Of the 206 isolates, 97 (47%) were identified as teschoviruses, 18 (9%) as sapeloviruses, and 7 (3%) as porcine adenoviruses using reverse transcription polymerase chain reaction (RT-PCR) methods. Neither Porcine enterovirus B nor Swine vesicular disease virus was found among the isolates (Buitrago *et al.*, 2010).

A total of 40 viral strains isolated on cell culture from gut contents, rectal swabs and faeces from apparently healthy pigs in Italy, during 2006–2007, were analysed by RT-PCR to detect the presence of porcine enteric picornavirus species. Of 40 samples, 21 were found positive to teschovirus RNA, whereas one and three were positive to Porcine Sapelovirus

(PSV) and PEV-B RNA, respectively. Mixed infections with two viruses were identified in the remaining 15 samples (PSV + PEV-B, n = 2; PSV + PTV, n = 11; PEV-B + PTV, n = 2). Therefore, a total of 55 viral RNAs were identified in the 40 samples tested; 34 (61.82%) of these were identified as PTV (serotype 1–11), 7 (12.73%) as PEV-B (serotype 9 and 10) and 14 (25.45%) as PSV (Sozzi *et al.,* 2010).

A new PTV strain (designated as JF613) was isolated from pigs showing symptoms of pyrexia, diarrhea, respiratory distress and nervous disorders (difficulty in standing, recumbent and hindlimb paralysis) in a commercial pig farm in Heilongjiang Province in China. It was confirmed by the specific CPE on IBRS-2 cells, RT-PCR and nucleotide sequencing. Analysis of its amino acids sequence of complete polyprotein indicated that the isolate belongs to serotype 2. The isolate exhibited highest similarities with strains of serotypes 2 and 5 in two crossover regions, suggesting the recombination event in PTV (Wang *et al.,* 2010).

Porcine Teschovirus (PTV, designated as PTV-8 Jilin/2003) was isolated from pooled samples of organs such as lungs, liver and intestine of 50-70 days old piglets showing signs such as acute diarrhea, respiratory distress, and death of pigs in a swine farm in Jilin Province, northern China. The presence of PTV was confirmed by the production of a specific cytopathic effect on susceptible cells and by the results of the immunoperoxidase monolayer assay (IPMA), polymerase chain reaction, and electron microscopy. The complete P1 sequence showed that this virus strain belongs to PTV-8. When inoculated into healthy pigs, PTV-8 Jilin/2003 caused the same symptoms as those observed in the affected herd. It was concluded that PTV-8 Jilin/ 2003 was the causal agent of the outbreak (Zhang *et al.,* 2010).

Sapelovirus was identified as the causative agent in an outbreak of gastroenteritis associated with respiratory disease and polioencephalomyelitis that occurred in a small commercial pig farm in Shanghai, China. The presence of PSV was confirmed by the specific cytopathic effects observed in susceptible cells and by the results of PCR, nucleotide sequencing and electron microscopy. When inoculated into healthy pigs, this isolate caused the same symptoms as observed in the affected herd (Lan *et al.,* 2011).

A novel PEV was identified from fecal samples of clinically healthy pigs in Hungary by RT-PCR using human enterovirus generic primer pairs for 5'UTR region, with subsequent

partial VP1 and complete genome sequencing and phylogenetic analysis. Among 45 fecal and blood sample pairs collected at the same farm from domestic pigs divided into three age groups (10 days, 4 weeks, and 3 months of age, N = 15 each group) six (40%) of the 15 fecal samples of 10-day-old pigs were enterovirus-positive. PEV was not detected in serum samples. The isolate named Swine/K23/2008/HUN had average of 77 and 75% amino acid identity in the P1 region, and only 61% in VP1 region to PEV9 and 10, respectively. It was proposed that PEV9 and PEV10 should be reclassified as PEV1 and PEV2. In this classification swine/K23/2008/HUN represents PEV3 (Boros *et al.,* 2011).

To study the genetic diversity of PTVs using PTV isolates collected between 2004 and 2009 in a wide territory in Spain phylogeny reconstructions were made using maximum likelihood and Bayesian inference methods, based on the 1D (VP1) gene, and including sequences available in public databases. The phylogenetic trees obtained indicated that PTVs present 12 main lineages, 11 corresponding to the PTV serotypes described earlier, and one lineage distinct from the rest which was tentatively named as PTV-12 (Cano-Gomez *et al.,* 2011).

Porcine teschovirus was identified in tissues from 4–7-week old pigs in western Canada which showed symptoms of bilateral hind-limb paresis or paralysis. Necropsies revealed no gross lesions, but microscopic lesions consisted of a non-suppurative polioencephalomyelitis, most severe in the brain stem and spinal cord. PTV was confirmed by immunohistochemistry, virus neutralization test, fluorescent antibody test, and nested reverse transcription polymerase chain reaction (Salles *et al.,* 2011).

PTV was identified in 58 fecal samples out of 525 faecal samples collected from each county and city in Taiwan. Infected pigs did not show any clinical symtoms. The results of the phylogenetic tree analysis indicated that the 58 isolated PTVs, were aligned among 10 different serotypes (PTV1-PTV4 and PTV6-PTV11) and these serotypes were distributed randomly around Taiwan (Yeou-Liang *et al.,* 2011).

PEV genome was identified in 5 of 10 (50%) faecal samples collected from wild boar piglets in Hungary and the complete genome was characterized by RT-PCR and pyrosequencing.

Wild boar/WBD/2011/HUN (JN807387) PEV showed only 67% amino acid identity in VP1 compared to the most closely related prototype PEV-3H/PEV-14. This Wild boar enterovirus represents a novel PEV genotype, provisionally called PEV-15 (Boros *et al.*, 2012a).

Porcine teschovirus type 1 was identified as the causative agent in an outbreak of a disease where 1,500 backyard pigs of variable age became sick, and approximately 700 of them died or were euthanized in Republic of Haiti. The main clinical sign was posterior ataxia followed by paresis and/or paralysis on the second or third day of illness. No gross lesions were observed at postmortem examinations. The morbidity and mortality were approximately 60% and 40%, respectively. Results of virus isolation, electron microscopy of virus particles, histopathological analysis on brain tissues, nucleic acid sequencing, and phylogenetic analysis of the viral isolate supported the diagnosis of teschovirus encephalomyelitis (Deng *et al.*, 2012).

In an epidemiological survey of porcine enteric picornaviruses belonging to the genera Teschovirus, Sapelovirus, and Porcine enterovirus B between years 2005 and 2011, Enterovirus B was the most prevalent virus detected in both domestic pigs and wild boars (50.2% and 69.4%, respectively), followed by Porcine teschovirus and Porcine sapelovirus. The majority of positive domestic pigs (69.4%) and wild boars (64.3%) were infected with two or three tested viruses. There was no significant difference in prevalences of teschoviruses, sapeloviruses, and enteroviruses among healthy and diarrhoeic pigs (Prodelalova, 2012).

The prevalence of PEV-9 in pig populations in middle and eastern China was studied using RT- PCR. All 14 sampled farms were positive for PEV-9 and the overall prevalence of infection in the studied pigs was 8.3% (37/447). There was a higher frequency of infection in pigs aged 10–15 weeks (12/119, 10.1%) than in pigs aged >20 weeks (5/103, 4.9%). Phylogenetic analysis based on the RdRp gene suggested that PEV-9 strains from China formed a new subgroup. Specific pathogen free (SPF) piglets were inoculated orally with the PEV-9 strain identified in this study. Although most experimental pigs showed no clinical signs, almost all carried PEV-9 in one or more tissues after 6 days post-inoculation. The results of tissue histologic examination suggested that PEV9 can cause pathological changes in cerebrum and lung (Yang *et al.*, 2013).

The prevalence of porcine teschovirus (PTV) in swine herds from 42 farms in northeast China was investigated by immunofluorescence assay using specific antibody against PTV-8. All 42 pig herds were positive for antibodies against PTV-8, and 61.3% of the serum samples were PTV-8 positive. During the survey, one PTV strain was isolated and named Fuyu/2009. Phylogenetic analysis showed that the PTV Fuyu/2009 belongs to the PTV-8 serotype. The serological results indicate that most if not all pig herds in northeast of China have been exposed to PTV. RT-PCR performed on 114 clinical samples from 2-3 months old animals showing symptoms of diarrhea, respiratory distress and death indicated a possible association between PTV and disease. According to genotyping based on partial VP1 sequences, four serotypes (PTV-2, -4, -6, and -8) were identified in northeast of China (Qiu *et al.,* 2013).

PTV and PSV were detected by RT-PCR in fresh faecal samples (n = 63) from wild boar in Donana Biological Reserve, Spain collected during 2007 and 2011, A total of 32 samples (50.8%) were positive for PTV, while PSV amplicons were detected in 4 samples (6.4%). All PSV-positive samples were also positive for PTV, which indicated co-infection with both viruses. Virus isolation was successful from 6 samples, 4 of which were identified as PTV by RT-PCR (Cano-Gomez *et al.,* 2013).

An EV-G was isolated for the first time in United States from a porcine diarrhea sample and complete genome of the designated isolated virus NP/2013/USA was characterized. The complete genome consists of 7,390 nucleotides excluding the 39 poly(A) tail, and has an open reading frame that encodes a 2,169 amino acid polyprotein. NP/2013/USA was most similar at the nucleotide (84%) and amino acid (95%) level to the HM131607, an EV-G1 type isolated from China in 2012 (Anbalagan *et al.,* 2014).

Forty randomly selected diarrhoeic and normal consistency faeces of suckling (n= 22) and nursery (n=18) pigs from farms located in 21 distinct cities of Brazil were evaluated by nested-RT-PCR assays. PTV and EV-G were identified in single and mixed infections in 40.9 % (9/22) of the faecal samples of suckling piglets. For nursery pigs, Porcine enteric picornaviruses amplicons were present in 77.8 % (14/ 18) of the faecal samples. PTV and EV-G were detected in single and mixed infections, while PSV was detected only in two samples in co-infection with PTV and EV-G in this age group (Donin *et al.,* 2014).

High rates of infection of Enterovirus G were recorded in pigs on all of the investigated farms, with detection frequencies of approximately 90% in recently weaned pigs but declining to 40% in those aged over 1 year. No differences in EV detection rates were observed between pigs with and without diarrhoea [74% (n=570) compared with 72% (n=5128)]. VP1 sequence comparisons identified six type 1 and seven type 6 variants of Enterovirus G, while four further VP1 sequences failed to group with any previously identified EV-G types. These have now been formally assigned as EV-G types 8–11 by the Picornavirus Study Group (Van Dung *et al.* 2014).

2.5.2. Swine vesicular disease Virus (SVDV)

Swine vesicular disease (SVD) was first observed in Italy in 1966, where it was clinically recognized as foot-and-mouth disease (Nardelli *et al.,* 1968). Later it was identified in Hong Kong (Mowat *et al.,* 1972), Great Britain (Dawe, 1973), Netherlands (Franssen, 1975) Austria, Italy and Poland (Dekkar, 2000). Currently it is prevalent in Eastern European and Asian countries like Taiwan, and China. North and South America are considered free of SVD (Dekkar, 2000). SVDV can infect humans (Brown *et al.,* 1973) and is lethal to new-born mice (Nardelli *et al.,* 1968). Based on host tropism and antigenic similarities it is thought to be related to Coxsackie B5 virus (Brown *et al.,* 1973, Graves, 1973). Graves (1973) suggested that SVDV was a swine-adapted Coxsackie B5 isolate. Now it is classified in genus Enterovirus and species Enterovirus B as swine variant of Coxsackie B5 virus along with other enteroviruses infecting human beings (ICTV, 2012, www.Picornaviridae.com). SVDV has approximately 75 - 85 % nucleotide homology with Coxsackie B5 virus (Knowles and McCauley, 1997).

2.6 Transmission

Transmission of enteric picornaviruses occurs mostly by faecal-oral route and indirect transmission is likely to occur as the viruses are relatively resistant (Knowles, 2006).

SVDV enters the pig through the skin or the mucosa of the digestive tract (Chu *et al.,* 1979, Lai *et al.,* 1979, Mann and Hutchings, 1980).

2.7 Pathogenesis

2.7.1 Pathogenesis of Porcine enteric Picornaviruses

Natural infection occurs by ingestion of the virus. Initial viral replication occurs in the tonsils and intestines (Long, 1985). The large intestines and ileum are infected with more frequency than the upper intestinal segments. Intestinal epithelial cells destruction is not a feature of these infections. Viremia follows initial replication, more regularly with virulent PTV-1 infection but less regularly with less virulent strains. Extra intestinal infections are relatively transient whereas the virus persists in large intestine for several weeks (Knowles, 2006).

The role PTVs in causing diseases was studied under the endemic and multi-infection situation, when most pigs in the herds are infected and immune. Based on the fecal-oral model of pathogenesis, a set of 15 organs were collected from 30 culled post-weanling piglets of 4–8 weeks old. The infection was most common in the intestines (averaged 61%) and lymphoid organs (averaged 59%), followed by visceral organs (averaged 37%) and the CNS (different parts varied from 17 to 47%). The correlation of PTVs detected by nested RT-PCR and histological lesions were analyzed by Chi-square test showing that in the field situation only non-suppurative encephalitis in the caudal part of the brain ($P = 0.054$) may be marginal significantly attributed to infection by PTVs (Chiu *et al.,* 2012).

To understand the pathogenesis of PTV in endemically infected pigs, a set of samples was studied by real time reverse transcription PCR (qRT-PCR) to quantitate viral loads in tissues and by in situ hybridization (ISH) to locate PTV signals in target cells, both targeting the 5'-NTR. Inguinal lymph node (LN) had the highest viral load of all (assuming 100%), followed by ileac LN (89–91%), tonsil (66–68%), ileum (59–60%), spleen (38–40%), and kidney (30–31%), with the least in brain (22.9%) of the inguinal LN. The 22.9% load in brain was higher than that anticipated from a simple fecal-oral-viremia operative model. The results suggested in addition that intranasal infection and retrograding axonal infection from the tonsils were equally operative and significant. PTV signals in ISH were noted most impressively in neurons of the cerebral cortex and hippocampus and in the dark zone of the germinal center and adjacent para cortex of regional LN (Chiu *et al*., 2014).

2.7.2 Pathogenesis of Swine vesicular disease virus

SVDV enters the pig through the skin or the mucosa of the digestive tract (Chu *et al.,* 1979, Lai *et al.,* 1979, Mann and Hutchings, 1980). Experimental SVDV infection can lead to clinical signs within 2 days. After contact of a pig with a SVDV-contaminated environment, viraemia can develop within 1 day, which is equal to the time needed when pigs are directly inoculated (Dekker, 2000). Virus titres in the spleen and kidney are often comparable to or higher than the titres in serum. The virus titres in the tonsil are very high. Lymph nodes also may contain high titres of SVDV after experimental infection. It is not known, however, whether lymph nodes are positive because of the drainage of virus or because of virus replication (Dekker, 2000). SVDV has a strong tropism for epithelial tissues but virus titres in the myocardium and the brain significantly exceed those in plasma. So, epithelial tissues, myocardium, and brain are probably the sites of virus replication (Chu *et al.,* 1979; Dekker *et al.,* 1995 and Lai *et al.,* 1979)

2.8 Clinical Signs

2.8.1 Porcine enteric Picornaviruses

The virus enters the animal via the oral or nasal cavity. The incubation period is about 14 days. The main signs of the prodromal stage are fever up to 41.5°C, lassitude, anorexia and locomotor disturbances. This stage is followed by hypersensitivity, tremors, clonic spasms of the legs, flaccid paralysis, opisthotonos and nystagmus; convulsions may be observed in young pigs. In the final clinical stage, paralysis proceeding from the hind part through the loins to the fore part of the body is observed. Paralysis of the thermoregulatory centre results in hypothermia. When respiratory muscles are paralysed, the animal dies of suffocation (OIE, 2009).

2.8.2 Swine vesicular disease virus

In swine vesicular disease in typical cases, lesions are first noticed at the junction of the heel and the coronary band. The whole of the coronary band may eventually be involved and the lesions may spread to the metatarsal and metacarpal regions. The horn and sole may be damaged so extensively that the claw(s) slough off. In lactating sows lesions on the udder and

teats can be seen also. Occasionally, the skin of the thorax and abdomen is involved. Lesions in the mouth, on the lips and snout occur in up to 10% of the cases. Those on the snout are mostly on the dorsal face of the rostrum and may be haemorrhagic in appearance. Tongue lesions are transient and heal rapidly (Hedger and Mann, 1989). In experimentally infected animals, non-suppurative meningoencephalitis may occur, but this does not result in signs of impaired central nervous system (Chu *et al.,* 1979).

2.9 Histo-pathological lesions

2.9.1 Porcine enterovirus/Teschovirus encephalitis

In a prolonged outbreak of infection with a group I porcine enterovirus in Indiana in USA characteristic histological lesions were described. Inflammation characterized by lymphocytic vasculitis/perivasculitis and focal gliosis was irregularly distributed throughout the brain but was most severe in midbrain, brainstem, and cerebellar peduncles and occasionally in cerebral cortex. Similar inflammation was observed in the gray matter in the spinal cord sections, occasionally following vessels into the more peripheral white matter. Inflammation varied from mild to severe between pigs, but all brains and all sections of the spinal cord in all pigs were affected. Lesions tended to be more severe in cervical and lumbar sections of the cord than in thoracic sections. Eosinophilic necrotic neurons were noted in some sections of the spinal cord with intense inflammation (Pogranichniy *et al.*, 2003).

Histologically lesions in the Teschovirus encephalitis have been described in CNS and peripheral nerve fibers. In an outbreak in Japan, all clinically affected piglets had similar histological changes of non-suppurative encephalomyelitis, characterized by perivascular cuffing of the mononuclear cells, focal gliosis, neuronal necrosis and neuronophagia. The spinal cord was severely affected, with the lesions seen along its full length. In the ventral horns, nerve cells were degenerated to severe degrees upto and including necrosis accompanied by neuronophagia, inflammatory or glial nodules, occasional hemorrhages and a rather diffuse infiltration of mononuclear cells. In the white matter of the spinal cord, perivascular cuffing and infiltration of mononuclear cells and focal gliosis were also observed. In addition to the infiltrative changes, severe vacuolar changes and axonal swelling were observed in the white matter of

the spinal cord. Infiltration of mononuclear cells was observed in the dorsal root ganglia, spinal nerves and sciatic nerves. In some dorsal root ganglia, degenerated ganglion cells and neuronophagia was observed. Swollen myelin sheaths and axonal spheroids were seen in spinal roots and in the peripheral nerves, including the brachial plexus and sciatic nerves. The cerebellar nuclei and the grey matter of the brainstem were also severely affected. In the cerebral hemisphere, only slight perivascular cuffing was present (Yamada *et al.*, 2004).

In a case of concurrent polioencephalomyelitis and PMWS in a piglet the principal histological lesions were observed in the CNS and were characterized by polioencephalomyelitis. A severe degree of changes was found in the pons and medulla oblongata, moderate changes in the spinal cord, spinal dorsal' root ganglia and cerebellum and mild changes in the diencephalon, mesencephalon and trigeminal ganglia. The lesions were distributed throughout the pons and medulla oblongata without any location affected preferentially, and consisted of neuronal degeneration, neuronophagia, glial accumulation and perivascular cuffing. Affected neurons were swollen with chromatolysis or shrunken with an increased eosinophilic affinity. Their nuclei were eccentrically located and often accompanied by karyopyknosis, karyorrhexis or karyolysis. Neuronophagia was often found and microglial accumulation was disseminated in the lesions. Perivascular cuffing was composed of two to three layers of cells in which lymphocytes were the predominant cell type. The involvement of the diencephalon and mesencephalon was limited to several nervous nuclei and adjoining white matter (Takahashi *et al.,* 2008).

2.9.2 Swine vesicular disease

In experimentally induced disease well developed acute necrotizing lesions appear in the epithelium of the tongue at one day post infection (PI). During the next 24 hours, the small necrotic foci get enlarged and involve almost all layers of the epithelium. Some of these necrotic foci develop into intraepithelial vesicles. Microabscesses develop from the necrotic foci and are characterized by their contents of neutrophils mixed with a few mononuclear cells. The earliest lesions in the epidermis of the snout are small necrotic foci occurring at two days PI and developing to multilocular intraepidermal vesicles by the third day. The affected epidermis is markedly hyperplastic and has focal areas of intracellular and intercellular edema. Multiple

small necrotic foci are seen in the stratum spinosum. Vesicles also are most often observed in this layer. The foci usually contain no inflammatory cells in this stage. However, a mild inflammatory response may already be seen in the adjacent superficial dermis. By the third day PI as the lesions develop, necrosis, swelling and rupturing of epidermal cells progress on to reticular degeneration with the formation of multilocular intraepidermal vesicle. The lesions in the skin of the coronary band are almost the same as those in the snout, except that they first appeared on the third day. The inflammatory response in skin of the coronary band is much more severe than it is in the snout (Chu *et al.,* 1979).

2.10 Detection of Porcine Enteroviruses

2.10.1 Isolation of the virus

Monolayer cultures of primary porcine kidney or established cell lines derived from porcine tissue are suitable for isolation of enteric Picornaviruses (Madr, 1959; Mayr and Schwoebel, 1957; Knowles *et al.*, 1979; OIE, 2009). Enterovirus G can be grown on established cell lines like IBRS-2, Vero cells, Hela cells and BHK21 (Knowles *et al.*, 1979).

Virus isolation on IBRS-2 cells is considered the most sensitive method for diagnosis of SVDV. In addition to IBRS-2 cells, SK6, PK-15 and primary or secondary porcine kidney cells are also susceptible to SVDV (Nardelli *et al.*, 1968; Dawe *et al*., 1973).

2.10.2 Immunohistochemistry

Porcine teschovirus (PTV) antigens were detected by a streptavidin-biotin complex method in formalin-fixed paraffin-embedded tissues of 3-week-old pigs that had been inoculated intravenously with PTV Talfan strain. PTV antigens were detected in cytoplasm of nerve cells, glial cells and endothelial cells in the cerebellar nuclei, the grey matter of the midbrain, pons and medulla oblongata and the ventral horn of the spinal cord and of ganglion cells in the spinal ganglion corresponding to those lesions characterized as non-suppurative encephalomyelitis and ganglionitis. The results of this study suggest that nerve cells of the brain stem and spinal cord and ganglion cells of the spinal ganglion permit PTV replication and represent the main target cell population of PTV (Yamada *et al.,* 2007).

A distribution of porcine teschovirus (PTV) antigens in pigs naturally infected with PTV was described by Yamada *et al.* (2008) using the method of immunohistochemistry. In the nervous system, PTV antigens were found in the cytoplasm of neuronal cells and glial cells distributed in the spinal ventral horn and brain stem, and also in the cytoplasm of ganglion cells in the spinal ganglion. No antigens were seen in the cerebral hemisphere. In the nervous system, the distribution of PTV antigens was consistent with lesions characteristic of non-suppurative encephalomyelitis. PTV antigens were also observed in bronchiolar epithelial cells in the lung, hepatocytes, epithelial cells in the tonsils and the myenteric nerve plexus in the small and large intestines.

Nonsuppurative encephalomyelitis with neurological signs expressed as flaccid paralysis of the hind limbs was experimentally induced in three-week-old piglets by a single intravenous injection of the Toyama 2002 strain of porcine teschovirus (PTV) isolated from field pigs in Japan. Lesions were characterized by perivascular cuffing of mononuclear cells, focal gliosis, neuronal necrosis and neuronophagia, mainly in the ventral horn of the spinal cord. Nonsuppurative ganglionitis of the spinal ganglion and neuritis of the spinal root were also detected. PTV antigens were detected immunohistochemically and the distribution of these antigens corresponded closely with the distribution of brain lesions. PTV antigens were observed in the ganglion cells before the appearance of the inflammatory changes 3 days post-inoculation (dpi) and were present in the dorsal root and spinal cord on 9 dpi. No lesions of the central nervous system were induced in pigs by oral or intranasal inoculation of this strain of PTV (Yamada *et al.,* 2009).

Seronegative specific-pathogen-free (SPF) pigs were inoculated with PTV1 intranasally to investigate localization of PTV-1 in various organs. Tissue specimens were collected on the 15^{th} and 50^{th} days postinfection (DPI) and processed for immunohistochemistry. In the nervous system PTV-1 antigen signals were noted within endothelial cells, cuffed lymphocytes, and astrocytes of a pig with neural signs, but with weaker signals noted within ganglion cells of well-developed ganglionitis site in a pig without neural signs. In the spleen PTV-1 antigen was noted abundantly within marginal zone macrophages of the white pulp and T lymphocytes of the red pulp. In the tonsil, signals were presented more apparent within T lymphocytes and

macrophages of the interfollicular area than those in the tonsillar epithelium and follicular center. This study indicates that, besides the commonly known fecal-oral route of infection, intranasal infection is able to establish infection (Chiu *et al.,* 2013).

2.10.3 Reverse transcriptase-Polymerase chain reaction (RT-PCR)

A nested RT-PCR protocol was described by Zell *et al.* (2000) to detect all known porcine enterovirus serotypes using three sets of primer pairs. These primer pairs were designed to amplify either highly conserved sequences of the 5'non-translated region (5'-NTR) or the polymerase gene region of the relevant virus species. All 13 acknowledged serotypes of three PEV species and several field isolates of clinical specimens were detectable. PEV PCR is more rapid and less laborious than the time-consuming virus isolation by tissue culture techniques over several passages and serotyping.

Palmquist *et al.*, (2002) described the development of a reverse transcription–polymerase chain reaction (RT-PCR) assay for the rapid and sensitive detection of PEVs of cytopathic effect groups I (now known as porcine teschoviruses [PTVs]) and II. The assay described not only detects the PTVs and CPE group II of PEVs but also allows them to be differentiated on the basis of the size of the amplification product, using the same set of oligonucleotide primers.

For reliable diagnosis of PTV infection an RT-PCR-based molecular strategy for serotyping was created that encompassed the dominant neutralizing antigenic site of PTV, followed by phylogenetic analyses of amplicons. By epitope mapping using MAbs followed by 3-dimensional modeling, one major antigenic site, the EF loop (puff) of VP2, and at least 2 additional antigenic sites, the GH loop of VP1 and the C terminus of VP1 were defined (Kaku *et al.*, 2007) .

Loop-mediated isothermal amplification (LAMP) is a sensitive method for DNA amplification. Wang *et al.* (2011) described the development of a single-tube, one-step, real-time accelerated reverse transcription (RT)-LAMP for the detection of Porcine teschovirus.

Reverse transcription followed by the PCR (RT-PCR) is a useful method to detect SVD viral genome in a variety of samples from clinical and subclinical cases. Several authors have described methods for detection of SVDV genome targeting conserved regions of

SVDV genome (Benedetti *et al*., 2010; Blomström *et al*., 2008; Callens and De Clercq, 1999; Hakhverdyan *et al*., 2006; Lin *et al*., 1997; McMenamy *et al*., 2011; Nunez *et al*., 1998; Reid *et al*., 2004a; 2004b).

2.10.5 Real time RT-PCR

A real-time RT-PCR protocol employing LightCycler technology to detect all known serotypes of the three porcine enterovirus (PEV) cytopathic effect (CPE) groups was established using three sets of primer pairs and group-specific hybridisation probes (Krumbholz *et al.*, 2003). The primer pairs were designed to amplify highly conserved sequences of the 5'non-translated region of the relevant virus species. The one-step real-time PCR based on the LightCycler technology is more rapid and less contamination-prone than the nested RT-PCR and allows the precise quantitation of the virus load in the tested specimens.

A real-time reverse transcriptase PCR (RT-PCR) method was developed by Jimenez-Clavero *et al.*, (2003) that allows the quantitative detection of pig teschovirus (PTV) RNA. The method is able to detect 92 fg of PTV RNA per ml of sample. The highly conserved 5' noncoding region (nucleotides 329 to 394, nucleotide numbering according to PTV-1 Talfan prototype strain sequence; GenBank accession no. AF231769) was chosen as the target for the forward and reverse primers and the fluorogenic TaqMan probe. Teschoviruses were used as indicators to detect porcine fecal contamination of surface water by real time RT-PCR method.

A real-time reverse transcription polymerase chain reaction (RT-PCR) based on TaqMan was established and evaluated for quantitative detection of porcine teschoviruses (PTVs). A pair of primers and a TaqMan probe targeting the highly conserved sequence of the 5'-untranslated region (5-UTR) of one to 11 serotypes of PTV were designed. The real-time RT-PCR was specific for detection of PTV with a detection limit of 10 copies/μL (Zhang *et al.,* 2013).

2.11 Enteroviruses as pathogens for human

2.11.1 Acute encephalitis syndrome (AES) and Acute flaccid paralysis (AFP) : Definition

Acute encephalitis syndrome (AES) is defined as the acute-onset of fever and a change in mental status (including signs and symptoms such as confusion, disorientation, delirium or

coma) and/or new-onset of seizures (excluding simple febrile seizures) in a person of any age at any time of the year (Solomon *et. al.,* 2008).

AFP is defined as sudden onset of weakness and floppiness in any part of the body in a child <15 years of age or paralysis in a person of any age in whom polio is suspected (Field Guide, Surveillance of acute flaccid paralysis, 2005.)

2.11.2 Occurrence in India

Concomitant with the phenomenal elimination of wild poliovirus transmission in India was an annual increase in the number of reported AFP cases from 2005 to date throughout the country (Rao *et al*., 2012). Although 8,103–9,705 cases of AFP were reported during 1998–2003, a total of 55,782 and 60,540, 60922, 54632 and 50672 cases were reported during from 2010 and 2014 December, respectively (WHO, 2014).

In an outbreak of viral encephalitis in eastern UP, India in 2006 enterovirus (EV) was identified as an etiologic agent in cerebrospinal fluid by using reverse transcription–PCR which showed positivity to Enteroviruses in 66 (21.6%) of 306 patients. Sequencing and phylogenetic analyses of PCR products from 59 (89.3%) of 66 specimens showed similarity with EV-89 and EV-76 sequences (Sapkal *et al.*, 2009).

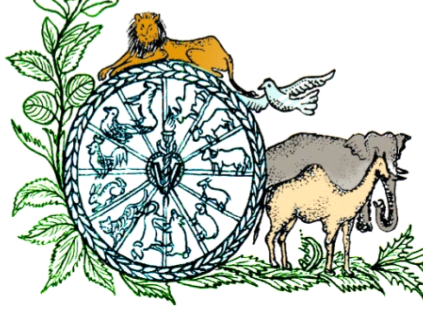
A case of acute encephalitis syndrome was reported in a 2-year-old patient from Japanese encephalitis endemic area in India which was provisionally diagnosed as JE viral encephalitis but subsequently worked up for enterovirus due to atypical clinical presentation such as moderate grade fever, hypotonia, and hepatosplenomegaly along with ECG findings suggestive of myocarditis, which was successfully treated with intravenous immunoglobulins (Bhatt *et al*., 2012)

A wide spectrum of non-polio enterovirus (NPEV) serotypes associated with Non Polio-Acute Flaccid Paralysis (NP-AFP) were detected from polio-endemic and -free regions in India in an investigation to study the antigenic diversity of NPEVs. Of fecal specimens from 2,786 children with NP-AFP in UP, Karnataka and Kerala states, 823 (29.5%) were positive for NPEVs in RD cells, of which 532 (64.6%) were positive by VP1 RT- PCR. Among 581

isolates, 66 serotypes were identified. Enterovirus 71 was the most frequently (8.43%) detected serotype, followed by Echovirus 13 (7.1%) and Coxsackievirus B5 (5.0%) (Rao *et al.*, 2012).

In a study of prevalence of enteroviruses from northern India based on molecular methods, the enterovirus genome was detected in 79 (24.7%) of 320 clinical specimens by real time PCR. Central nervous system syndrome (CNS) was the most common clinical manifestation (n=32, 62.74%), followed by respiratory tract infection (n=8, 15.69%), acute febrile illness (n=7, 13.73%), and gastrointestinal disease (n=4, 7.84%). A total of 32 different serotypes were identified with the predominance of coxsackievirus B5 and echovirus 6 (Kumar *et al.,* 2013).

Materials and Methods



3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Sample collection

Samples for the present study consisted of faecal samples from apparently healthy animals as well as from animals showing signs of diarrhoea and tissue samples collected from post-mortem facility (Division of Pathology, IVRI), during the period of July 2013 to December 2014. Tissue samples comprising brain, tonsil, inguinal and other lymph nodes, heart, lung, liver and spleen from 52 pigs with GI tract or CNS involvement were collected. The present study covered Swine production farm (IVRI) maintaining cross bred pigs and pure Landrace pigs, one organised farm in Meerut, one organized farm in Chiraudi, Ghaziabad, and one slaughter house in Bareilly. Samples were also collected from backyard farms in Muzzafarpur district in Bihar and one organized farm in Bihar Veterinary college, Patna maintaining mostly cross bred pigs.

3.1.2 Data on animals

The study was carried out from July 2013 to December 2014. During the study period disease investigations were conducted on outbreaks and all the relevant data pertaining to general health status of animals, clinical records, morbidity and mortality pattern were recorded.

3.1.3 Cell lines

3.1.3.1 Porcine kidney (PK15) cell line

PK-15 cell line for viral culture was kindly provided by Dr. K.K. Rajak IVRI, Mukteshwar as a fully confluent monolayer in a 25 cm^2 flask at 44^{th} passage level and maintained under standard cell culture conditions using Dulbecco's minimum essential medium (DMEM) containing 5% fetal calf serum (FCS) as culture medium.

3.1.3.2 IB-RS-2 cell line

IB-RS-2cell line for viral culture was kindly provided by Dr. B. Pattnaik, Director, PDFMD, Mukteshwar as a fully confluent monolayer in a 25 cm^2 flask at 15^{th} passage level and maintained under standard cell culture conditions using Dulbecco's minimum essential medium (DMEM) containing 10% fetal calf serum (FCS) as culture medium.

3.1.3.3 Vero cell line

Vero cells for viral culture was kindly provided by Dr. G. Taru Sharma, Division of Physiology, IVRI, Izatnagar as a fully confluent monolayer in a 25 cm2 flask and maintained under standard cell culture conditions using Dulbecco's minimum essential medium (DMEM) containing 5% fetal calf serum (FCS) as culture medium.

3.1.4 Glasswares and plasticwares

All the glasswares used in the present study were procured from Schott Duran (Germany) and Borosil (India). The glass wares were cleaned as per standard procedures and autoclaved after overnight treatment with 0.01% diethyl pyrocarbonate (DEPC) treated water to render them DNase, RNase and DEPC free.

All the plasticwares used in the present study were procured from Nunc (Denmark), Greiner Bio-One (Germany), Corning (USA), Millipore (Ireland) and ThermoFisher Scientific (USA). The plastic wares were either certified to be free from DNase and RNase activity or were DEPC treated and autoclaved to render them DNase, RNase and DEPC free.

3.1.5 Equipments and instruments

Standard equipments and instruments available in divisional laboratories and Central Instrumentation Facility (CIF), modular laboratory building (MLB) and divisional laboratory of Physiology and Climatology were used for requisite purposes. Specifications of the instruments/equipments are given at appropriate places in the text wherever necessary.

3.1.6 Buffers, media and reagents

The list of buffers, media and reagents used in this study with their chemical compositions and specifications is given in the appendix. Buffers and reagents were prepared either in RNase and DNase free water or in water rendered RNase and DNase free by diethyl pyrocarbonate (DEPC) treatment and autoclaving thereafter as and when required.

3.1.7 Chemicals, molecular biology reagents, enzymes, conjugates and kits

All the chemicals, molecular biology reagents, enzymes, conjugates and kits used in this study were obtained from Sigma, Amresco, Merck, Fermentas, Invitrogen, ThermoScientific, New England Biolabs, Qiagen, Vector, Gibco, KAPA Biosystems and Genetix. Wherever necessary, molecular biology grade chemicals and biochemicals were used. Specifications of the chemicals, molecular biology reagents, enzymes and kits are given at appropriate places in the text wherever necessary.

3.1.8 Oligonucletotide primers and probes

Oligonucleotide primers used in the present study were obtained commercially synthesized from IDT and Eurofins. The nucleotide sequence and other relevant details are given in Table 1 as follows :

Table 1. Sequences of oligonucleotide primers and probes used in this study

Sl No.	Primer Name	Sequence	Region	Target virus	References
1.	PEV5'UTRF	5'- GTGGCGACAGGGTACAGAAGA G -3'	5' UTR	PTV, PSV	Palmquist *et al.* (2002)
2.	PEV5'UTRR	5'- GGCCAGCCGCGACCCTGTCAG -3'	5' UTR	PTV, PSV	Palmquist *et al.* (2002).
3.	PEV-1a	5'-AGTTTTGGATTATCTTGTGCCC-3'	5' UTR	PTV	Zell *et al.* (2000)
4.	PEV-1b	5'-CCAGCCGCGACCCTGTCAGGCAGCAC-3'	5' UTR	PTV	Zell *et al.* (2000)
5.	PEV-1c	5'-TGAAAGACCTGCTCTGGCGCGAG-3'	5' UTR	PTV	Zell *et al.* (2000)
6.	PEV-1d	5'-GCTGGTGGGCCCCAGAGAAATCTC-3'	5' UTR	PTV	Zell *et al.* (2000)
7.	PTV VP1 F	5-ATGTTTACACARTGGCGHGG-3	VP1	PTV	Lab designed
8.	PTV VP1 R	5-AGAGCATCAACHGCYGGRTC-3	VP1	PTV	Lab designed
9.	PTV F1	5- GGCACCTATTGAGATTTCTCTGG-3	VP4	PTV	Lab designed
10.	PTV R1	5-CAGTGAACAGTCACATCDGTWAT-3	VP2	PTV	Lab designed
11.	PTV R2	5-GTTGCTGATGAATTDGTYCT-3	VP2	PTV	Lab designed
12.	Talfan-3D-386F	5-CAAAGACTGGTCCTTCATTG-3	3D Polymerse	PTV	Kaku *et al.* (2001)
13.	Talfan-3D-1269R	5-ATACGCCGAGCGCGGAAGAT-3	3D Polymerse	PTV	Kaku *et al.* (2001)
14	PTV-F1	5'-GAGCTAAAGCGCAATTGTCA- 3'	5' UTR	PTV	Lab designed
15	PTV-R1	5'-AGTCCCATTACCCAGTCAGG- 3'	5' UTR	PTV	Lab designed
16	PTV- P1	5'-TGTCGCCACCATTGGTGCAA- 3' (5'-FAM-TAMRA-3')	5' UTR	PTV	Lab designed
17	PTV- 2	5' Texas red® –x (NHS ester) – TGTCGCCACCATTGGTGCAA- 3'	5' UTR	PTV	Lab designed
18.	PEV-8a	5'-CCCTGGGACGAAAGAGCC TG-3'	3D Polymerse	PSV	Zell *et al.* (2000)
19.	PEV-8b	5'-CCTTTAAGTAAGTAGTAAAGG G-3'	3D Polymerse	PSV	Zell *et al.* (2000)
20.	PEV-8c	5'-CCAAGATTAGAAGTTGATTTG-3'	3D Polymerse	PSV	Zell *et al.* (2000)
21.	PEV-8d	5'-GGGTAGCCTGCTGATGTAGTC-3'	3D Polymerse	PSV	Zell *et al.* (2000)
24	PEV-9a	5'-GTACCTTTGTACGCCTGTTTT A-3'	5' UTR	PEV	Zell *et al.* (2000)
25	PEV-9b	5'-ACCCAAAGTAGTCGGTTCCGC-3'	5' UTR	PEV	Zell *et al.* (2000)
26	PEV-9c	5'-CAAGCACTTCTGTTTCCCCGG-3'	5' UTR	PEV	Zell *et al.* (2000)
27	PEV-9d	5'-GTTAGGATTAGCCGCATTCA-3'	5' UTR	PEV	Zell *et al.* (2000)
28	WBev 2626 F	5'GCATGCTGGAAACTAGACATG 3'	VP1	PEV	Boros *et al.*, (2012).
29	WBev 3394 R	5'TCTTCATGGGTTGCAAGGTGT3'	VP1	PEV	Boros *et al,* (2012).
30	K23 545F	5' GTCGTAACGGGTAACTCTGT 5'	5' UTR	PEV	Boros *et al.* (2011).
31	K23 2905R	5' GGCAAATACATAACCTGTA 3'	VP1	PEV	Boros *et al.* (2011).
32	PEV 9 F1	5-CTACTTGATGAGGTGTGGGTGGAC-3	VP2	PEV	Lab designed
33	PEV 9 R1	5-TTCTTGGGTGGGGTAATCTGG-3	VP3	PEV	Lab designed
34	PEV 9 F2	5-TGATGACACTATGCCAGGAAAGAG-3	VP2	PEV	Lab designed
35	PEV 9 R2	5-AGACCCGGTAAACATAAAAGTGAG-3	VP3	PEV	Lab designed
36	EVG-F1	5'-AGATGAACCCGTCCGTTATC- 3'	5' UTR	PEV	Lab designed
37	EVG-R1	5'-ACGCCAAGACAGATCTTTGA- 3'	5' UTR	PEV	Lab designed

Table 1. Contd...

Sl No.	Primer Name	Sequence	Region	Target virus	References
38	EVG-P1	5'-CGGCCAGCTACTTCGAGAAGCC-3' (5'-FAM-TAMRA-3')	5' UTR	PEV	Lab designed
39	EVG-2	5' Alexa Fluor 488(NHS ester)-GGCTTCTCGAAGTAGCTGGCCG-3'	5' UTR	PEV	Lab designed
40	SVSV SS4	TTCAGAATGATTGCATATGGGG	Polymerse gene	SVDV	Nu´nez *et al*. (1998)
41	SVSV SA2	CACGTTTGTCCAGGTTACC	Polymerse gene	SVDV	Nu´nez *et al*. (1998)
42	GSVD-3 (F)	5'ACACCCTTTATAAAACAGG -3'	1C	SVDV	Lin *et al.* (1997)
43	NK45 (R)	5'GCCAACGTACACGGCACC-3'	2A	SVDV	Lin *et al.* (1997)
44	LSVD-I (F)	5'TTCTTTCAAGGGCCCCCAGGAG-3'	1C/1D	SVDV	Lin *et al.* (1997)
45	GSVD-5 (R)	5'AACATGCTGTATGCGTTGCCTAT-3'	1D	SVDV	Lin *et al.* (1997)

3.1.9 Software for phylogenetic analysis

'EditSeq' programme of Lasergene version.6 (DNASTAR Inc, USA) was used for editing the raw sequences obtained after sequencing. 'Megalign' programme of Lasergene version 6 was used for aligning and joining the truncated sequences to obtain genome sequences of PTV/PSV/EVG and phylogenetic analysis in the present study. Phylogenetic analysis was carried out using Molecular Evolutionary Genetics Analysis (MEGA) software version 6 (Tamura *et al.*, 2013).

3.2 METHODS

3.2.1 Pathological studies

3.2.1.1 Post mortem examination and recording of gross lesions

A detailed systematic necropsy examination was conducted on dead animals of various age groups and animals which were presented in moribund stage and gross findings were recorded.

3.2.1.2 Collection of tissue samples

Tissue samples from brain, tonsil, intestines, spleen, mesentertic lymph nodes and other carcass lymph nodes were collected on ice and in 10% buffered neutral formalin fixative. Viable tissue samples were collected aseptically in sterile, screw capped polypropylene vials

using sterile scissors and forceps, transported on ice and stored at -80^{0}C with proper labelling till further processing.

3.2.1.3 Histopathological examination

Fixed tissues were subjected to histopathological processing and staining as per standard procedures (Bancroft, 2008). Haematoxylin and eosin (H&E) stained individual sections were microscopically examined and the histopathological alterations were digitally photographed and recorded (Olympus BX41).

3.2.2 Diagnostic assays

3.2.2.1 Polymerase chain reaction (PCR)/Reverse transcription-polymerase chain reaction (RT-PCR) amplification, cloning and sequencing of viral genome of Porcine teschovirus, Porcine sapelovirus and porcine entrovirus; amplification and sequencing of fragments of various regions of enteric Picornaviruses

3.2.2.1.1 Total RNA extraction from tissues

Total RNA was extracted from tissues using commercial TRIzol® Reagent (Life technologies) which is an improved and modified version of single step RNA isolation method developed by Chomcynski and Sacchi (Chomcynski and Sacchi, 1987), employing guanidium isothiocyanate and phenol as monophasic solution. To approximately 100 mg tissue, 500 µl of TRIzol reagent was added and homogenized thoroughly with a nuclease free mircopestle in a sterile nuclease free microcentrifuge tube kept on ice. After proper homogenization, 500 µl more of TRIzol reagent was added and mixed the contents properly. The homogenized sample was incubated for 5 minutes at room temperature to permit complete dissociation of the nucleoprotein complex. 200 µl of chloroform (Amresco, USA) was added to the mixture after incubation and mixed properly by shaking the tube vigorously by hand for 15 seconds, thereafter incubated for 3 minutes at room temperature. After centrifugation at 13,000 rpm for 15 minutes at 4^{0}C, the colourless upper aqueous phase was pipetted out into a new sterile 1.5 ml MCT without disturbing the lower interphase and organic layer. To this, 500 µl of 100% isopropyl alcohol was added to precipitate RNA. The contents were centrifuged at 13,000 rpm for 10 minutes after incubating at 4^{0}C for 10 minutes. The supernatant was discarded, leaving only

the RNA pellet. The pellet was washed with 1 ml of 75% ethanol by vortexing the sample briefly and then centrifuging at 8,000 rpm for 5 minutes at 4^0C. The wash was discarded and the RNA pellet was air dried for 10 minutes. The partially dried RNA pellet was resuspended in nuclease free water and incubated in water bath for 10 minutes at 60^0C. All the RNA samples extracted were quantified by NanoVue plus and stored at -80^0C till further use. The RNA from cell culture supernatant were extracted using QIAmp® Viral RNA Mini kit (Qiagen) as per manufacturer's recommendation.

3.2.2.1.2Total RNA extraction from faecal samples/rectal contents

Total RNA was extracted from faecal sample using commercial TRIzol LS® Reagent (Life technologies). 100 mg faeces was made into a 20% w/v suspension in PBS(pH 7.2-7.4) After proper homogenization, suspension was clarified by centrifugation at 5000 rpm for 10 minutes. 250 µl of this clarified supernatant was used for RNA extraction. 750 µl of TRIzol LS reagent was added to 250 µl of faecal supernatant and the contents were mixed properly. The homogenized sample was incubated for 5 minutes at room temperature to permit complete dissociation of the nucleoprotein complex. 200 µl of chloroform (Amresco, USA) was added to the mixture after incubation and mixed properly by shaking the tube vigorously by hand for 15 seconds, thereafter incubated for 3 minutes at room temperature. After centrifugation at 13,000 rpm for 15 minutes at 4^0C, the colourless upper aqueous phase was pipetted out into a new sterile 1.5 ml MCT without disturbing the lower interphase and organic layer. To this, 500 µl of 100% isopropyl alcohol was added to precipitate RNA. The contents were centrifuged at 13,000 rpm for 10 minutes after incubating at 4^0C for 10 minutes. The supernatant was discarded, leaving only the RNA pellet. The pellet was washed with 1 ml of 75% ethanol by vortexing the sample briefly and then centrifuging at 8,000 rpm for 5 minutes at 4^0C. The wash was discarded and the RNA pellet was air dried for 10 minutes. The partially dried RNA pellet was resuspended in 20 µl nuclease free water and incubated in water bath for 10 minutes at 60^0C. All the RNA samples extracted were quantified by NanoVue plus and stored at -80^0C till further use.

3.2.2.1.3 First strand complementary DNA (cDNA) synthesis (Reverse Transcription)

First strand cDNA was synthesized by random priming using genetically modified Molony murine leukemiavirus reverse transcriptase (M-MLV-RT) (RevertAid H Minus RT, Thermo Scientific) in a 20 µl standard reaction volume. Briefly, 1 µl of random primer (100 picomole/ µl), 1µg of RNA (volume varies depending on the concentration) and nuclease free water were added in a sterile, nuclease free tube on ice to make the volume 12.5 µl. After gentle mixing and brief centrifugation, the mixture was incubated at 65^0C for 5 minutes followed by quick chill on ice. To this, 4 µl of 5X Reaction buffer, 0.5 µl of RiboLock RNase Inhibitor (Thermo Scientific), 2 µl of 10 mM dNTP (Thermo Scientific) and 1 µl of RevertAid H Minus reverse trancriptase were added and incubated at 25 ^{0}C for 10 minutes and then at 50^0C for one hour. The reaction was terminated by inactivating the enzyme by heating at 70^0C for 10 minutes. The synthesized cDNA was stored at -20^0C till further use.

3.2.2.1.4 Polymerase chain reaction amplification

Polymerase chain reaction (PCR) conditions for various genomic regions were similar to those described earlier with minor modifications, and variations in primer annealing temperatures for the lab designed primer sets. Amplification was performed in a thermal cycler (S1000™ Thermal cycler, Bio-Rad) for 40 cycles. The reaction mix and thermal cycling conditions were as described below:

A. Porcine Teschovirus

1. PEV1a and PEV1b (Zell *et al.*, 2000; Expected product size-321bp)

PCR reaction set up (Standard Taq DNA Polymerase, New England BioLabs)

PCR reaction contents	Quantity
10X Standard Taq Reaction Buffer	2.5 µl
10 mM dNTP	0.5 µl
PEV1a (10µM)	1.0 µl
PEV1b (10µM)	1.0 µl
Taq DNA Polymerase (5µ/ µl)	0.125 µl
Template DNA	2.0 µl
Nuclease-free water	17.875 µl

THERMAL PROFILE

Initial Denaturation	95^{0}C, 4 min.
Denaturation	95^{0}C, 30S
Annealing	51^{0}C, 50S
Extension	68^{0}C, 50S
Final Extension	68^{0}C, 7 min.

2. PEV 1c and PEV 1d (Zell *et al.*, 2000; Expected product size-158bp)

PCR reaction set up

PCR reaction contents	**Quantity**
10X Standard Taq Reaction Buffer	2.5 µl
10 mM dNTP	0.5 µl
PEV1c (10µM)	1.0 µl
PEV1d (10µM)	1.0 µl
Taq DNA Polymerase (5u/ µl)	0.125 µl
Template DNA	2.0 µl
Nuclease-free water	17.875 µl

THERMAL PROFILE

Initial Denaturation	95^{0}C, 4 min.
Denaturation	95^{0}C, 30S
Annealing	53^{0}C, 45S
Extension	68^{0}C, 50S.
Final Extension	68^{0}C, 7 min.

3. PEV5'UTRF and 5'UTR R (Palmquist et al. 2002, Expected product size-180bp)

PCR reaction set up

PCR reaction contents	**Quantity**
10X Standard Taq Reaction Buffer	2.5 µl
10 mM dNTP	0.5 µl
PEV5'UTRF (10µM)	1.0 µl
PEV5'UTRR (10µM)	1.0 µl
Taq DNA Polymerase (5u/ µl)	0.125 µl

Template DNA	2.0 µl
Nuclease-free water	17.875 µl

THERMAL PROFILE

Initial Denaturation	95^{0}C, 4 min
Denaturation	95^{0}C, 40S
Annealing	56^{0}C, 40S
Extension	68^{0}C, 50S
Final Extension	68^{0}C, 7 min.

4. PTV VP1 F and PTV VP1 R (Lab designed, Expected product size-1263bp)

PCR reaction set up

PCR reaction contents	Quantity
5X Phusion Buffer	5 µl
10 mM dNTP	0.5 µl
PTV VP1 F (10µM)	1.0 µl
PTV VP1 R (10µM)	1.0 µl
DMSO	0.75 µl
Phusion Taq Polymerase (5u/ µl)	0.25 µl
Template DNA	3.0 µl
Nuclease-free water	12.5 µl

THERMAL PROFILE

Initial Denaturation	98^{0}C, 4 min
Denaturation	95^{0}C, 10S
Annealing	62^{0}C, 40S
Extension	72^{0}C, 1min.
Final Extension	72^{0}C, 7 min.

5. PTV F1 and R1/R2 (Lab Designed, Expected product size-1243bp)

PCR reaction set up

PCR reaction contents	Quantity
10X Standard Taq Reaction Buffer	2.5 µl
10 mM dNTP	0.5 µl

PTV F1 (10µM)	1.0 µl
PTV R1/R2 (10µM)	1.0 µl
Taq DNA Polymerase (5u/ µl)	0.13 µl
Template DNA	3.0 µl
Nuclease-free water	16.87 µl

THERMAL PROFILE

Initial Denaturation	95^0C, 30S
Denaturation	95^0C, 40S
Annealing	48^0C, 40S
Extension	68^0C, 1min 20S
Final Extension	68^0C, 10 min.

6. TaqMan real time PCR for PTV [In house developed (TaqMan) assay]

PCR reaction contents	**Quantity**
Kapa qPCR Probe Fast Buffer	10 µl
PTV F1(10µM)	0.5µl
PTV R1 (10µM)	0.5 µl
PTV P1(10µM; Probe)	1.0 µl
Template DNA	3.0 µl
Nuclease-free water	10.0 µl

THERMAL PROFILE

Initial Denaturation	95^0C, 3 min
Denaturation	95^0C, 3S
Annealing	53^0C, 20S
Extension	72^0C, 20S

7. PEV8a & PEV8b (Zell *et al.,* 2000; Expected product size-383bp)

PCR reaction set up

PCR reaction contents	**Quantity**
10X Standard Taq Reaction Buffer	2.5 µl
10 mM dNTP	0.5 µl
PEV8a (10µM)	1.0 µl

PEV8a (10µM)	1.0 µl
Taq DNA Polymerase (5u/ µl)	0.125 µl
Template DNA	2.0 µl
Nuclease-free water	17.875 µl

THERMAL PROFILE

Initial Denaturation	95^{0}C, 4 min.
Denaturation	95^{0}C, 40S
Annealing	45^{0}C, 40S
Extension	68^{0}C, 50S
Final Extension	68^{0}C, 7 min.

8. PEV9a & PEV9b (Zell *et al.,* 2000; Expected product size-491bp)

PCR reaction set up

PCR reaction contents	**Quantity**
10X Standard Taq Reaction Buffer	2.5 µl
10 mM dNTP	0.5 µl
PEV9a (10µM)	1.0 µl
PEV9b (10µM)	1.0 µl
Taq DNA Polymerase (5u/ µl)	0.125 µl
Template DNA	2.0 µl
Nuclease-free water	17.875 µl

THERMAL PROFILE

Initial Denaturation	95^{0}C, 4 min
Denaturation	95^{0}C, 40S
Annealing	50^{0}C, 40S
Extension	68^{0}C, 50S
Final Extension	68^{0}C, 7 min.

9. PEV9c & PEV9d (Zell *et al.,* 2000; Expected product size-313bp)

PCR reaction set up

PCR reaction contents	**Quantity**
10X Standard Taq Reaction Buffer	2.5 µl
10 mM dNTP	0.5 µl

PEV9c (10µM)	1.0 µl
PEV9d (10µM)	1.0 µl
Taq DNA Polymerase (5u/ µl)	0.125 µl
Template DNA	1.0 µl
Nuclease-free water	18.875 µl

THERMAL PROFILE

Initial Denaturation	95^0C, 4 min
Denaturation	95^0C, 40S
Annealing	50^0C, 40S
Extension	68^0C, 50S
Final Extension	68^0C, 7 min.

10. WBev 2626 F & WBev 3394 R (Boros *et al.*, 2012; Expected product size 768bp).

PCR reaction set up

PCR reaction contents	**Quantity**
5X Phusion Buffer	5.0 µl
10 mM dNTP	0.5 µl
WBev 2626 F (10µM)	1.0 µl
WBev 3394 R (10µM)	1.0 µl
DMSO	0.75 ml
Phusion Taq DNA Polymerase(5u/ µl)	0.25µl
Template DNA	2.0 µl
Nuclease-free water	14.50 µl

THERMAL PROFILE

Initial Denaturation	98^0C, 3 min
Denaturation	98^0C, 10S
Annealing	50^0C, 1 min.
Extension	72^0C, 1 min.
Final Extension	72^0C, 7 min.

11. K23 545F & K23 2905R (Boros *et al.*, 2011; Expected product size-2360bp)

PCR reaction set up

PCR reaction contents	Quantity
10X Platinum Taq Hi fidelity buffer Buffer	2.5 µl
50mM $MgSO_4$	1.0 µl
10 mM dNTP	0.5 µl
K23 545F (10µM)	1.0 µl
K23 2905R (10µM)	1.0 µl
Platinum Taq DNA Polymerase (5u/ µl)	0.10 µl
Template DNA	2.0 µl
Nuclease-free water	16.90µl

THERMAL PROFILE

Initial Denaturation	94^0C, 45S
Denaturation	95^0C, 20S
Annealing	50^0C, 30S
Extension	68^0C, 85S
Final Extension	68^0C, 10 min.

13. PEV 9 F2 & PEV 9 R2 (Lab designed; Expected product size-650bp)

PCR reaction set up

PCR reaction contents	Quantity
10X Standard Taq Reaction Buffer	2.5 µl
10 mM dNTP	0.5 µl
PEV 9 F2 (10µM)	1.0 µl
PEV 9 R2 (10µM)	1.0 µl
Taq DNA Polymerase (5u/ µl)	0.125 µl
Template DNA	2.0 µl
Nuclease-free water	17.875 µl

THERMAL PROFILE

Initial Denaturation	95^0C, 4 min
Denaturation	95^0C, 40S
Annealing	50^0C, 40S

Extension	68^{0}C, 50sec.
Final Extension	68^{0}C, 7 min.

13. TaqMan real time PCR for enterovirus G [In house developed (TaqMan) assay]

PCR reaction contents	**Quantity**
Kapa qPCR Probe Fast Buffer	10 µl
EV G F1(10µM)	0.5µl
EV G R1 (10µM)	0.5 µl
EV G P1(10µM; Probe)	1.0 µl
Template DNA	3.0 µl
Nuclease-free water	10.0 µl

THERMAL PROFILE

Initial Denaturation	95^{0}C, 3 min
Denaturation	95^{0}C, 3S
Annealing	55^{0}C, 20S
Extension	72^{0}C, 20 S

14. SVSV SA2 & SVSV SS4 for SVDV(Nu´nez *et al*. 1998; Expected product size-154bp)

PCR reaction set up

PCR reaction contents	**Quantity**
10X Standard Taq Reaction Buffer	2.5 µl
10 mM dNTP	0.5 µl
SVSV SS4 (10µM) (10µM)	1.0 µl
SVSV SA2 (10µM)	1.0 µl
Taq DNA Polymerase (5u/ µl)	0.125 µl
Template DNA	2.0 µl
Nuclease-free water	17.875 µl

THERMAL PROFILE

Initial Denaturation	95^{0}C, 4 min
Denaturation	95^{0}C, 40S
Annealing	50^{0}C, 40S
Extension	68^{0}C, 50S
Final Extension	68^{0}C, 7 min.

3.2.2.1.5 Analysis of PCR/RT-PCR amplicons

The PCR/RT-PCR amplicons (7 µl) were electrophoresed along with 100 bp plus molecular weight marker (Thermo Scientific) on a 1.5% agarose gel in 1X Tris borate EDTA (TBE) buffer (90 mM Tris borate, 2 mM DTA, pH- 8.0) containing 0.5 ug/ml ethidium bromide for 50 min at 70 V. Following electrophoresis, the gel was visualized under UV light and photographed in a gel documentation system (GelDoc- It ™, UVP).

3.2.2.1.6 Purification of PCR amplicons

PCR/RT-PCR amplicons from the target regions of PTV and EV G were purified using DNA extraction kit (GeneJET™ Gel Extraction Kit, Fermentas) as per manufacturer's recommended protocol. The DNA was recovered from agarose gels. Briefly, the PCR products were loaded on 1.7% agarose gel and run to separate the reaction products. The gel slice containing the desired DNA band was excised using a clean razor minimizing the time of UV exposure and placed the gel slice into a pre-weighed 1.5 ml MCT and weighed again. The weight of the gel slice was calculated. An approximate volume of gel slice was determined by weight (1 mg equals approximately 1 µl) and an equal volume of binding buffer was added to the tube completely covering the gel slice. The gel mixture was incubated for 10 min at 60^0C to dissolve the gel completely. All the centrifugation steps were carried out at 13,000 rpm for 1 minute at room temperature. The entire solubilised gel solution was transferred to the GeneJET™ purification column and centrifuged. The flow-through was discarded and 100 µl of binding buffer was added to the column and centrifuged. After discarding the filtrate, 700 µl of wash buffer was applied to the column and centrifuged. To avoid the residual ethanol in the purified DNA solution, the empty column was once again centrifuged. The column was placed in a new clean 1.5 ml MCT and 50 µl of elution buffer was added to the centre of the purification column membrane. The purified column bound DNA was retrieved by centrifugation. The purified PCR products were stored at -20°C till further use.

3.2.2.1.7 Cloning of PCR amplicons

PCR amplicons from target regions of PTV and EV G were cloned in pJET1.2/blunt cloning vector using CloneJET™ PCR product cloning kit (Fermentas) for sequencing as per manufacturer's recommended procedure as given below:

3.2.2.1.8 CloneJET™ ligation reaction and transformation

In the ligation reaction, the purified DNA fragment and pJET1.2/blunt cloning vector were used in 3:1 molar ratio. To a sterile 0.2 ml tube on ice, the following components were added, mixed gently and centrifuged briefly:

2X Reaction Buffer	10 µl
Purified PCR product	3 µl (0.15 pmol ends)
DNA Blunting Enzyme	1 µl
Nuclease-free water	4 µl

The above mixture was incubated at 70^{0}C for 5 minutes and then chilled on ice. Thereafter, the following contents were added to the blunting reaction mixture, mixed gently and centrifuged briefly:

pJET1.2/blunt Cloning Vector (50ng/ µl)	1 µl (0.05 pmol ends)
T4 DNA Ligase	1 µl

The ligation mixture was incubated at 22^{0}C for 15 minutes followed by 4^{0}C for one hour. The transformation was carried out by using frozen stock of competent *E.coli* cells of DH5α strain. Briefly, 10 µl of ligation mixture was added to 100 µl of competent cells and mixed gently on ice. The resultant mixture was incubated at 4 ^{0}C for 45 minutes. This was followed by heat shock at 42^{0}C for 90 seconds and then quick chill on ice for 3 minutes. 900 µl of SOC (Super Optimal broth with Catabolite repression) medium was added to the transformed bacterial mixture after bringing the mixture to room temperature and incubated at 37^{0}C in an orbital shaker (150 rpm) for 90 minutes. The grown bacterial culture was pelleted by centrifuging at 10,000 rpm for 10 minutes. The supernatant was discarded and the pellet was resuspended properly in 100 µl of LB media. The pre-warmed LB-ampicillin (50µg/ml, usb) agar plate was inoculated with the above bacterial culture by streak plate method and incubated overnight at 37^{0}C till the white colonies appear. Here blue/white screening was not required since only the recombinant clones containing the insert was propagated.

3.2.2.1.9 Analysis of recombinant colonies for selection of positive recombinant clones

White recombinant colonies appearing at 12-16 hr on ampicillin agar plates were picked up and inoculated in LB-ampicillin broth for analysis. 5 ml of LB-ampicillin broth was inoculated with the recombinant colony and incubated overnight at 37^0C in an orbital shaker (150 rpm). Colony PCR was performed using overnight grown culture as template to confirm and select the recombinant clones. The positive recombinant clones were identified and inoculated into 1.5 ml sterile MCT containing LB-ampicillin agar. After incubation at 37^0C overnight, the clones were stored at 4^0C till further use.

3.2.2.1.10 Plasmid DNA isolation and confirmation of orientation by plasmid PCR

The recombinant plasmid DNA was extracted from the clones having desired gene insert using Qiagen Plasmid Miniprep kit following the manufacturer's protocol.

For plasmid isolation, QIAGEN plasmid mini kit (Catalogue No. 12123) was used.

Procedure

1. Harvested the overnight bacterial culture by centrifuging at 6000xg for 15 min at 4 ÚC
2. Resuspended the bacterial pellet in 0.3 ml buffer P1
3. Added 0.3 ml Buffer P2, mix thoroughly by vigorously inverting 4-6 times, and incubated at room temperature (15-25ÚC) for 5 min.
4. Added 0.3 ml prechilled buffer P3, mixed thoroughly by vigorously inverting 4-6 times. Incubated on ice for 5 min. Mixed the solution until it was colorless.
5. Centrifuged at 14000-18000xg for 10 min at 4ÚC.
6. Equilibrated a QIAGEN – tip 20, by applying 1 ml buffer QBT, and allowed column to empty by gravity flow.
7. Applied the supernatant from step 5 to the QIAGEN tip and allowed it to enter the resin by gravity flow
8. Washed the QIAGEN –tip 20 with 2x2 ml buffer QC then allowed the buffer QC to move through the QIAGEN-tip by gravity flow.
9. Eluted DNA with 0.8 ml buffer QF into a clean 2 ml vessel.

10. DNA was precipitated by adding 0.56 ml room temperature isopropanol to the eluted DNA and mixed and centrifuged at 15000xg for 30 min at 4°C. Carefully decanted the supernatant.
11. Washed The DNA Pellet with 1 ml room temperature 70% ethanol and centrifuged at 15000xg for 10 min. Carefully decanted supernatant.
    Air dried the pellet for 5- 10 min and redissolved in a suitable volume of TE buffer.

3.2.2.1.11 Sequencing of PCR/ RT-PCR amplicons

The recombinant plasmids containing gene fragments of PTV/PSV/EVG isolate were subjected to DNA sequencing using pJET 1.2 forward and reverse sequencing primer Eurofins genomics Pvt Ltd. Bangalore, to determine the nucleotide sequence of the isolates. The identities of the amplified products were confirmed by direct sequencing of purified PCR products using respective forward and reverse primers used for PCR amplification at DNA sequencing facility of Eurofins, Banglore. The sequence data generated was received as colored electropherograms and text files.

3.2.2.1.12 Phylogenetic/molecular analysis

Nucleotide sequences of the gene fragments of the isolates were studied, analyzed using 'EditSeq' programme of 'Lasergene' version.6 (DNASTAR Inc, USA) software. The nucleotide sequences were aligned separately by using ClustalW method of 'MEGA6' programme. To establish the genotypes of the sequenced PTV/PSV/EV G strains of this study, a phylogenetic analysis based on VP 1/ P1 gene as well as complete gene sequence was performed. Apart from Indian isolates (from this study), sequences of isolates corresponding to different genotypes from various countries were retrieved from GenBank and used as input sequences. Multiple sequence alignment was carried out using ClustalW programme of MEGA v.6 software (Tamura *et al.,* 2013). Phylogenetic tree was constructed using MEGA v.6 software with p-distance as nucleotide substitution model. Neighbor-joining (NJ) was applied as the statistical method and reliability of the constructed tree was determined by 1000 bootstrap replicates. A similar approach was adopted for molecular characterisation of PTV/PSV strains based on complete genome.

3.2.2.2 TaqMan PCR assay

TaqMan PCR assay were performed using KAPA™ PROBE® FAST Universal qPCR Kit (KAPABiosystems) in Cepheid SmartCycler- real - time PCR cycler as per manufacturer's recommendation. The details of reaction set up and thermal profile is given in the previous section (polymerase chain reaction amplification).

3.2.2.2.1. Creating a standard curve for Quantitative TaqMan PCR assay:

For preparation of the standard curve, plasmid DNA (isolation described in section 3.2.2.1.10) containing the 321 bp and 491 bp fragment from 5' UTR region of PTV and EV G respectively, was used. The detailed procedure is described here for PTV. The plasmid DNA was first quantified (92.7 ng/µl) in nanodrop spectrophotometer and then required dilution was prepared in nuclease free water so that the plasmid would be at a workable concentration of 9.27 e-0.8 µg/ul. The PCR reaction was set up such that 3 ul plasmid DNA (serial dilutions) was pipetted into each PCR reaction.

Background: We have to prepare a standard curve in which the cloned NS5B target sequence is present at 100000000, 10000000, 1000000, 100000, 10000, 1000, 100,10 copies. The stock of plasmid DNA was determined to be 0.66 µg/µl by spectrophotometric analysis. The PCR reactions were set up such that, 3 µl of plasmid DNA were pipette into each PCR reaction.

Step 1: Calculation of the mass of a single plasmid molecules

Plasmid size = 2886 bp

Inserted target size =321 bp

So, the size of the entire plasmid (plasmid + insert) = 3207 bp

M= (n) (1.096 e-21µg/ bp)

Where

M = mass

n = plasmid size (bp)

e-21 = 10^{-21}

Hence, the mass of one plasmid molecule = 3207 bp x 1.096 x 10^{-21} g/bp= 3.514e-18 g

Step 2: Calculation of mass of plasmid containing the copy #s of interest, which is 100000000 to 10 copies

(Copy No. of interest) x (mass of single plasmid) = mass of plasmid DNA needed

So, (3.514e-18g) x (100000000copies) =3.514e-10g

The following table presents the calculated plasmid masses needed to achieve the copy numbers of interest:

Table 2. Calculation of mass of plasmid containing the desired copy #s

Copy#	Mass of Plasmid	Mass of plasmid DNA(g)
1000000000		3.514e-10g
100000000		3.514e-11g
1000000		3.514e-12g
100000	x 3.514e-18 g	3.514e-13g
10000		3.514e-14g
1000		3.514e-15g
100		3.514e-16g
10		3.514e-17g

Step 3: Calculation of the concentrations of plasmid DNA needed to achieve the copy #s of interest:

The mass needed (calculated in step - 2) is divided by the volume to be pippeted into each reaction.

In the present study, 3 µl plasmid DNA solutions were used in each reaction.

Table 3: Calculation of the concentrations of plasmid DNA needed to achieve the copy #s of interest

Copy#	Mass of Plasmid DNA needed (g)		Final concentration of plasmid DNA (g/µl)
1000000000	3.514e-10g		1.71e-10
100000000	3.514e-11g		1.71e -11
1000000	3.514e-12g		1.71e -12
100000	3.514e-13g	÷3 µl	1.71e -13
10000	3.514e-14g		1.71e -14
1000	3.514e-15g		1.71e -15
100	3.514e-16g		1.71e -16
10	3.514e-17g		1.71e -17

Step-4: Preparation of the serial dilution of plasmid DNA

Cloned sequences are extremely concentrated in purified plasmid DNA stocks. Hence, a series of serial dilutions were prepared to achieve a working stock of plasmid DNA for quantitative PCR applications.

The following formula was used to calculate the volume needed to prepare the 100000000 copy standard dilution (Dilution #2)

$$C1VI = C2V2$$

$$9.2e\text{-}08 \text{ X } V1 = 1.71e\text{-}10 \text{ X } 100$$

$$\text{So, } V1 = 0.12 \text{ µl}$$

Volume of diluents = 100 - 0.203 µl = 99.88 µl

Table 4: Preparation of the serial dilution of plasmid DNA

Dilution#	Source of plasmid DNA for dilution	Initialconc (g/ µl) C1	Volume of plasmid DNA (µl)	Volume of diluent (µl)	Final volume (µl) V2	Final conc (g/ µl) C2	Resulting copy #/3 µl
1	Dilution -1	9.27e-08	0.203	99.8	100	1.71e-10	100000000
2	Dilution -2	1.71e-10	10	90	100	1.71e-11	10000000
3	Dilution -3	1.71e-11	10	90	100	1.71e-12	1000000
4	Dilution -4	1.71e-12	10	90	100	1.71e-13	100000
5	Dilution -5	1.71e-13	10	90	100	1.71e-14	10000
6	Dilution -6	1.71e-14	10	90	100	1.71e-15	1000
7	Dilution -7	1.71e-15	10	90	100	1.71e-16	100
8	Dilution -8	1.71e-16	10	90	100	1.71e-17	10

In the above table, dilutions 1 to 8 were used for preparation of standard curve in the quantitative PCR application

3.2.3.4 Fluorescent *In situ* hybridization (FISH) employing Texas Red-X (NHS ester) labelled oligonucleotide probe for detection of PTV genome/Alexafluor 488 labelled probe for detection of EVG genome in formalin-fixed paraffin-embedded tissue sections

3.2.3.4.1 Tissue preparation and sectioning

10% buffered formalin-fixed tissues were paraffin embedded using standard histopathological procedures and paraffin-embedded blocks were processed by cutting 5-8 µ

thin sections on a rotary microtome (Leica) onto labeled, APES treated glass slides rendered nuclease free by baking at 120^{0}C for 8 hr. After tissue sections were taken on the sildes, the slides were let dry at RT for one hour followed by incubation at 56^{0}C for 30 min. Slides were subsequently stored at RT in a sealed container till further use.

3.2.3.4.2 Detection of PTV viral genome by FISH

In-house designed oligonucleotide probe labelled with Texas red (NHS ester) was got commercially synthesized from IDT, USA.

For entire FISH procedure, standard procedures to avoid nuclease contamination such as use of certified nuclease free reagents, preparation of all reagents in diethyl pyrocarbonate (DEPC) treated water or rendering them free of nuclease activity by DEPC-treatment and autoclaving and use of gloves throughout the protocol etc were followed.

3.2.3.4.3 Rehydration of formalin fixed-paraffin embedded tissue sections

Paraffin-embedded sections were deparaffinized and rehydrated by incubation at 56^{0}C for one hour followed by successive treatment with xylene thrice for 5 min each, xylene: absolute ethyl alcohol (1:1; once for 5 minutes), absolute ethanol thrice for 5 min each and 90%, 80%, 70% and 50% ethanol for 5 min and finally PBS bath twice for 5 min each.

3.2.3.4.4 Prehybridization

After rehydration, the sections were treated with 0.3% Triton X-100 in PBS for 15 minutes. Triton X-100 was poured off and the sections were washed in PBS bath twice for 5 minutes each. Excess PBS was shaken off and wiped around the sections using lint free tissue paper (KimWipes) and permeabilised the sections with 50-60 µl of Proteinase-K (10 µg/ml of 0.1M Tris-HCl EDTA buffer) for 30 minutes at 37^{0}C in a humidified chamber. After incubation, Proteinase-K was removed and its action was stopped by treating with 100mM glycine in PBS twice for 5 minutes each. Following digestion, the sections were post fixed in 4% paraformaldehyde in PBS for 10 min at RT followed by rinsing twice with PBS. In order to minimise non-specific binding, acetylation was performed with 50-60 µl of 0.25% v/v acetic anhydride in 0.1 M Triethanolamine-HCl buffer (pH-8) twice for 5 minutes. Excess acetic anhydride was poured off, wiped around the sections and the hybridisation chamber (SecureSeal^{TM}, Grace biolabs) was fixed over the sections so that the sections come to center of the circle. Pre-hybridization was carried out in the Hybridizer (Dako). For this, 55 µl of

hybridisation buffer (PerfectHyb Plus™ buffer, Sigma) and sheared salmon sperm DNA (Sigma) at a concentration of 1 µl/10 µl of hybridisation buffer were added in Secure Seal hybridisation chamber without producing bubble and incubated at 37^{0}C for 2 hours.

3.2.3.4.5 Hybridization

Hereafter all the steps were carried out in darkness or with minimum light. After prehybridization incubation, the buffer was removed from the sections by pipetting out. The hybridization reaction was carried out by applying 55µl hybridization buffer containing 15 picomole of probe and 5 µl of sheared salmon sperm DNA and the sections were incubated at 45^{0}C for 12 hrs in the Hybridiser.

3.2.3.4.6 Post hybridization washes

After the completion of incubation, the frames were removed from the slides and the slides were transferred to the glass container (HYBAID) containing 2X Saline Sodium Citrate (2XSSC) (NaCl-15 mM, Sodium citrate-1.5 mM, pH-7.2) with 0.1%SDS and washed with continuous rotation in the hybridisation oven (HYBAID) at room temperature for 15 minutes followed by washing at 40^{0}C for 15 minutes. Further washing was done in buffer with medium and high stringency at 40^{0}C for 15 minutes. First, washed in 1X SSC with 0.1%SDS followed by washing in 0.5X SSC with 0.1% SDS respectively. After washing, the slides were rinsed in PBS with 3-4 quick dips. Excess PBS was shaken off and wiped around the sections with lint free tissue paper and the sections were mounted with VECTASHIELD containing DAPI as fluorescent counter stain. Slides were allowed to dry at 37^{0}C for 10 minutes and observed under fluorescent microscope (Olympus BX41).

3.2.3.6 Cell culture isolation and identification of enteric picornaviruses

3.2.3.6.1 Cultivation of PK15 cells/IB-RS-2 cells

Precautions like disinfection of culture room by fumigation, ultra violet light irradiation, keeping the laminar hood dry and clean, mopping the working area with 70% ethanol etc. were taken to avoid bacterial contamination of the cell line.

For routine propagation, PK15/IB-RS-2 cells were cultured in 25 cm^2 (50 ml) plastic culture flasks (Nunc) with 5 ml Dulbecco's minimum essential medium (DMEM) containing 10% fetal bovine serum (FBS) as culture medium in a water jacketed humidified CO_2 incubator (Galaxy 170 R, New Brunswick) with 5% CO_2 environment. Two passages weekly with a

splitting ratio of 1:3 were done for routine propagation of cells. The passaging procedure is briefly outlined as follows. Splitting was carried by using HyQTase (Thermo Scientific) which is an ultra filtered, non- mammalian formulation used as a safe replacement for Trypsin/EDTA. Medium was removed from a 100% confluent 25cm^2 culture flask and the monolayer was washed with 5 ml of phosphate buffered saline (PBS) solution twice for 30 seconds. After washing, PBS was removed and 200 µl of undiluted HyQTase was spreaded over the monolayer for 30 seconds. 800 µl of fresh undiluted HyQTase was added after removing previously used detachment solution and incubated at 37^{0}C for five minutes until cells were detached from the surface and then the culture flask was filled up with DMEM containing 5% FCS to requisite volume depending upon the splitting ratio and type of dishes to be seeded.

3.2.3.6.2 Inoculation of PK 15/ IB-RS-2 cell culture with PTV/PSV/EV G infected faecal/tissue samples

Twenty four hour grown, 50-80% confluent PK15/ IB-RS-2 cell cultures were used for inoculation with infected faecal or intestinal samples. Approximately 1 g organ sample (pooled samples of intestines/lymph node) was homogenized in a sterile mortar in 9 mL cell culture medium containing antibiotic solution (Sigma) to produce a 10% organ suspension. Similarly a 10% faecal sample suspension was prepared in PBS (pH 7.2-7.4). The suspension was mixed with antibiotic antimycotic solution (Sigma, 100X) in 1: 100 ratio and left at RT for one hour and then centrifuged for 15 min at 2,500 g followed by filtration through a sterile 0.22 µm filter (Millipore). The resultant sterile filtrate was used for inoculation of cell cultures. 1 ml of the sterile test suspension was inoculated on a 50-80% confluent cell culture in 25 cm^2 flask to cover the monolayer. The cell cultures were incubated at 37^{0}C for 1 hour for virus adsorption and then washed once with PBS and overlaid with 4 ml of fresh DMEM containing 2% FCS and 0.5% antibiotic mix. The cell cultures were then incubated for 5 hrs at 37^{0}C in a 5% CO_2 incubator. Negative controls were processed in the same way. The cell cultures were frozen at -70^{0}C for one hour and then thawed (freeze and thaw cycle) three times and centrifuged at 2,500 g and the supernatant was again passaged. After four passages, the monolayer of PK-15/IBRS-2 was screened for nucleic acid by PCR.

3.2.3.6.3 Detection of PTV replication by RT-PCR

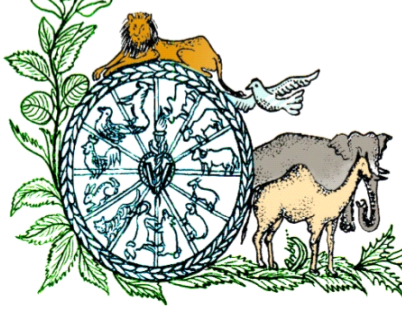
Control as well as virus inoculated flasks containing fourth passage cells were freeze thawed three times. 2 ml of the cell lysate from each flask was used for RNA extraction. The

harvested cell lysates were centrifuged at 13,000 rpm for 5 minutes at RT and the RNA was extracted from the 250 µLvolume of resultant supernatant by Trizol method as described in the section 3.2.2.1.2. Later, PCR was carried out to confirm the replication of PTV with PEV 1a and PEV 1b primer set, amplifying a 321 bp fragment. The reaction set up was same as that mentioned in the earlier section (Polymerase chain reaction amplification).

3.2.3.7 Next generation Sequencing

To obtain complete genome sequence one isolate of each PTV and PSV virus was used for next generation sequencing at PDFMD, Mukteshwar. Breifly, total RNA was extracted from 460 µl of cell culture supernatant using RNeasy Mini Kit (Qiagen) according to manufacturer's protocols. Total RNA was eluted in 50 µl of nuclease-free water and quantified using the Qubit RNA High Sensitivity (HS) Assay Kit (Life Technologies) according to manufacturer's instructions. Genomic DNA was removed from extracted total RNA samples TURBO DNA-*free*™ Kit (Life Technologies). First-strand cDNA synthesis (reverse transcription) was performed using Superscript III First-Strand Synthesis System (Life Technologies) according to the manufacturer's instruction. Second-strand synthesis of cDNA was performed using NEBNext® mRNA Second Strand Synthesis Module (NEB) as per manufacturer's instructions. 100 nanogram of each dsDNA sample was used to prepare sequencing libraries using the Ion AmpliSeq™ Library Kits v2.0 (Life Technologies) according to manufacturer's instructions. Size selection was performed followed by library amplification using AmpliSeq™ Library Kits v2.0. Smear analysis was performed on the Agilent® Bioanalyzer® instrument with the Agilent High Sensitivity DNA Kit. For template preparation, the library was hybridized to the Ion Sphere particles (ISPs) in a process that involves emulsion PCR, bead breaking, and enrichment on the Ion One Touch System. Enriched ISPs were loaded on the Ion PI™ Chip v2 and sequencing was performed on Ion Proton™ Platform (Life Technologies). The run analysis was analyzed by FastQC tools and the short sequences were aligned on the Server using Alignment tools with reference to the consensus sequence of the virus available in public domain. The aligned sequence was visualized by using Integrative Genomic Viewer tool from Broad Institute (www.broadinstitute.org/igv). From the obtained sequences phylogenetic tree was constructed using MEGA v.6 software with p-distance as nucleotide substitution model. Neighbor-joining (NJ) was applied as the statistical method and reliability of the constructed tree was determined by 1000 bootstrap replicates.

Results



4 RESULTS

4.1 Sample collection for study of prevalence

Faecal sample were collected from piggeries in Uttar Pradesh and Bihar. Farms sampled in UP were Swine Production Farm in IVRI, Izzatnagar, Premnagar slaughterhouse, Bareilly, Star Piggeries, Meerut, AVS pig farm at Chiraudi, Ghaziabad, and from backyard pig farms around Bareilly. In Bihar the samples were collected from backyard pigs in Saraiya and Marwan blocks in Muzzaffarpur district of Bihar, Bihar veterinary college, Patna. From Swine production farm, IVRI, 90 samples were collected (coded as SPF followed by no. of animals). From Premnagar slaughter house 20 samples were collected coded as IC 1-20 and then pooled into 4 samples and further processed. 5 representative samples were collected from an organized farm in Meerut. Six representative samples were from an organized farm in Ghaziabad (coded as GZB 1-6). Forty samples were from Muzzafarpur district of Bihar (coded as MZPR 1-40), Bihar veterinary college 17 samples were collected and coded as BVC1-17.

4.2 Result of Molecular Diagnostic Assays

4.2.1 Total RNA extraction from faecal/tissue samples

Spectrophotometric (Nanovue, GE, USA) quantitation of total RNA extracted from faecal or tissue samples collected during the study is presented in Table 5.

Table 5: Concentration of total RNA

Sl.No.	Sample ID	A_{260}/A_{280}	Concentration(µg/mL)	Types of samples
1	IC-1	1.793	184.6	Faecal sample
2	IC-2	1.877	173.9	Faecal sample
3	IC-3	1.254	529	Faecal sample
4	IC-4	1.779	158	Faecal sample
5	SPF-6	2.111	76	Faecal sample
6	SPF-7	1.876	72.8	Faecal sample
7	SPF-11	2.041	40.0	Faecal sample
8	SPF-9	2.174	40.0	Faecal sample
9	SPF-27	1.81	148.4	Faecal sample
10	SPF-37	1.408	67.6	Faecal sample
11	SPF-37A	1.706	81.2	Faecal sample
12	SPF- 60A	1.424	71.2	Faecal sample
13	SPF-63	1.716	159.2	Faecal sample
14	SPF-7c	1.75	67.2	Faecal sample
15	357/13-H	1.237	1030	Heart
16	358/13-H	1.544	1012	Heart
17	357/13-B	1.358	994.8	Brain
18	358/13-B	1.493	976	Brain
19	357/13-SP	0.933	977.2	Spleen
20	358/13-SP	1.522	1016	Spleen
21	379/13-I	1.418	1018	Intestines
22	379/13-B	0.925	993.2	Brain
23	379/13-SPLN	0.911	1016	Spleen , LN
24	379/13-LG	1.305	1063	lungs
25	379/13-T	1.55	855.6	tonsil
26	379/13-B2	1.452	979	Brain
27	380/13-I	1.771	949.6	Intestines
28	381/13-I	0.947	986.4	Intestines
29	381/13-B	1.119	750	Brain
30	381/13-LN	1.443	1013	Lymph nodes
31	348/13-LN	0.894	964.4	Lymph nodes
32	352/13-B	1.404	122	Brain
33	348/13-B	1.178	922.4	Brain
34	348/13-SP	0.879	919.6	Spleen
35	348/13-IC	1.545	906.8	Faecal samples
36	348/13-SK	0.914	980	Skin
37	352/13-LN	1.011	448	Lymph nodes
38	352/13-SP	1.099	957.2	Spleen
39	352/13-I	1.479	950	Intestines
40	348/13-I	1.414	346	Intestines
41	353/13-LN	1.225	994	Lymph nodes

42	359/13-L	1.218	930	Lungs
43	359/13-SK	1.31	920.8	Skin
44	353/13-I	1.2	1009	Intestines
45	353/13-SP	1.208	203	Spleen
46	359/13-B	1.005	694	Brain
47	359/13-IC	1.66	811.2	Faecal samples
48	359/13-I	1.21	2378	Intestines
49	359/13LN	1.45	928	Lymph nodes
50	359/13-SP	1.311	520.4	Spleen
51	353/13-B	1.721	353	Brain
52	453/13-IC	1.978	641.5	Faecal samples
53	453/13-F	1.682	688.4	Faecal samples
54	453/13-I	1.278	366	Intestines
55	454/13-IC	1.392	233	Faecal samples
56	454/13-F	1.887	220.4	Faecal samples
57	SPF15-1	2.27	104.4	Faecal samples
58	SPF15-2	1.778	134.4	Faecal samples
59	SPF-16	2.14	72.2	Faecal samples
60	SPF-28	2.20	124.4	Faecal samples
61	SPF-48	2.45	102	Faecal samples
62	92/14 C	0.993	2279	Colon
63	93/14 FS	1.496	918.4	Faecal samples
64	92/14 J	1.612	860	Jejunum
65	92/14 MLN	1.324	890.8	Lymph nodes
66	93/14 J	1.72	924.8	Jejunum
67	99/14 I	1.682	942	Intestines
68	99/14 C	0.992	923	Intestines
69	99/14 J	1.782	56	Intestines
70	100/14 C	1.665	867	Intestines
71	99/14 MLN	0.874	891.6	Lymph nodes
72	133/14 C	1.581	912.4	Brain
73	133/14 P	1.426	890.8	Brain
74	133/14 SC	1.58	839.6	Spinal cord
75	135/14 C	1.626	1610	Brain
76	135/14 P	1.803	1118	Brain
77	135/14 SC	1.421	903.6	Spinal cord
78	135/14 T	1.421	842.8	Tonsil
79	139/14 C	1.321	916.4	Brain
80	139/14 P	1.803	599.2	Brain
81	139/14 SC	1.661	547.6	Spinal cord
82	139/14 T	1.341	908.8	Tonsil
83	133/14 T	1.671	945	Tonsil
84	190/14 T	1.48	867.2	Tonsil
85	190/14 SP	1.56	810	Spleen
86	190/14 MLN	1.286	886	Lymph nodes
87	190/14 I	1.41	887.6	Intestines

88	190/14 L	1.457	615.6	Lungs
89	196/14 T	1.437	898.4	Lungs
90	196/14 SP	0.953	896.0	Spleen
91	196/14 LN	1.488	883.6	Lymph nodes
92	196/14 L	1.742	432	Lungs
93	GZB 2FS	2.22	111.2	Faecal samples
94	GZB 2 ES	1.731	391.2	Skin
95	GZB 6 FS	2.112	143.6	Faecal samples
96	GZB 1FS	2.118	200.8	Faecal samples
97	GZB 1ES	1.889	339.2	Skin
98	GZB 3 FS	1.998	394.8	Faecal samples
99	GZB 4FS	1.797	169.6	Faecal samples
100	GZB 5FS	1.837	454	Faecal samples
101	M 1F	1.794	139.2	Faecal samples
102	M 2 F	2.488	42.8	Faecal samples
103	M 4 F	2.082	131.6	Faecal samples
104	M 5 F	2. 10	52.8	Faecal samples
105	30/14 I &IC	1.85	202.4	Faecal samples
106	455/13 SK	1.61	850.2	Skin
107	446/13 B	1.78	430.2	Brain
108	446/13 CRB	1.46	505	Brain
109	446/13 I	1.92	398.2	Intestines
110	446/13 F	1.982	120.4	Faecal samples
111	449/13 B	2.02	312	Brain
112	449/13 I	1.88	148	Intestines
113	449/13 F	2.137	93	Faecal samples
114	101/14 I	1.025	227.8	Intestines
115	102/14 I	1.498	117.6	Intestines
116	103/14 I	1.656	382.4	Intestines
117	105/14 I	1.883	442.6	Intestines
118	220/14 I	1.506	563.4	Intestines
119	107/14 I	1.728	553	Intestines
120	107/14 B	1.826	390	Brain
121	109/14 I	1.195	787	Intestines
122	109/14 B	1.248	477	Brain
123	120/14 I	1.77	930	Intestines
124	120/14 B	1.472	622	Brain
125	130/14 B	1.664	624	Brain
126	130/14 I	1.384	530	Intestines
127	257/14 F	1.79	473	Faecal samples
128	257/14 Co	1.635	502.4	Intestines
129	257/14 B	1.428	794.8	Brain
130	257/14 IL	1.46	672	Intestines
131	258/14 F	1.159	836	Faecal samples
132	258/14 Co	1.577	756	Intestines
133	258/14 B	1.437	363	Brain

134	258/14 IL	1.468	1320	Intestines
135	309/14MLN	1.324	898	Lymph nodes
136	309/14 LN	1.71	836	Lymph nodes
137	309/14 Co	1.604	748.4	Intestines
138	309/14 B	1.117	540	Brain
139	309/14 F	1.721	126	Faecal samples
140	322/14 LN	1.37	725.6	Lymph nodes
141	322/14 B	1.334	1974	Brain
142	322/14 SI	1.579	400	Intestines
143	322/14 LI	1.678	827.6	Intestines
144	322/14 F	1.526	236.8	Faecal samples
145	322/14 T	1.026	799.2	Tonsil
146	83/14 F	1.7	185.6	Faecal samples
147	318/14 F	1.682	220.8	Faecal samples
148	318/14 I	1.76	690	Intestines
149	320/14 F	1.821	108.8	Faecal samples
150	321/14 F	1.892	122.7	Faecal samples
151	323/14 F	1.769	165.6	Faecal samples
152	323/14 I	1.518	772	Intestines
153	352/14 F	1.804	70	Faecal samples
154	352/14 I	1.28	764.8	Intestines
155	374/14 F	1.839	169.2	Faecal samples
156	374/14 I	1.668	887	Intestines
157	SPF 865	1.928	107.3	Faecal samples
159	221/14 I	1.662	690.2	Intestines
160	264/14 I	1.65	795.4	Intestines
161	264/14 IL	1.828	880.6	Intestines
162	264/14 F	2.08	98.2	Faecal samples
163	264/14 B	1.787	690.2	Brain
164	264/14 MLN	1.624	740.4	Lymph nodes
165	MZPR-1	1.78	95.4	Faecal samples
166	MZPR-2	2.250	71.2	Faecal samples
167	MZPR-3	1.625	110.2	Faecal samples
168	MZPR-4	2.00	63.2	Faecal samples
169	MZPR-5	1.549	44.0	Faecal samples
170	MZPR-6	1.898	44.8	Faecal samples
171	MZPR-7	1.672	77.6	Faecal samples
172	MZPR-8	1.866	131.6	Faecal samples
173	MZPR-9	1.785	116.2	Faecal samples
174	MZPR-10	1.543	144.4	Faecal samples
175	MZPR-11	1.831	90.8	Faecal samples
176	MZPR-12	1.378	78.8	Faecal samples
177	MZPR-13	1.577	94.0	Faecal samples
178	MZPR-14	1.979	78.0	Faecal samples
179	MZPR-15	1.544	79.2	Faecal samples
180	MZPR-16	1.498	123.6	Faecal samples

181	MZPR-17	1.586	121.7	Faecal samples
182	MZPR-18	1.53	80.8	Faecal samples
183	MZPR-19	1.781	78.8	Faecal samples
184	MZPR-20	1.582	89.2	Faecal samples
185	MZPR-21	1.741	87.2	Faecal samples
186	MZPR-22	1.984	115.8	Faecal samples
187	MZPR-23	1.851	110.2	Faecal samples
188	MZPR-24	1.662	96.4	Faecal samples
189	MZPR-25	2.18	124.0	Faecal samples
190	MZPR-26	1.92	106.8	Faecal samples
191	MZPR-27	1.866	119.3	Faecal samples
192	MZPR-28	1.721	133.7	Faecal samples
193	MZPR-29	1.75	78.0	Faecal samples
194	MZPR-30	1.646	127.0	Faecal samples
195	MZPR-31	1.983	89.6	Faecal samples
196	MZPR-32	1.664	82.8	Faecal samples
197	MZPR-33	1.78	110.5	Faecal samples
198	MZPR-34	1.65	135.4	Faecal samples
199	MZPR-35	2.128	118.0	Faecal samples
200	393/14 I	1.459	713.2	Intestines
201	393/14 LN	1.461	675.2	Lymph nodes
202	393/14 F	1.488	76.8	Faecal samples
203	394/14 I	1.704	610.8	Intestines
204	394/14 LN	1.676	525.2	Lymph nodes
205	394/14 B	2.037	178.4	Brain
206	394/14 F	1.902	280.0	Faecal samples
207	BVC-1	1.723	97.4	Faecal samples
208	BVC-2	1.988	109.2	Faecal samples
209	BVC-3	1.682	134.8	Faecal samples
210	BVC-4	1.923	112.3	Faecal samples
211	BVC-5	2.112	137.5	Faecal samples
212	BVC-6	1.773	102.8	Faecal samples
213	BVC-7	1.844	142.4	Faecal samples
214	BVC-8	1.913	161.7	Faecal samples
215	BVC-9	1.738	122.8	Faecal samples
216	BVC-10	1.948	98.4	Faecal samples
217	BVC-11	1.526	157.5	Faecal samples
218	BVC-12	1.813	118.3	Faecal samples
219	BVC-13	1.774	98.1	Faecal samples
220	BVC-14	1.94	146.4	Faecal samples
221	BVC-15	2.023	127.5	Faecal samples
222	BVC-16	1.856	116.4	Faecal samples
223	BVC-17	1.668	107.2	Faecal samples
224	SPF54 F	1.75	171.6	Faecal samples
225	SPF54 F2	2.262	132.0	Faecal samples
226	SPF 770 F	1.767	76.0	Faecal samples

227	SPF770 F2	2.17	81.6	Faecal samples
228	SPF770 F3	1.882	96.0	Faecal samples
229	SPF 770 F4	2.06	118.0	Faecal samples
230	454/14 I	1.456	1228.0	Intestines
231	457/14 I	1.280	330.0	Intestines
232	SPF C-1	1.469	102.8	Faecal samples
233	SPF C-6	2.17	80.0	Faecal samples

A_{260}= Absorbance at 260 nm wavelength
A_{280}= Absorbance at 280 nm wavelength

4.2.2. Result of polymerase chain reaction

4.2.2.1 PCR result of faecal samples/rectal swabs

Faecal samples/rectal swabs were screened for PTV, EV G and PSV by primers described earlier in material and methods sections. PTV positive samples yielded a 321 bp product specific for PTV (Fig 1) whereas EVG positive samples amplified a 491 bp product (Fig. 2). PSV positive samples yielded a 383 bp product (Fig. 3). Out of 190 faecal samples/ rectal swabs, 13 samples were found positive for PTV and 21 samples were found positive for EV G. Out of 70 faecal samples/rectal swabs, 5 samples were found positive for PSV. SVDV was not detected in any of the samples tested. The details of the PTV, PSV and EVG has been presented in table 6, 7 and 8 respectively. The prevalence of enteric picornaviruses have been presented in table 9. Faecal samples collected from muzzaffarpur, Meerut and backyard farm were negative for any of the virus under study. Remaining farms were found positive for one or more enteric picornaviruses. SPF, IVRI samples were positive for PTV, PSV as well as EVG. SVDV was not found in any of the samples.

Table 6: Faecal samples/Intestinal/rectal contents positive for Porcine teschovirus

Sl.No.	Sample ID	Age	Breed	Status of animals at time of collection	Signs, lesions
1	SPF 60	Adult	CB	Live	Apparently healthy
2	SPF 63	4 weeks	CB	Live	Apparently healthy
3	453/13	13 weeks	Local	Dead	Hepatitis, enteritis
4	454/13	13 weeks	CB	Dead	Hepatitis
5	SPF 15-2	5 weeks	CB	Live	Diarrhoea
6	SPF 48	5 weeks	CB	Live	Diarrhoea
7	309/14	Adult	Local	Dead	Septicaemia
8	320/14	6 weeks	CB	Necropsy	Enteritis

9	SPF 11	4 weeks	Local	Live	Diarrhoea
10	SPF C-1-2	5 weeks	CB	Live	Diarrhoea
11	SPF 751	9 weeks	CB	Live	Apparently healthy
12	SPF 7-2	6 weeks	CB	Live	Apparently healthy
13	SPF 7-3	6 weeks	CB	Live	Apparently healthy

Table 7: Faecal samples positive for Porcine Sapelovirus

Sl.No.	Sample ID	Age	Breed	Status of animals at time of collection	Signs, lesions
1	SPF C-6	5 weeks	CB	Live	Diarrhoea
2	323/14	9 weeks	CB	Dead	Autolysed
3	SPF 532	6 weeks	CB	Live	Diarrhoea
4	GZB 1	13 weeks	CB	Live	Diarrhoea
5	SPF 61-2	6 weeks	CB	Live	Apparently healthy

Table 8: Faecal samples positive for EV G Positive

Sl.No.	Sample ID	Age	Breed	Status of animals at time of collection	Signs, lesions
1	SPF 7-2	6 weeks	CB	Live	Apparently healthy
2	SPF 7-3	6 weeks	CB	Live	Apparently healthy
3	SPF C-6	5 weeks	CB	Live	Diarrhoea
4	SPF 9	5weeks	CB	Live	Apparently healthy
5	SPF 11	4 weeks	Desi	Live	Diarrhoea
6	SPF 37	5 weeks	CB	Live	Diarrhoea
8	SPF 751	9 weeks	CB	Live	Apparently healthy
9	SPF 758	9 weeks	CB	Live	Apparently healthy
10	SPF 774	9 weeks	CB	Live	Apparently healthy
11	SPF 781	9 weeks	CB	Live	Apparently healthy
12	SPF C-1-2	5 weeks	CB	Live	Diarrhoea
13	SPF 825	9 weeks	CB	Live	Apparently healthy
14	323/14	9 weeks	CB	Dead	Autolysed
15	374/14	22 weeks	CB	Dead	Haemopericardium
16	453/13	13weeks	Desi	Dead	Hepatitis, enteritis
17	454/13	13 weeks	CB	Dead	Hepatitis
18	BVC-4	6 weeks	CB	Live	Apparently healthy
19	BVC -9	6 weeks	CB	Live	Apparently healthy
20	BVC- 10	8 weeks	CB	Live	Apparently healthy
21	BVC -14	5 weeks	CB	Live	Apparently healthy

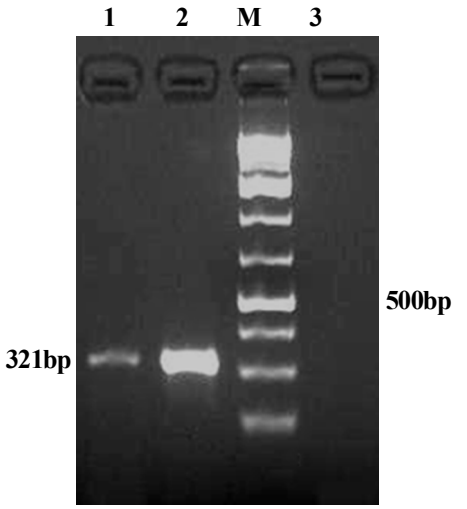


Fig.1: Ethidium bromide stained 1.5% agarose gel showing amplification of 321 bp fragment of PTV
Lane 1 : SPF 60
Lane 2 : SPF 63
Lane M: Marker
Lane 4 : NTC

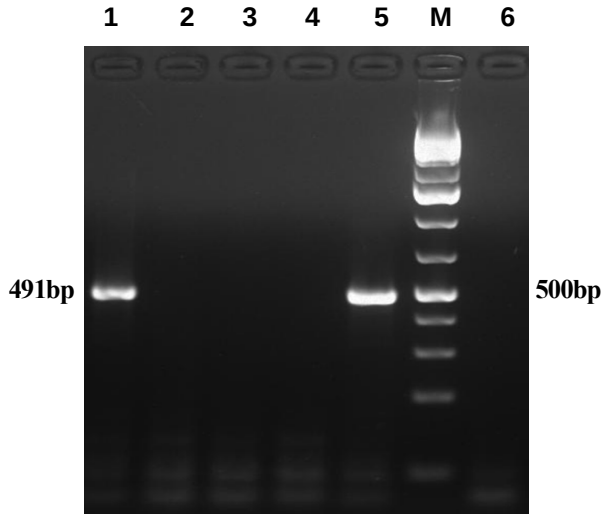


Fig.2: Ethidium bromide stained 1.5% agarose gel showing amplification of 491 bp fragment of EVG
Lane 1 : SPF 37
Lane 2 : SPF 63
Lane 3 : SPF 9
Lane 4 : SPF 27
Lane 5 : SPF 11
Lane M: Marker
Lane 6 : NTC

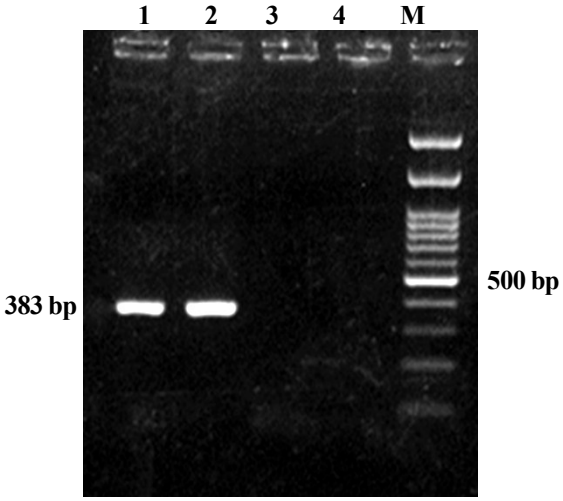


Fig. 3: Ethidium bromide stained 1.5% agarose gel showing amplification of 383 bp fragment of PSV.
Lane 1 : SPF C6
Lane 2 : GZb1
Lane 3 : SPF 37
Lane 4 : NTC
Lane L : Marker

Table 9: Prevalence of enteric picornaviruses based on RT-PCR

Organism	PTV	PSV	EV G	PTV+EV G	PSV+EV G	SVDV
No. Positive	13	5	21	7	2	0
No of cases tested	190	70	190	190	70	15
Percentage	6.84 %	7.14%	11.05%	3.68%	2.85%	0

4.2.2.2 PCR result of tissue samples from necropsied cases

Out of 52 necropsied cases only one intestinal and mesenteric lymph node sample was found positive for porcine teschovirus. No other organ was found positive for any of the enteric picornaviruses. However intestinal contents from 4 necropsied animals were found positive for PTV and intestinal contents from three animals were found positive for EVG. Details of PTV/EV G PCR positive cases are mentioned in table 10.

Table 10: Details of necropeied cases positive for enteric picornaviruses by PCR

Sl. No.	Necropsy No.	Breed	Age	Sex	Brand No.	Date of necrispy	Virus identified
1.	453 A/13	Desi	3 months	F	-	28.12.13	PTV, EVG
2.	454 A/13	CB	3 months	F	835	28.12.13	PTV, EV G
3.	309 A/14	Desi	Adult	M	33	7.7.14	PTV
4.	320 A/14	CB	43 days	M	272	16.7.14	PTV
5.	323 A/14	Landrace	64 days	F	865	18.7.14	EV G

4.3. Result of Virus isolation

4.3.1. Isolation of PTV

PTV was isolated successfully in IB-RS-2 cells from PTV positive faecal samples. The CPE was observed first in 2nd or 3rd passage in IB-RS-2 cells. Control flask kept in each passage showed no CPE (Fig. 4 and 5) The CPE was characterized by round, refractile cells grouped in foci, followed by detachment in 3-5 days(Fig. 6 & 7). In 6th passage the CPE reached about 90 % after which the cell culture supernatant was harvested and preserved

as viral stocks. A total of three isolates of PTV could be isolated out of which two were determined as PTV 5 and one isolate was determined as PTV-8 by sequence analysis. Presence of PTV was confirmed by RT-PCR and sequencing. Three isolates of Teschovirus were designated as IVRI/PTV/SPF 63/2013, IVRI/ PTV/SPF 15-2/2014 and IVRI/ PTV/SPF11-2/2015. First two isolates were determined as PTV 5 where as third isolate was designated as PTV-8 based on nucleotide sequences.

4.3.2 Isolation of PSV

PSV was isolated from PCR positive faecal samples in IB-RS-2 cells. The CPE was noticed in 1st or 2nd passage. The CPE was characterized by refractile cells, enlargement, rounding, increased cytoplasmic granularity, rosette-like appearance due to cytoplasmic protrusions through the cell membrane and detachment of the cells. Clear CPE (Fig. 8 to 10)was first observed on 3rd day PI in 2nd passage, and at day 4, the cells completely detached from the surface of the culture flask. In the subsequent passages, complete detachment of cells was seen within 24 hours. The growth of the virus in culture was confirmed by RT-PCR and sequencing. Three isolates of Porcine Sapelovirus were named as IVRI/PSV/ SPF C-6/ 2015, IVRI/PSV/ 323-14/2015 and IVRI/ PSV/GZB 1/2015.

4.3.2 Isolation of EV G

EV G could not be isolated even after repeated attempts in cultures such as IB-RS-2, PK-15 and Vero cell lines.

4.4 Development of Real time RT-PCR

Primers and probes were designed for PTV and EV G from the sequences identified in the laboratory during the study to develop TaqMan based assays for rapid and specific detection of PTV and EV G in clinical or necropsy specimen. The primer and probe sequences have been given in Material and methods section with thermal cycling condition. Since sapelovirus was identified later in the study so the sequences for PSV was not available so the real time assay could not be developed for this virus.

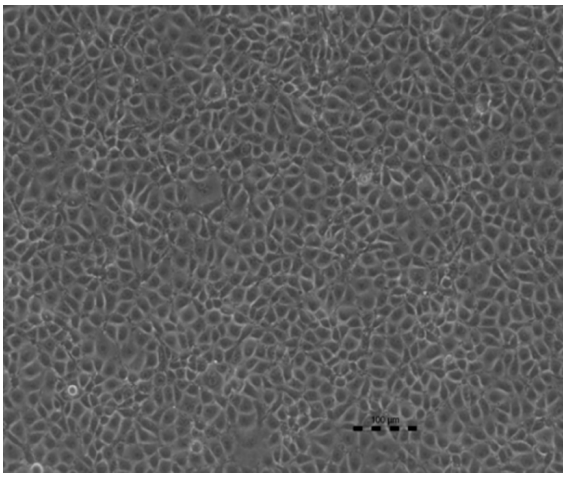


Fig. 4: Mock infected IB-RS-2 control cells, 3rd day, 10x

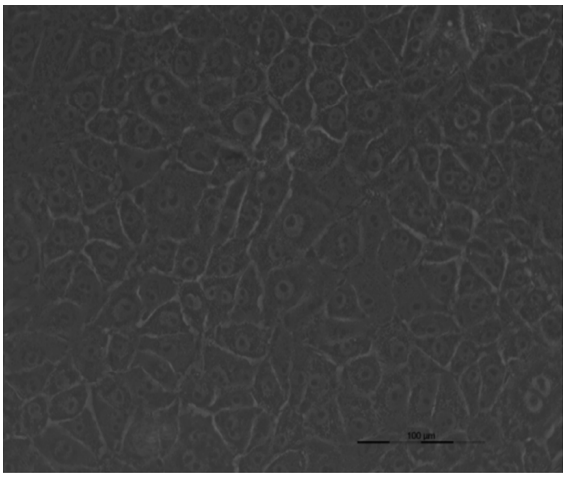


Fig. 5: Mock infected IB-RS-2 control cells, 3rd day, 20x

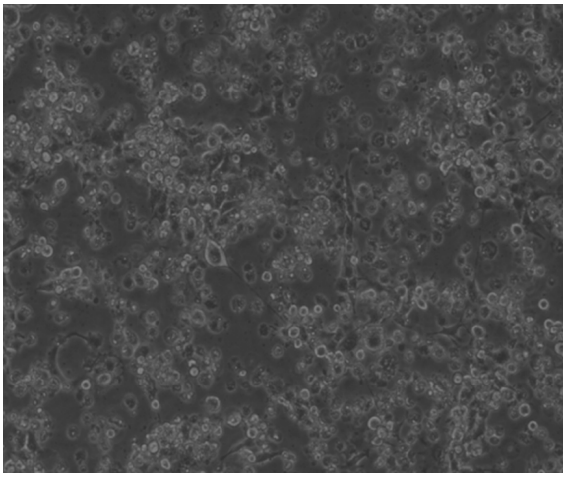


Fig. 6: Round refractile cells grouped in foci. PTV, SPF 63, 4th Passage, 3rd day PI, 10x

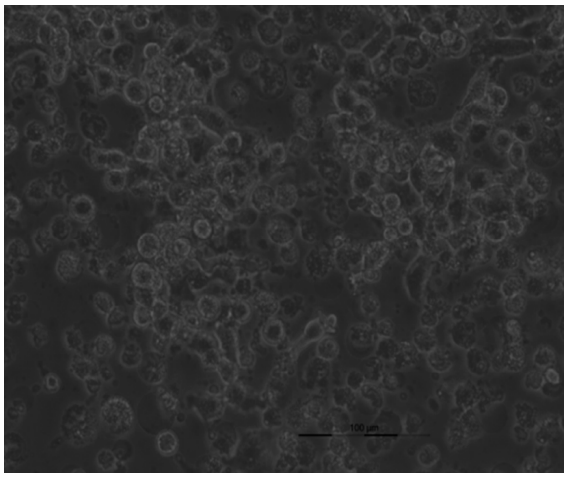


Fig. 7: Enlarged round refractile cells grouped together. PTV, SPF 63, 4th Passage, 3rd day PI, 20x

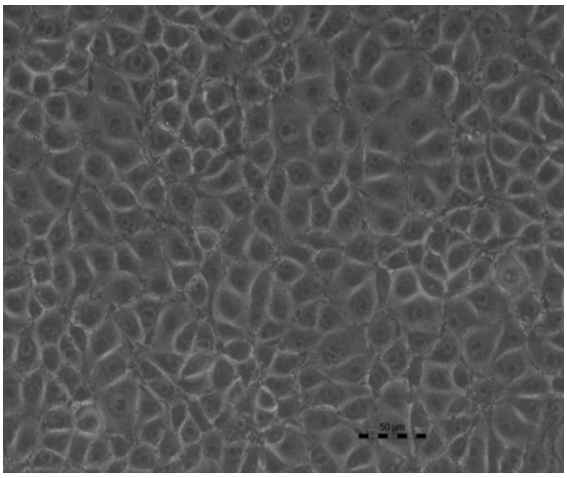


Fig.8: Mock infected IB-RS-2 control cells, 3rd day, 10x

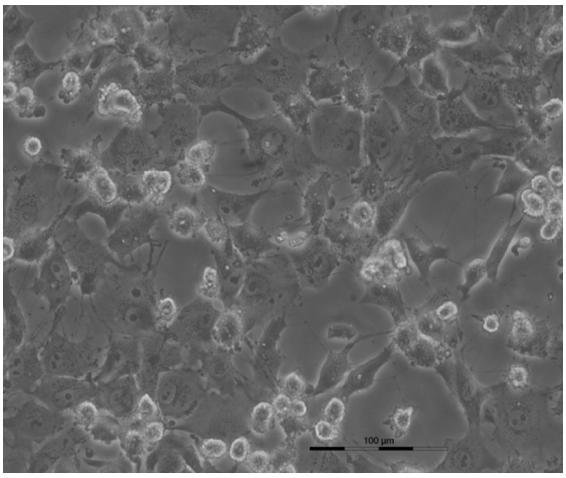


Fig.9a: Round enlarged refractile cells with cytoplasmic extension PSV, GZB 1, 2nd Passage, 4 hours PI, 10x

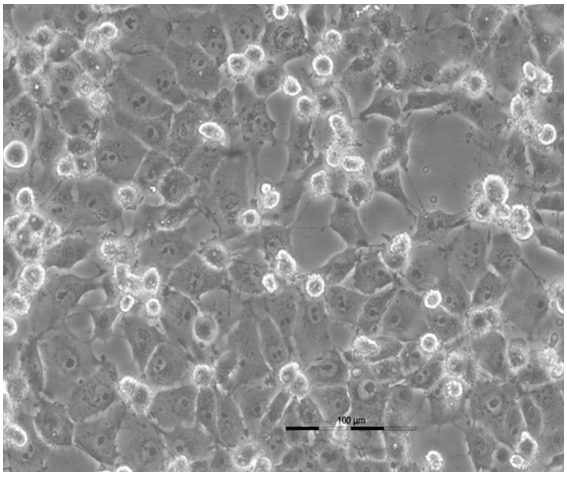


Fig.9b: Round enlarged refractile cells with increased granularity and rosette like appearance PSV, SPF C-6, 3rd Passage, 4 hours

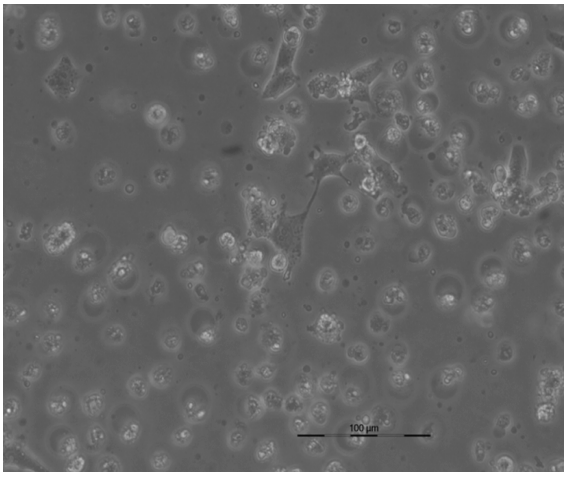


Fig.10: Detchement of cells from flask surface PSV, SPF C-6, 3rd Passage, 24 hours PI, 10x

4.4.1 PTV

4.4.1.1 Sensitivity

The standard curve was created using 10 fold dilution of plasmid containing 5' utr region of the PTV genome. The assay was able to detect from 107 to 102 copies of plasmid containing viral genome. A straight line was obtained as the standard curve with r2 value of 0.992 (Fig 11). The Ct values against plasmid concentration is given in the table 11.

Table 11: Real time PCR result for creation of standard curve for detection of PTV

Sl. No.	Plasmid conc.	FAM Ct
1	10000000	20.54
2	1000000	25.59
3	100000	28.59
4	10000	33.72
5	1000	36.84
6	100	39.89

4.4.1.2 Specificity

The assay was able to detect only PTV to the exclusion of related viruses such as porcine enterovirus (EV G), porcine Sapelovirus and other common viruses present in porcine such as classical swine fever (CSFV) and porcine circovirus 2 (PCV 2) (Fig. 12).

4.4.1.3 Identification of cell culture isolates

All the three isolates available in the laboratory were tested and identified with the assay developed in this study (Fig. 13). The Ct values obtained are given in the table 12

Table 12: Real time PCR results of the PTV isolates

Sl. No.	Sample ID	FAM Ct
1	PTV/SPF 63 CC	18.75
2	PTV/SPF 15	19.52
3	PTV/SPF 11-2	23.71
4	SPF 63 FS	29.72

4.4.2 Real time assay for porcine enterovirus (EV G)

4.4.2.1 Sensitivity

The standard curve was created using 10 fold dilution of plasmid containing 5' utr region of the enterovirus G genome. The assay was able to detect from 107 to 101 copies of plasmid containing viral genome. A straight line was obtained as the standard curve with r2 value of 0.979 (Fig 14). The Ct values against plasmid concentration is given in the table 13.

Table 13: Real time PCR result for creation of standard curve for detection of EV G

Sl. No.	Plasmid conc. (Copy No.)	FAM Ct
1	10000000	12.42
2	1000000	16.06
3	100000	19.86
4	10000	21.92
5	1000	26.06
6	100	27.71
7	10	29.28

4.4.2.2 Specificity

The assay was able to detect only porcine enteroviruses to the exclusion of related viruses such as porcine teschovirus (PTV), porcine Sapelovirus (PSV). Enterovirus G1 and EV G6 (two samples from different sources) available in the lab could be detected by this assay (Fig 15).

4.5. Gross Pathological findings

Enteric picornaviruses were identified in six necropsied cases. Of these cases, in only one case PTV could be identified in tissue samples. The gross lesions of GI tract and CNS found in these cases are described here. In live animals, these viruses were detected in animals with diarrhoea as well as in apparently healthy animals. The piglets with diarrhea had whitish to grayish yellow colored faeces (Fig 16). On necropsy examination the intestine was sometimes thin walled and filled with yellowish liquid contents (Fig 17), frequently congested visible from serosal layer as well as mucosa (Fig. 18, 19and 20) with whitish semi-solid or mucoid contents. Patchy areas of petechial haemorrhoges were also found in ileum mucosa (Fig 21). Gross lesions were visible mostly in small intestine portion of the GI tract where as caecum, colon

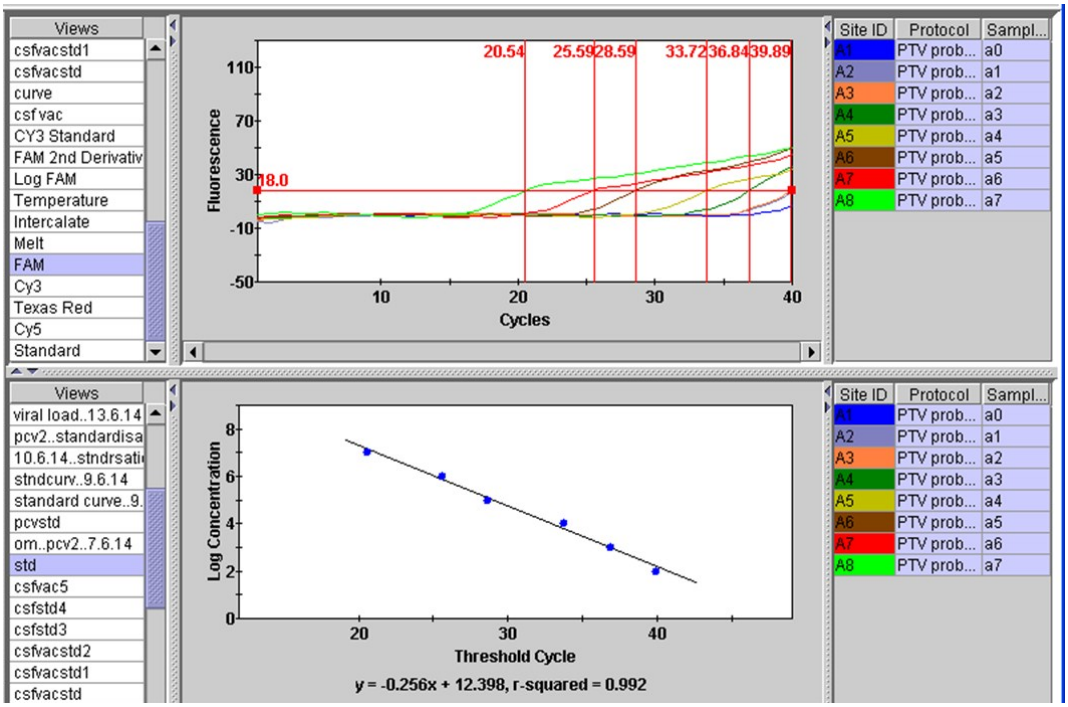


Fig.11: Amplification plot (above) and standard curve of serial dilutions of plasmid for testing sensitivity of TaqMan based Real-time PCR assay for PTV

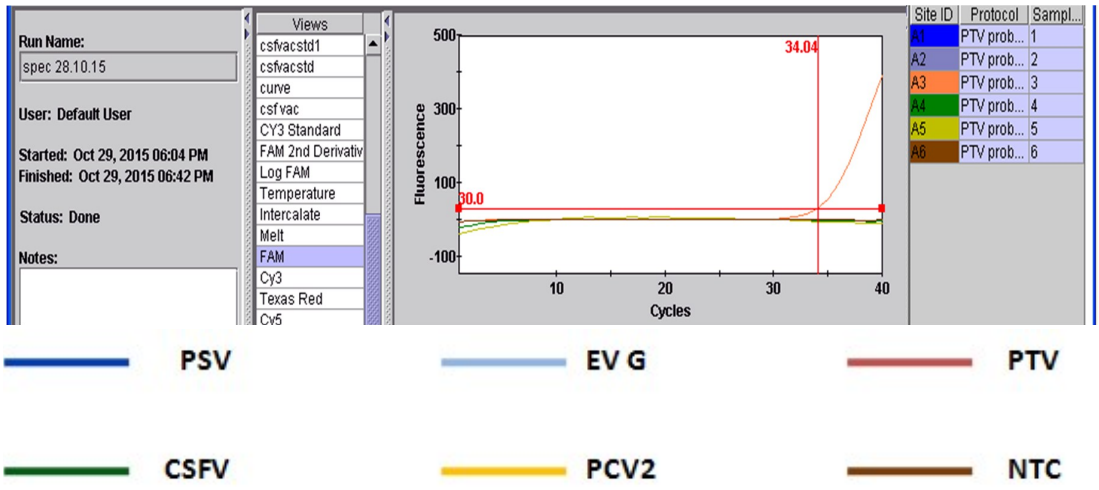


Fig.12: Amplification plot to determine specificity of TaqMan based Real-time PCR assay for PTV

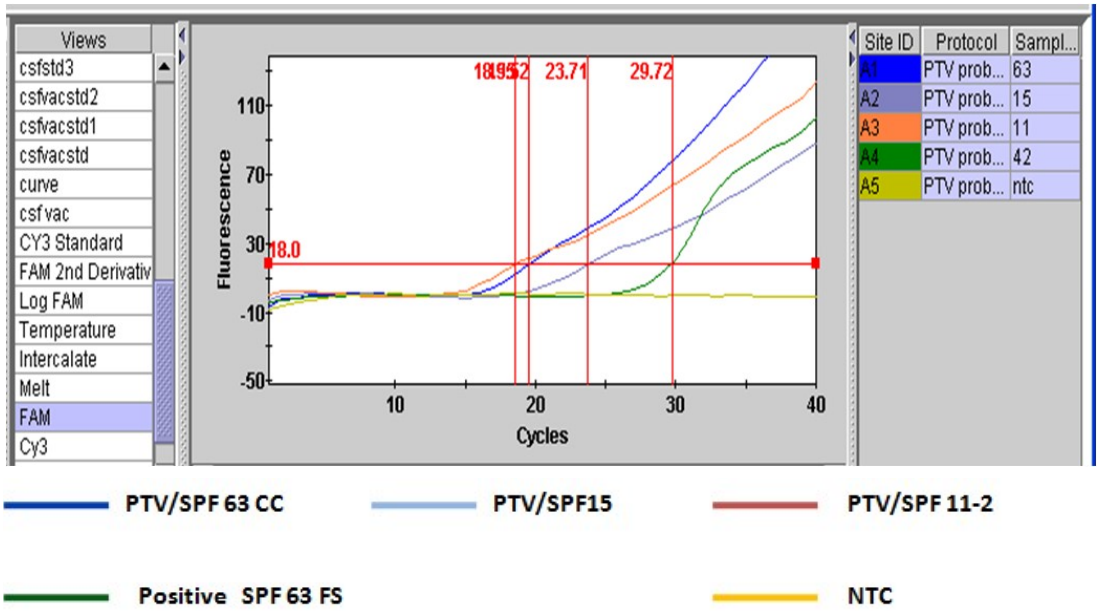


Fig.13: Amplification plot of real-time PCR of faecal samples for detection of cell culture isolates of PTV

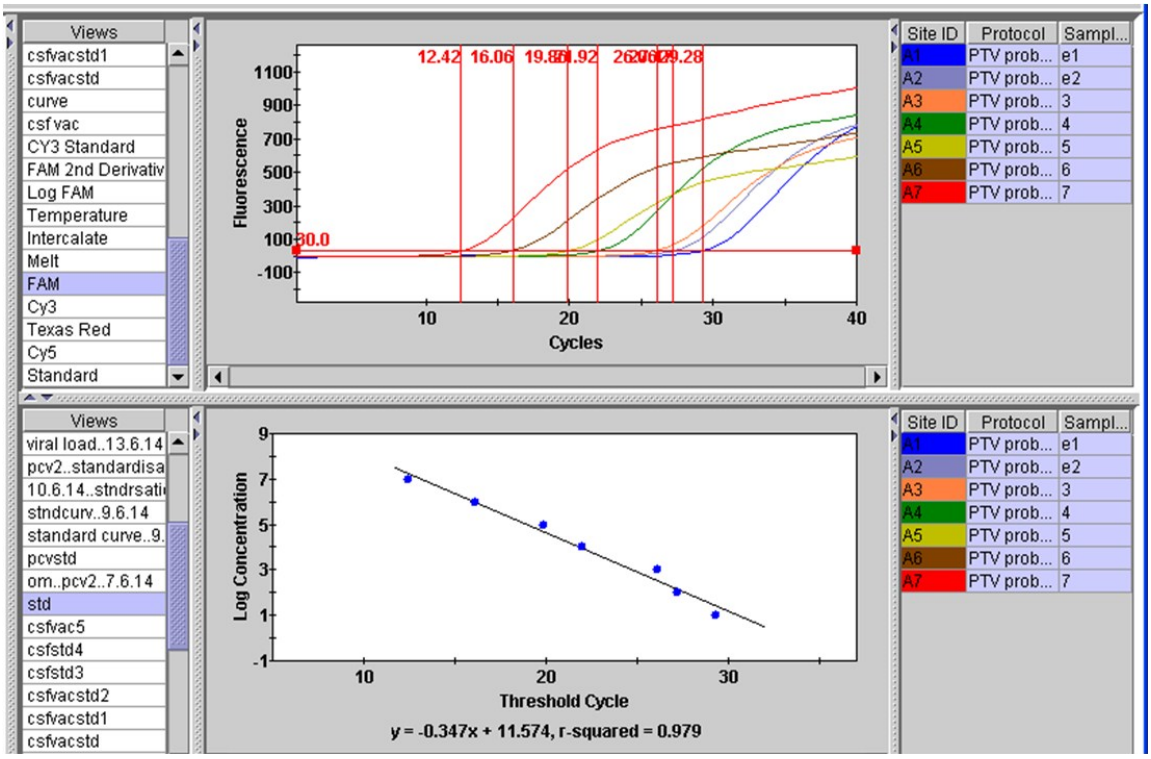


Fig.14: Amplification plot (above) and standard curve of serial dilutions of plasmid for testing sensitivity of TaqMan based Real-time PCR assay for Enterovirus G.

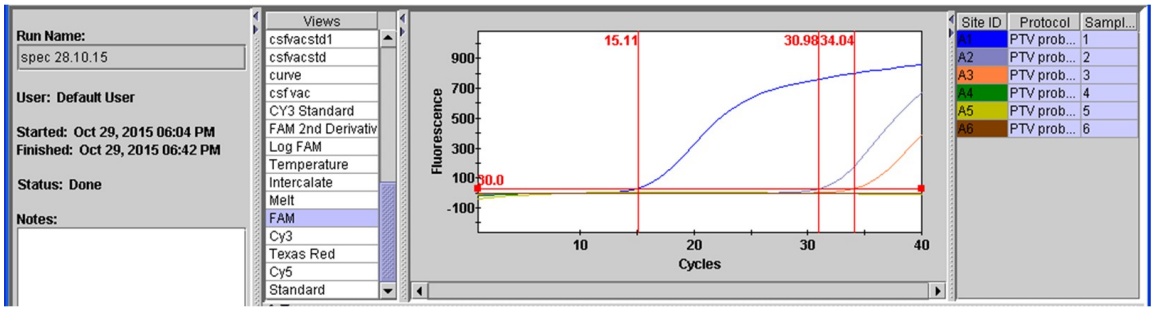


Fig.15: Amplification plot of real-time PCR of faecal samples for specific detection of EV G.

Fig.16: Grayish yellow diarrheic feces of a piglet, positive for PSV

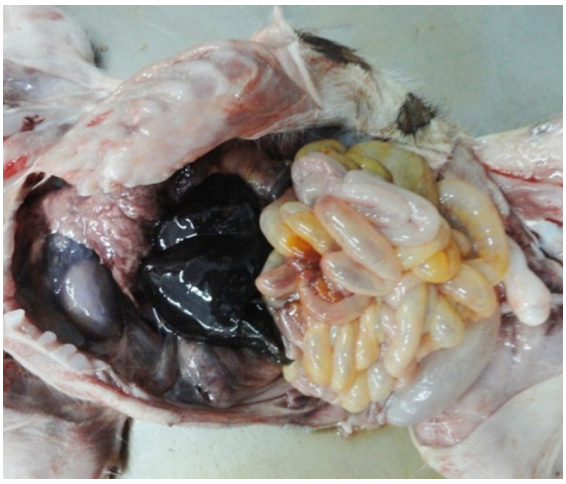


Fig.17: Distended thin walled intestines with yellowish contents, positive for EV G

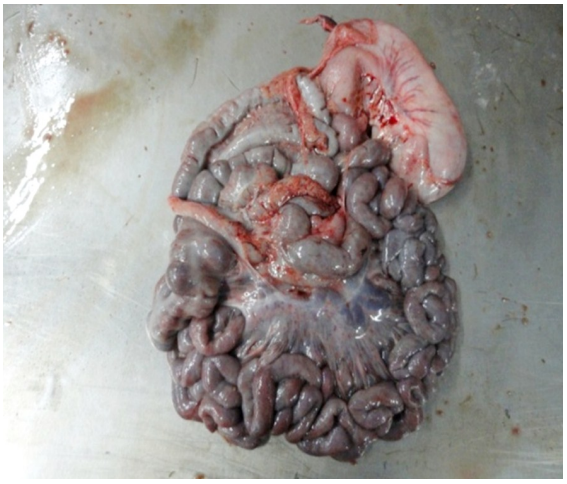


Fig.18: 309A/14: Congested intestines and mesenteric lymph nodes (PTV Positive)

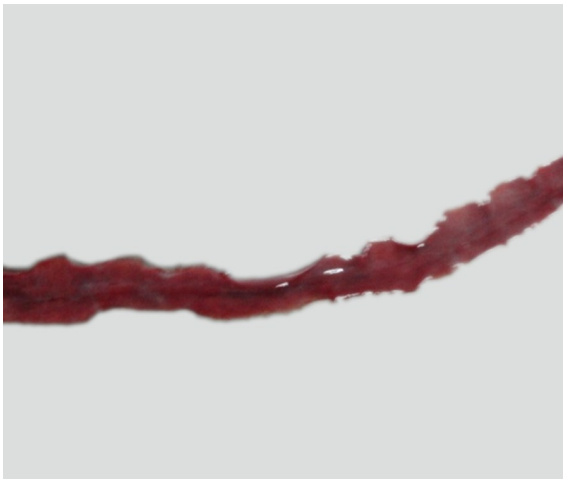


Fig.19: 309 A/14: Intestinal mucosal congestion (PTV Positive)

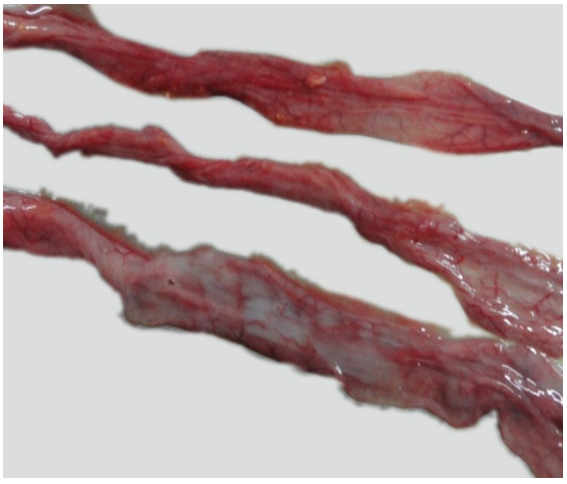


Fig.20: 320A/14: Intestinal mucosal congestion with mucoid content (PTV Positive)

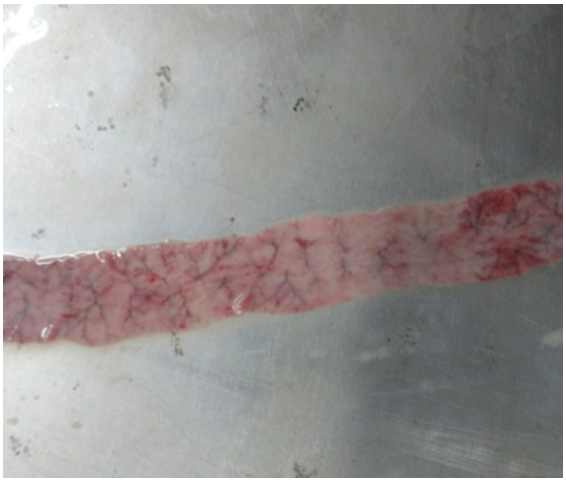


Fig.21: 320A/14: Intestinal mucosa showing petechial and echymotic hemorhhage (PTV Positive)

and rectum were free of gross lesions. Jejunum was the most affected portion (Fig 22 and 23). Mesenteric lymph nodes were congested (Fig 24). Extra intestinal lesions in liver was also found in one case where liver was enlarged and mottled with areas of haemorrhages interspersed with pale areas (Fig. 25 & 26) which might be due to non- infectious or infectious causes not of interest to this study. In CNS, only gross lesions of note were congestion of meningeal blood vessels (Fig. 27 & 28). In a farm in Chiraudi village in Ghaziabad which had history of two pigs dying of nervous symptoms grossly showed vesicular lesions in skin of ventral stomach, ears and around hooves of feet (Fig.29 & 30). The faecal samples of these animals were found positive for porcine sapelovirus.

4.6. Histo-Pathological Lesions

The major histo-pathological lesions in gastro-intestinal tract associated with PTV or EV G positive cases were inflammation of intestines along with desquamation of villi epithelium mainly in jejunum and ileum. There was severe congestion of intestinal blood vessels and destruction of villous epithelium of jejunum (Fig 31 & 33). Mucosal blood vessel congestion, desquamation of villus epithelium, goblet cell hyperplasia, infiltration of mononuclear cells in the ileal region of intestine (Fig. 32) were important lesions. Congestion of blood vessels and infiltration of mononuclear cells were also seen in duodenal mucosa (Fig. 34). Mononuclear cells infiltration in lamina propria and sub-mucosal blood vessel congestion was present in cardiac region of stomach (Fig 35, 36 & 37). Lymphoid cells depletion in follicles of cortical region and eosinophilic infiltration with mild vascular congestion of was seen in mesenteric lymph nodes (Fig 38 and 39). Loss of villous structures in jejunum (Fig. 40) and submucosal vascular engorgement, lymphoid depletion in Payer's patches in ileum was also present (Fig. 41).

CNS lesions were present in only two necropsy cases. The CNS lesions were characterized by perivascular cuffing with mononuclear cells and eosinophils (Fig 42, 43, 44 and 45), neuronal degeneration in spinal ganglion (Fig. 46), meningeal and parenchymal blood vessel congestion (Fig 47 & 48) and gliosis (Fig. 49). The lesions were present mainly in cerebrum, hippocampus and to a lesser extent in pons and medulla.

4.7 In situ hybridization

Probes were designed in an attempt to demonstrate PTV or PSV in tissue samples found positive for these viruses by RT-PCR. But these viruses could not be demonstrated in tissue by ISH even after repeated attempts.

4.8. Molecular Characterization

4.8.1. Molecular Characterization of PTV

The nucleotide sequences of VP1 gene of 4 isolates from this study were aligned with reference PTV sequence to derive 783 bp sequence of VP1 gene. BLAST analysis of VP1 gene sequence of two isolates named as SPF-15-2/2014 and SPF 63/CC/2015 showed maximum identity (81-84%) with PTV 5 with highest identity with PTV-5 strain F 26 (Accession No. AF296090). Third isolate designated in the study as SPF 11-2/2015 clustered with PTV- 8. The nucleotide identity of this isolate with PTV 8 ranged from 85-91% with highest identity of 91% with PTV 8 strain 25-&-VII (Accession No. AF296118). The fourth sequence designated as SPF 63/ 2013 shared 72% sequence identity with PTV-13 strain wild boar/ WB2C-TV/2011/HUN (Accession No. JQ425405). Other serotypes shared 70-72% identity with this strain indicating the possibility of a new serotype of PTV.

Phylogenetic analysis based on VP1 gene and construction of a phylogenetic tree was done by the maximum likelihood method with 1,000 bootstrap replicates. Four VP1 region sequences were obtained in this study and were analyzed along with 63 sequences retrieved from NCBI database to construct a phylogenetic tree. Phylogenetic analysis showed 13 clades corresponding to 13 genotypes of the PTV (Fig. 50). Two of the isolates (SPF-15-2/2014 and SPF 63/CC/2015) clustered with PTV-5. The third isolate designated in the study as SPF 11-2/2015 clustered with PTV- 8. The fourth sequence designated as SPF 63/ 2013 formed a clade with PTV-13 having 72% sequence identity with strain wild boar/WB2C-TV/2011/ HUN.

To further characterize the PTV one isolate namely SPF 63/CC/2015 was sequenced by Next generation sequencing (NGS) on Ion Torrent platform. The NGS yielded 7009 bp long sequence with 5'UTR, polyprotein and partial 3' UTR region. This isolate contained 6712 bp long polyprotein gene. Phylogenetic analysis of this isolate based on polyprotein gene was done along with polyprotein gene sequences of 39 PTVs representing 13 different serotypes. The phylogenetic tree is shown in Fig. 51. Based on polyprotein gene also this isolate clustered

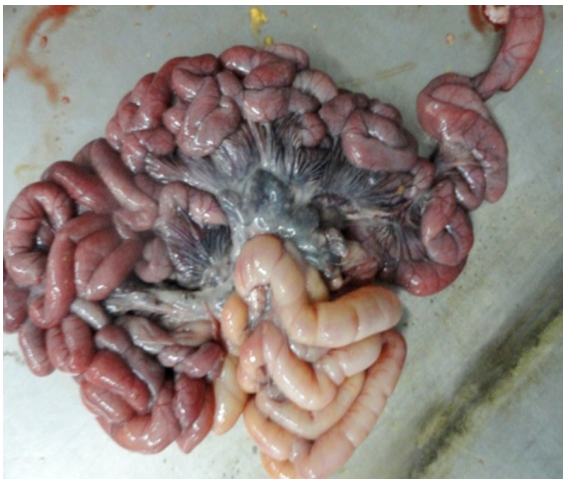


Fig.22: 453 A/13: Congestion of intestines and mesenteric lymph nodes (PTV and EV G Positive)

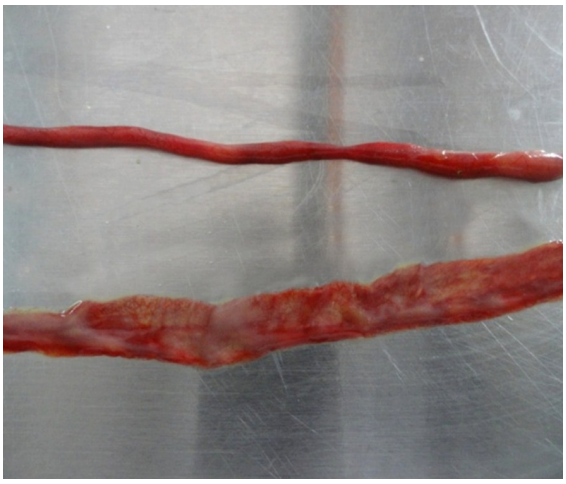


Fig.23: 453 A/13: Congestion of jejunum mucosa (PTV and EV G Positive)

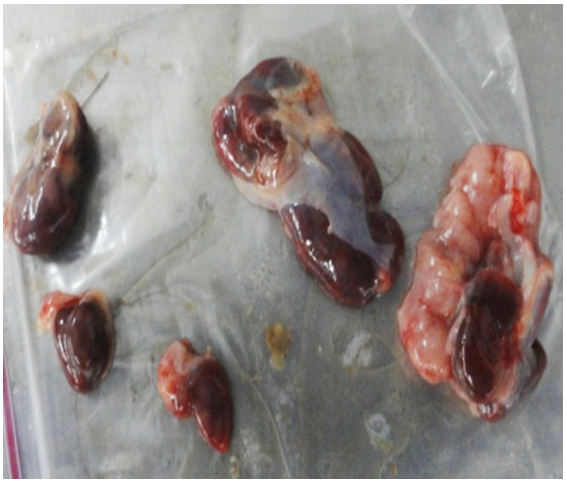


Fig.24: 320A/14: Congestion of mesenteric lymph nodes (PTV Positive)

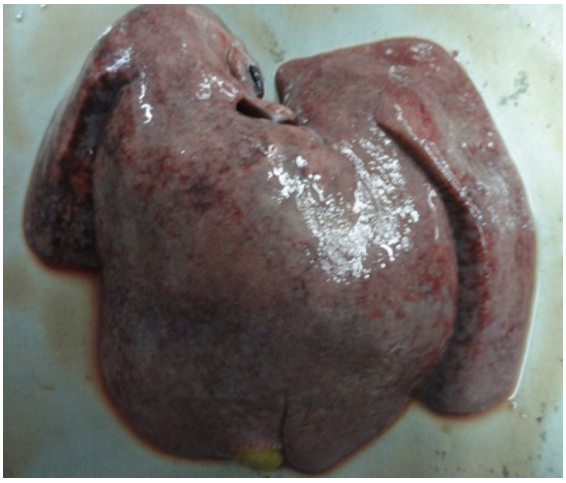


Fig.25: 454 A/13 : Mottled liver (Dorsal surface) with area of haemorrhages interspersed with non haemorrhagic areas.

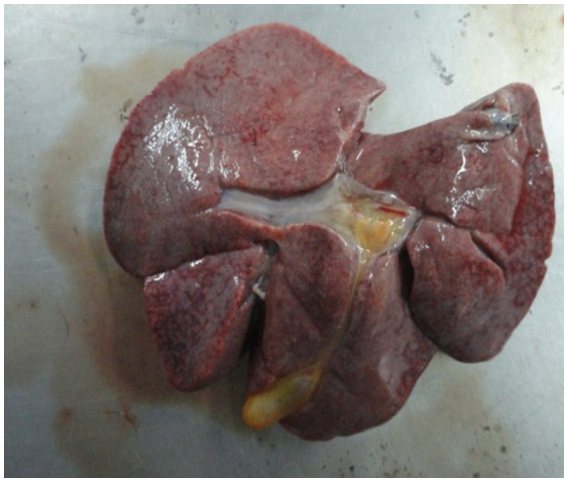


Fig.26: 454 A/13 : Mottled liver (Ventral surface) with area of haemorrhages interspersed with non haemorrhagic areas.

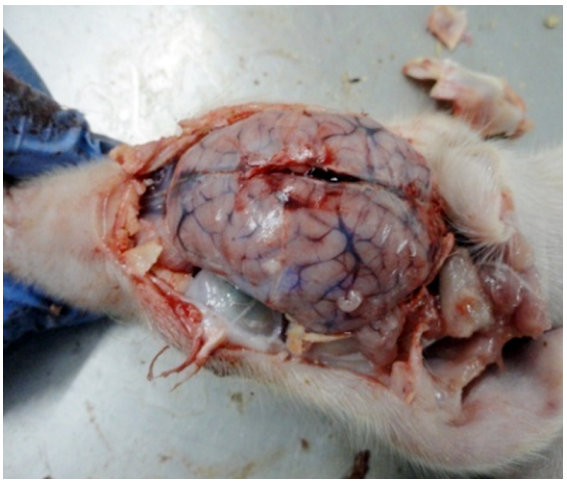


Fig.27: 320A/14: Congestion of meningeal blood vessels (PTV Positive)

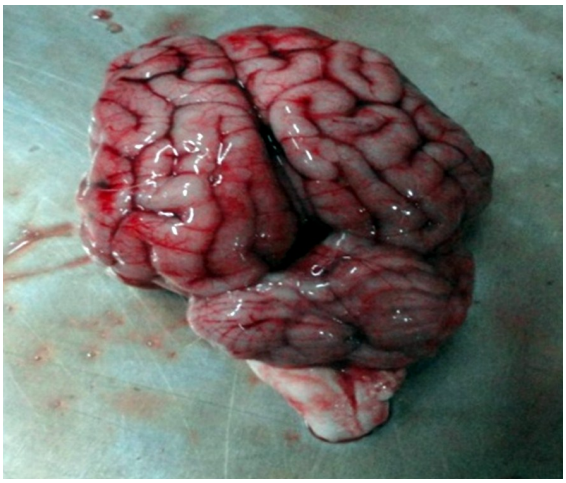


Fig.28: 320A/14: Congestion of brain blood vessels (PTV Positive)

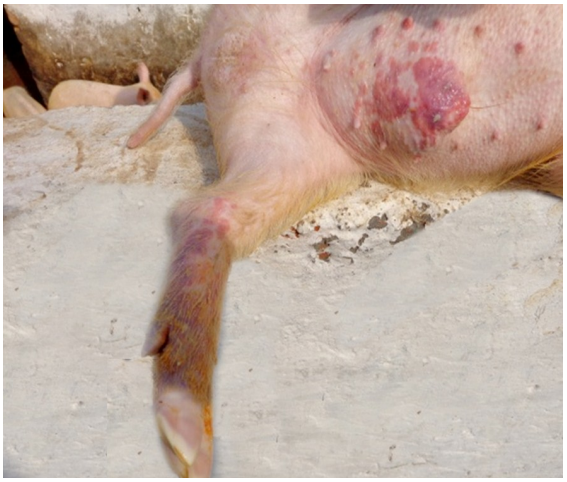


Fig.29: Vesicles in the ventral surface of abdomen of a pig (PSV Positive)

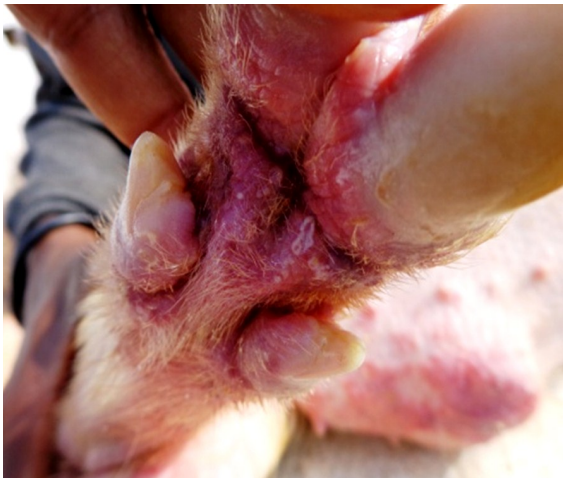


Fig.30: Broken vesicles on the plantar surface of feet and inter digital space of a pig (PSV Positive)

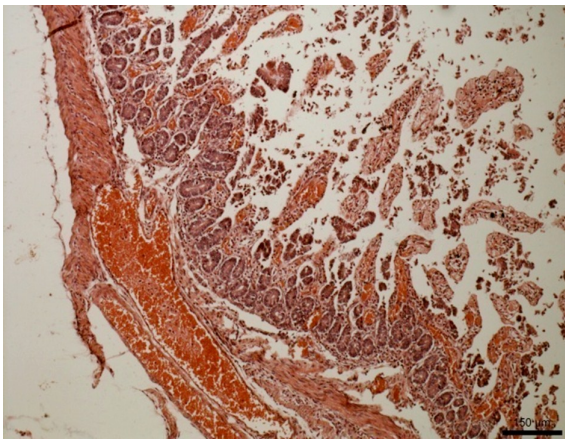


Fig.31: 320 A/14: Severe congestion of intestinal blood vessels and destruction of villous epithelium of jejunum. 10X, H&E (PTV Positive)

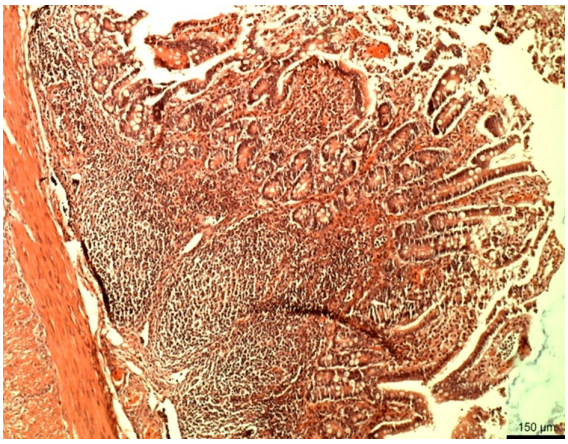


Fig.32: 320 A/14: Mucosal blood vessel congestion, desquamation of villus epithelium, goblet cell hyperplasia, infilteration of mononuclear cells in ileum region of intestine. 10 X, H & E (PTV Positive)

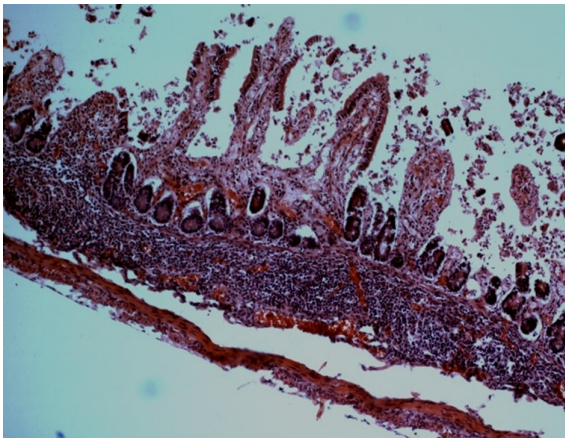


Fig.33: 453 A/14: Congestion, denudation of villus epithelium and infiltration of mononuclear cells. 10 X, H & E (PTV and EVG Positive)

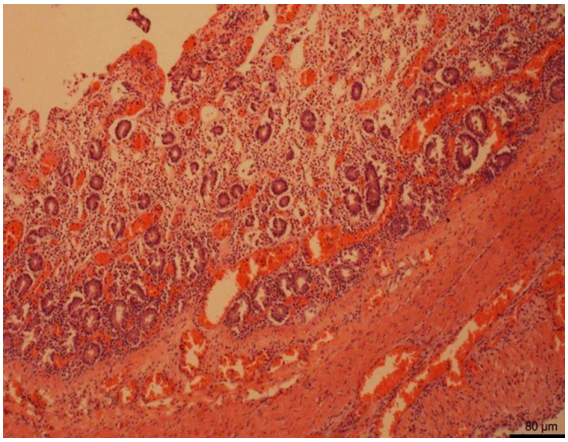


Fig.34: 453 A/14: Congestion of blood vessels and infiltration of mononuclear cells in duodenal mucosa. 10 X, H & E (PTV and EVG Positive)

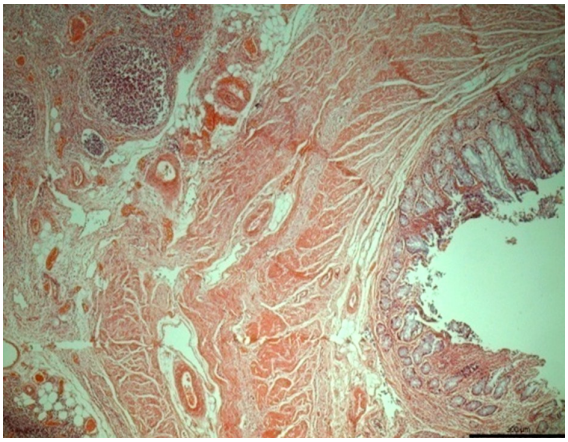


Fig.35: 309 A/14: Sub-mucosal blood vessel congestion of stomach cardiac region. 10 X, H & E (PTV Positive)

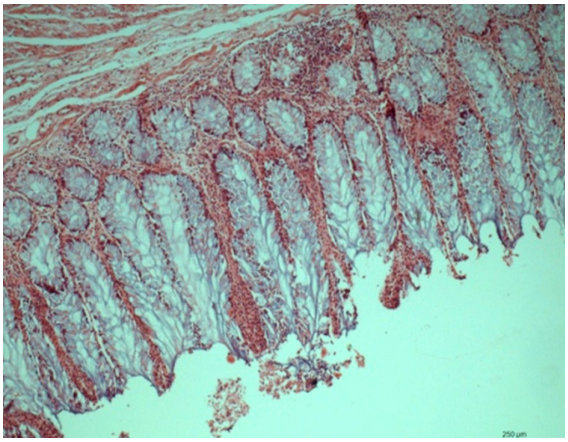


Fig.36: 309 A/14: Mononuclear cells infiltration in lamina propria of cardiac region of stomach. 10 X, H & E (PTV Positive)

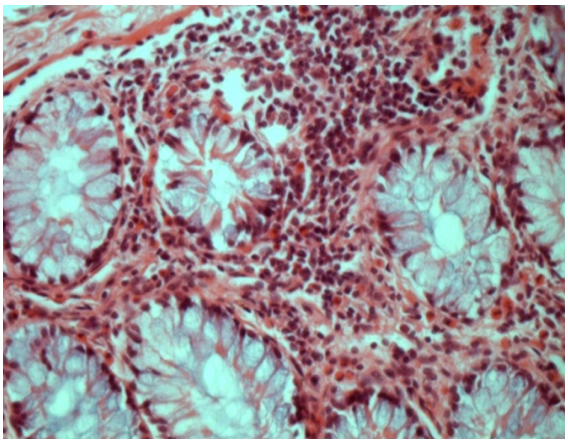


Fig.37: 309 A/14: Mononuclear cells infiltration in lamina propria of cardiac region of stomach. 40 X, H & E (PTV Positive)

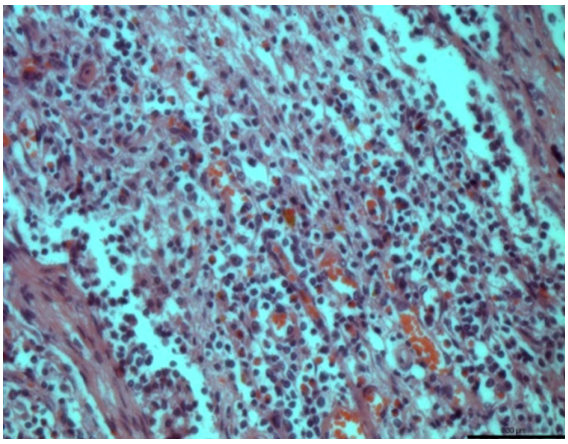


Fig.38: 309 A/14: Eosinophilic infiltration in mesenteric lymph nodes with mild vascular congestion. 10 X, H & E (PTV Positive)

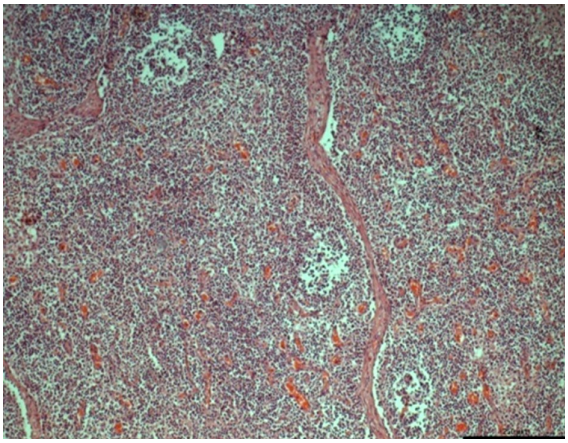


Fig.39: 309 A/14: Lymphoid cells depletion in follicles of cortical region of mesenteric lymph nodes. 10X, H & E (PTV Positive)

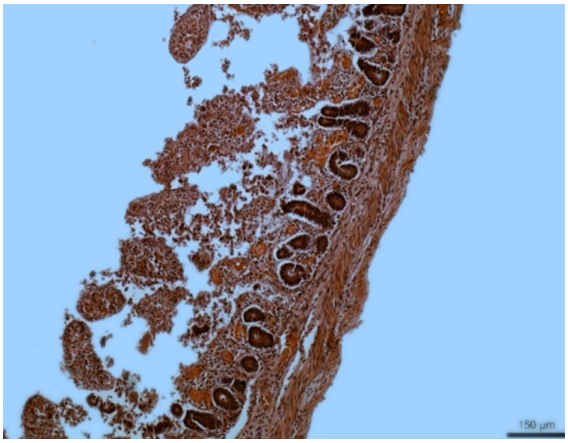


Fig.40: 309 A/14: Jejunum: Severe vascular congestion, loss of villous structure and desquamation of villi. 10 X, H & E (PTV Positive)

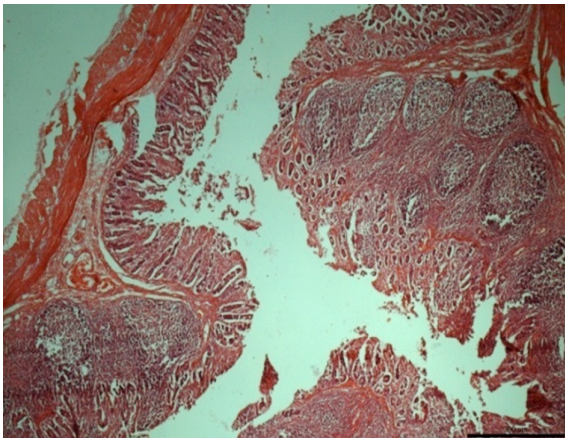


Fig.41: 309 A/14: Ileum: Submucosal vascular engorgement and lymphoid depletion in payer's patches. 10 X, H & E (PTV Positive)

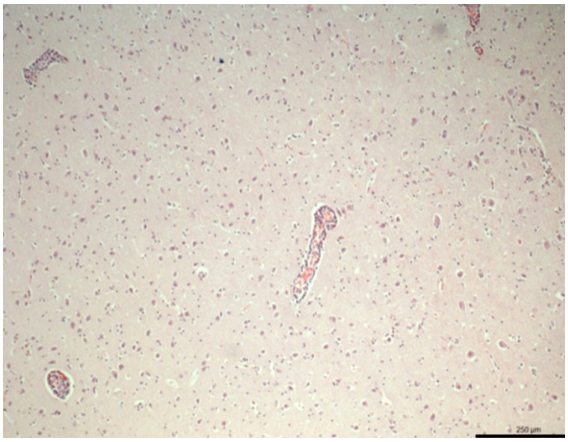


Fig.42: 309 A/14: Perivascular cuffing in pons region of brain. 10 X, H & E (PTV Positive)

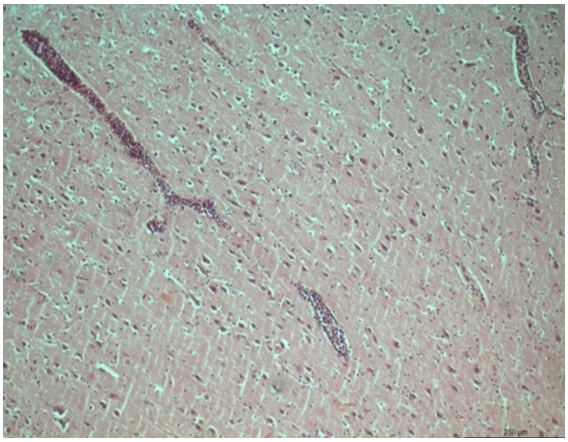


Fig.43: 309 A/14: Severe perivascular cuffing in cerebrum. 10 X, H & E (PTV Positive)

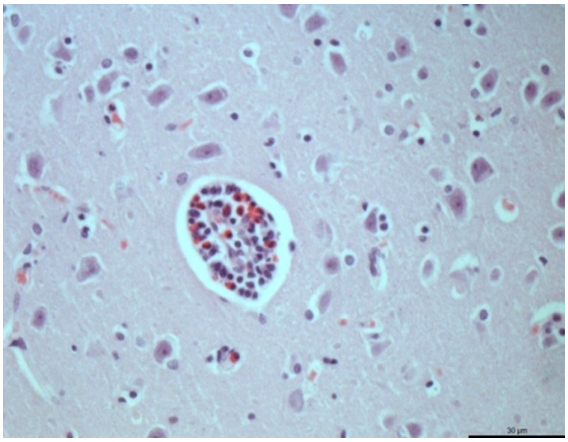


Fig.44: 309 A/14: Perivascular cuffing with mononuclear cells and eosinophils in pons region of brain. 40 X, H & E (PTV Positive)

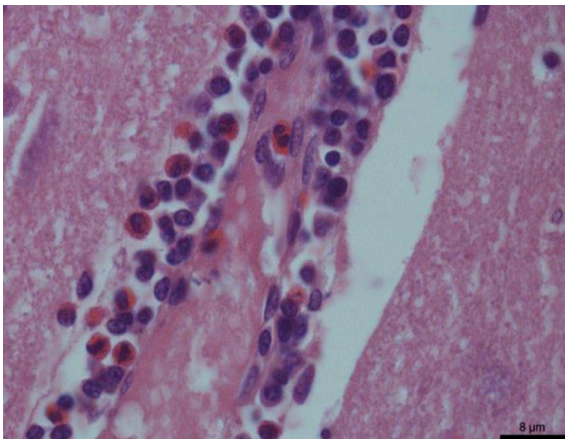


Fig.45: 309 A/14: Perivascular cuffing with mononuclear cells and eosinophils in pons region of brain. 100 X, H & E (PTV Positive)

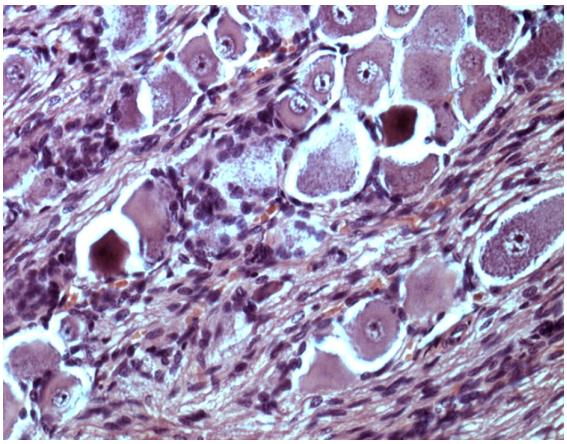


Fig.46: 309 A/14: Neuronal degeneration in spinal ganglion. 40 X, H & E (PTV Positive)

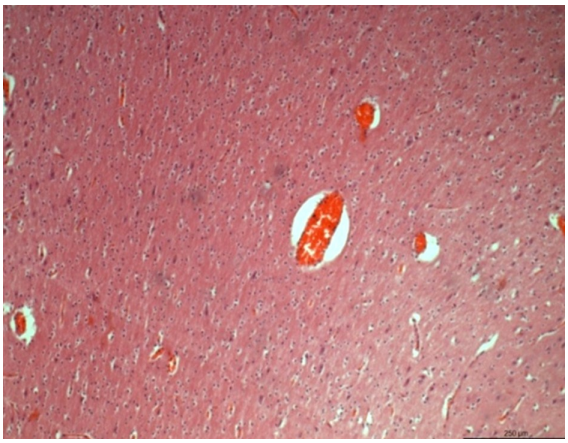


Fig.47: 320 A/14: Vascular congestion in brain capillaries. 10 X, H & E (PTV Positive)

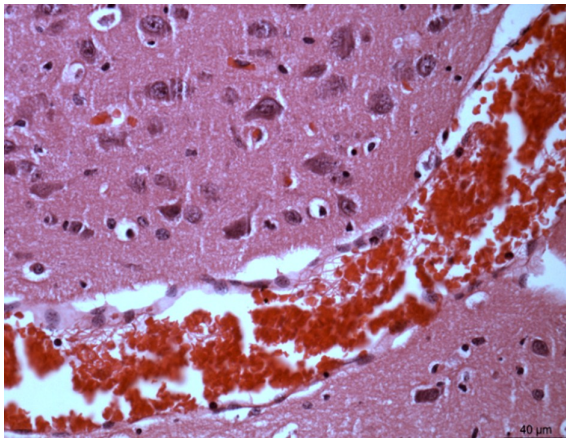


Fig.48: 320 A/14 Severe vascular congestion of blood vessel in cerebrum of brain. 40 X, H & E (PTV Positive)

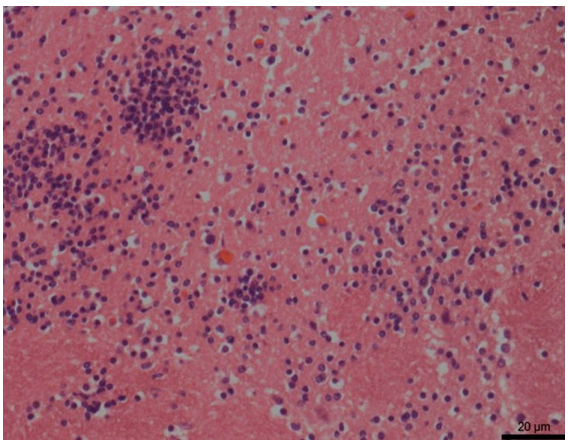


Fig.49: 320 A/14: Gliosis in white matter of brain. 10 X, H & E (PTV Positive)

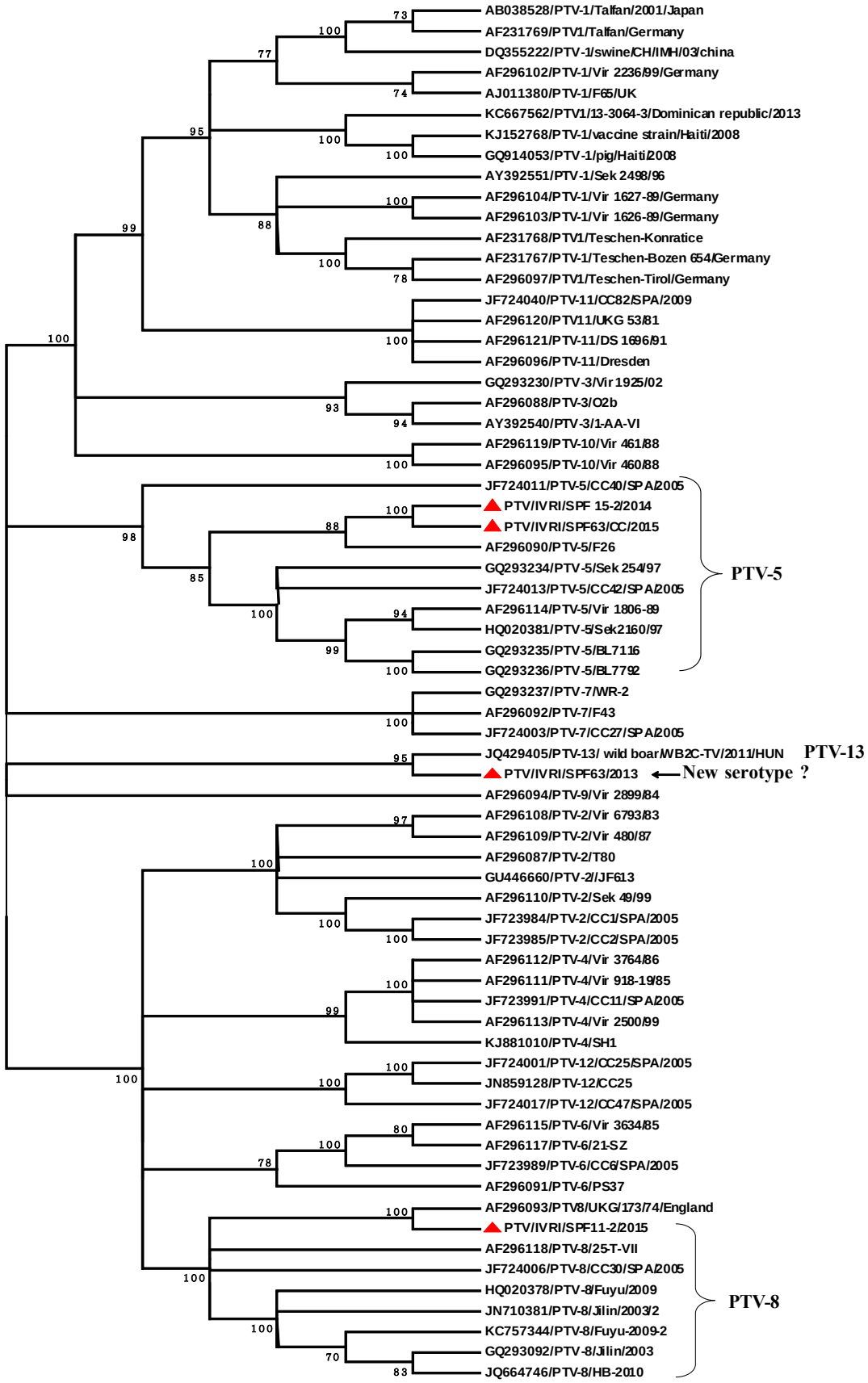


Fig.50: Phylogenetic tree based on sequence of VP1 gene region of the PTV isolates, using the neighbor-joining method with MEGA 6. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The isolates from this study is marked with a solid triangle.

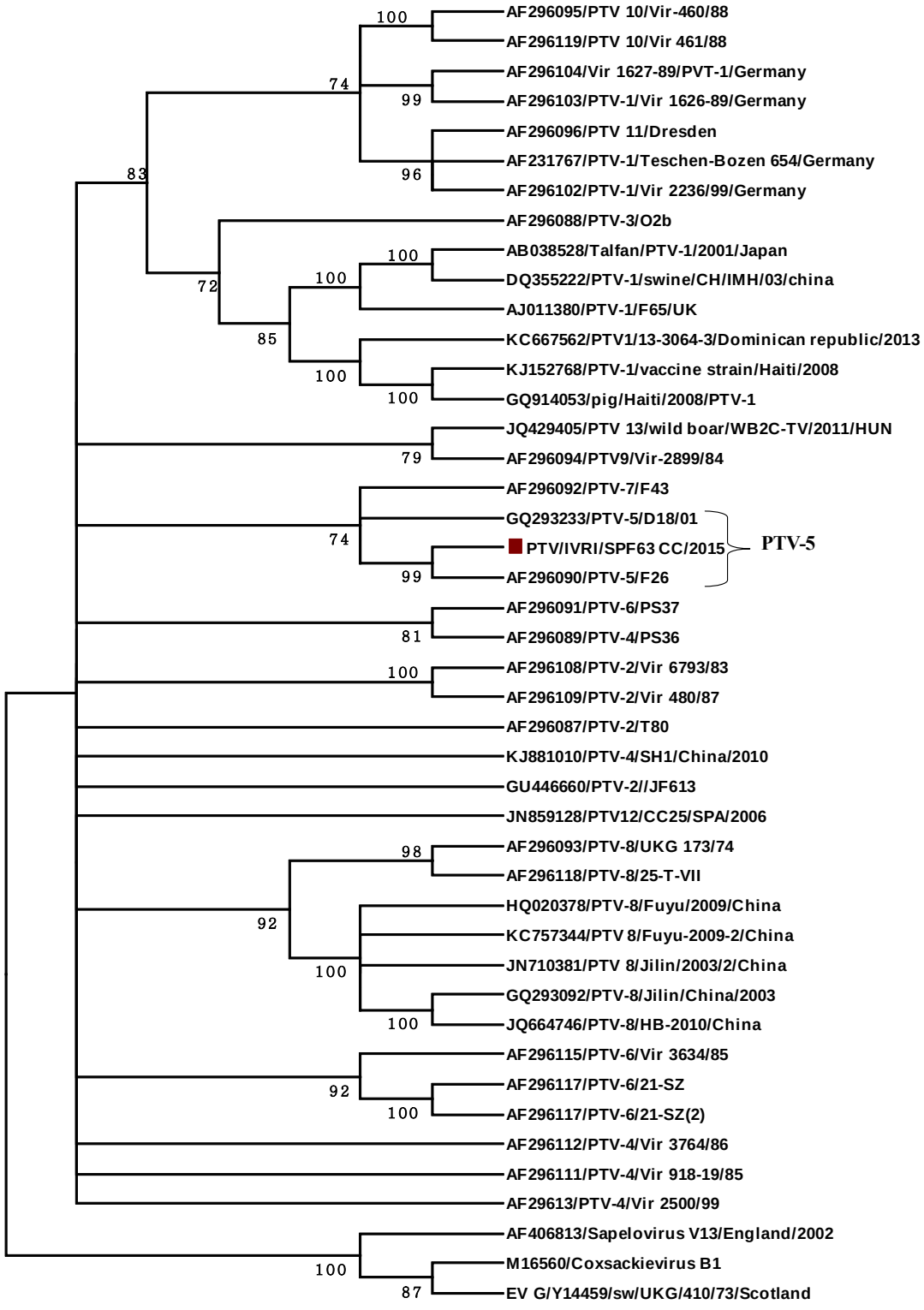


Fig.51: Phylogenetic tree based on sequence of polyprotein gene region of the isolates, using the neighbor-joining method with MEGA 6. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method [3] and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The isolates from this study is marked with a solid quadrangle.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. PTV/IVRI/SPF63 CC/2015		0.203	0.181	0.264	0.009	0.008	0.011	0.011	0.009	0.007	0.007	0.008	0.005	0.008	0.009	0.007	0.009
2. M16560/Coxsackievirus B1	3.622		0.050	0.093	0.182	0.220	0.182	0.198	0.187	0.210	0.209	0.199	0.207	0.195	0.202	0.207	0.198
3. Enterovirus G/Y14459/UKG/410/73/Scotland	3.581	1.119		0.100	0.199	0.195	0.191	0.199	0.187	0.203	0.178	0.204	0.185	0.184	0.192	0.199	0.195
4. AF406813/Sapelovirus V13/England/2002	4.457	2.295	2.226		0.258	0.258	0.262	0.287	0.254	0.265	0.245	0.275	0.273	0.276	0.277	0.279	0.265
5. JQ429405/PTV 13/wild boar/WB2C-TV/2011/HUN	0.282	3.454	3.484	4.394		0.009	0.010	0.009	0.008	0.010	0.009	0.009	0.009	0.010	0.009	0.009	0.010
6. JN859128/PTV12/CC25/SPA/2006	0.253	3.602	3.424	4.186	0.268		0.010	0.009	0.010	0.007	0.008	0.009	0.010	0.008	0.009	0.008	0.009
7. AF296096/PTV 11/Dresden	0.274	3.516	3.465	4.389	0.277	0.276		0.008	0.010	0.011	0.010	0.010	0.009	0.010	0.007	0.009	0.007
8. AF296095/PTV 10/Vir-460/88	0.273	3.556	3.515	4.555	0.265	0.277	0.219		0.009	0.009	0.010	0.010	0.011	0.009	0.007	0.010	0.009
9. AF296094/PTV9/Vir-2899/84	0.272	3.557	3.472	4.509	0.246	0.266	0.268	0.242		0.009	0.008	0.010	0.009	0.009	0.011	0.009	0.010
10. AF296093/PTV-8/UKG 173/74	0.245	3.584	3.514	4.373	0.275	0.176	0.286	0.285	0.280		0.006	0.009	0.008	0.008	0.009	0.006	0.009
11. AF296092/PTV-7/F43	0.204	3.671	3.492	4.268	0.281	0.232	0.277	0.287	0.264	0.208		0.007	0.006	0.008	0.008	0.005	0.007
12. AF296091/PTV-6/PS37	0.230	3.539	3.488	4.258	0.280	0.197	0.269	0.270	0.272	0.194	0.216		0.009	0.007	0.007	0.007	0.008
13. AF296090/PTV-5/F26	0.131	3.725	3.580	4.387	0.271	0.232	0.268	0.274	0.263	0.220	0.178	0.219		0.009	0.009	0.007	0.008
14. AF296089/PTV-4/PS36	0.239	3.584	3.538	4.236	0.288	0.194	0.286	0.288	0.273	0.196	0.221	0.169	0.230		0.009	0.007	0.010
15. AF296088/PTV-3/O2b	0.239	3.552	3.599	4.483	0.279	0.261	0.215	0.224	0.266	0.268	0.225	0.216	0.217	0.223		0.008	0.007
16. AF296087/PTV-2/T80	0.225	3.648	3.457	4.482	0.287	0.183	0.291	0.291	0.280	0.165	0.172	0.172	0.201	0.181	0.242		0.008
17. AJ011380/PTV-1/F65/UK	0.223	3.505	3.506	4.417	0.292	0.240	0.199	0.232	0.267	0.228	0.185	0.230	0.196	0.238	0.180	0.200	

Fig.52: Estimates of Evolutionary Divergence between PTV and related Sequences based on polyprotein gene. The number of base substitutions per site from between sequences are shown. Standard error estimate(s) are shown above the diagonal. Analyses were conducted using the Maximum Composite Likelihood model. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1).

with PTV5 strain F 26. Related viruses coxsackievirus B1, Porcine enterovirus EV G and sapelovirus were used as outgroup sequences. Evolutionary divergence between different serotypes was calculated in MEGA 6 programme shown in figure 52. The least divergence of 13.1 % base substitution was seen with PTV 5 strain F 26. These isolates did not show any similarity with viruses of human origin in BLAST analysis.

4.8.2. Molecular Characterization of PSV

For molecular characterization of PSVs 383 bp sequence of 3D polymerase gene was amplified. Three sequences obtained (named IVRI/PSV/ SPF C-6/2015, IVRI/PSV/ 323-14/2015 and IVRI/ PSV/GZB 1/2015) in this study were analysed in MEGA 6 programme with 9 other PSV sequences retrieved from NCBI database. In phylogenetic tree, the isolates from this study clustered together and were closely related to PSV strain V13/ England/57 and PSV strain V13/ England/2000 (Fig. 53).

To further characterize the PSV one isolate namely IVRI/SPF C6/2015 was sequenced by Next generation sequencing (NGS) on Ion Torrent platform. The NGS yielded 7491 bp long sequence with partial 5'UTR, polyprotein and partial 3' UTR region. This isolate contained 6969 bp long polyprotein gene. Phylogenetic analysis of this isolate based on polyprotein gene was done along with polyprotein gene sequences of 8 PSVs retrieved from NCBI. The phylogenetic tree is shown in Fig. 50. Based on polyprotein gene also this isolate was most closely related to PSV strain V13/ England/2000. The pairwise distance of this isolate with other sequences is shown in Fig. 454. The lowest divergence was seen with PSV strain V13/ England/2000 (13.3%).

4.8.3. Molecular Characterization of Enterovirus G (EV G)

For molecular characterization of EV G, 768 bp sequence of VP1 gene was amplified with primers WBev 2626 F & WBev 3394 R. From it 672 bp long sequence of EV G VP1 sequence was obtained after editing of the sequence. Four sequences obtained (named EVG/ IVRI/SPF 11/2013, EVG/IVRI/SPF 37/2013 and EVG/IVRI/ SPF 453/2013 and EVG/ IVRI/BVC 9/2013) in this study were analysed in MEGA 6 programme with 39 other EVG sequences retrieved from NCBI. In phylogenetic tree (Fig. 56) the isolates from this study

EVG/IVRI/SPF 11/2013, EVG/IVRI/SPF 37/2013 and EVG/IVRI/BVC 9/2013 clustered together and were closely related to EV G6 isolated from Vietnam (Accession No. KJ156436, KJ156449, KJ156450) and Korea (Accession No. JQ818253). The fourth isolate showed highest similarity with EV G1 strain Ch-ah-f1 ((Accession No. HM 131607) isolated from China. In BLAST analysis it showed 83% identity with strain Ch-ah-f1. P1 region of EV G virus for isolate EVG/IVRI/SPF 11/2013 was sequenced after amplification with primers pairs WBev 2626 F & WBev 3394 R and K23 K23 545F & K23 2905R (Fig. 57&58). A 2505 bp long sequence was obtained after editing and joining the two set of sequences. Phylogenetic tree was constructed using Neighbour-joining method with P1 sequences of other porcine and human enteroviruses (Fig 59). This isolate clustered with Enterovirus G6 isolated from Korea earlier named as PEV 16.

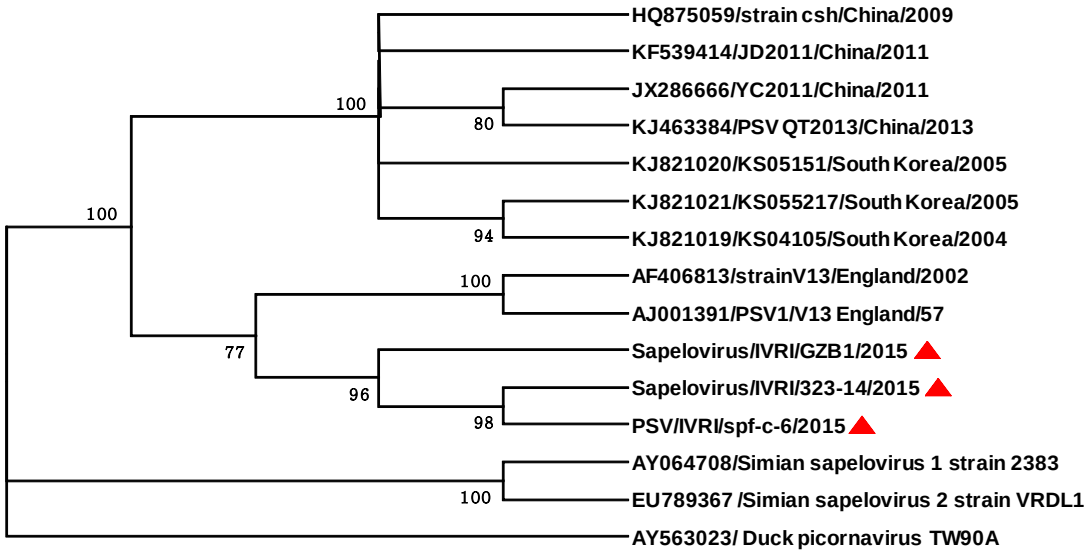


Fig.53: Phylogenetic tree based on sequence of 3 D polymerase gene (partial) of the PSV isolates, using the neighbor-joining method with MEGA 6. The evolutionary history was inferred using the Neighbor-Joining method The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method [3] and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The isolates from this study is marked with a solid triangle.

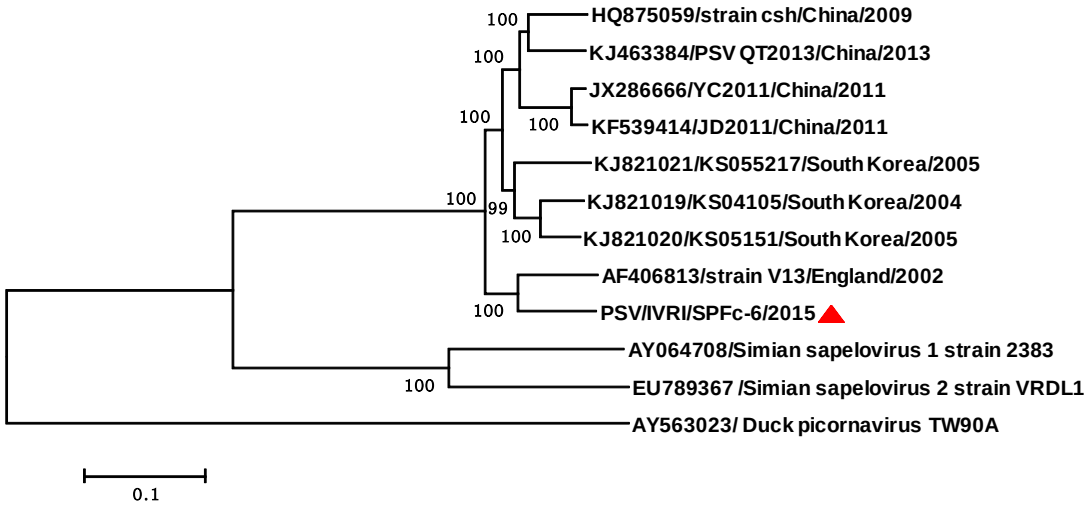


Fig.54: Phylogenetic tree based on sequence of 3 D polymerase gene (partial) of the PSV isolates, using the neighbor-joining method with MEGA 6. The evolutionary history was inferred using the Neighbor-Joining method The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown next to the branches. Simian sapelovirus and Duck Picornavirus sequences were used as outgroup. The isolates from this study is marked with a solid triangle.

	1	2	3	4	5	6	7	8	9
1. AF406813/strain V13/England/2002		0.006	0.006	0.006	0.006	0.006	0.006	0.005	0.004
2. HQ875059/strain csh/China/2009	0.182		0.004	0.005	0.003	0.004	0.004	0.004	0.005
3. JX286666/YC2011/China/2011	0.186	0.111		0.002	0.004	0.004	0.005	0.005	0.005
4. KF539414/JD2011/China/2011	0.189	0.111	0.025		0.004	0.004	0.005	0.005	0.005
5. KJ463384/PSV QT2013/China/2013	0.183	0.097	0.107	0.110		0.004	0.004	0.004	0.005
6. KJ821019/KS04105/South Korea/2004	0.185	0.141	0.137	0.139	0.147		0.003	0.004	0.006
7. KJ821020/KS05151/South Korea/2005	0.182	0.129	0.125	0.128	0.133	0.068		0.004	0.005
8. KJ821021/KS055217/South Korea/2005	0.179	0.147	0.144	0.144	0.143	0.110	0.128		0.005
9. PSV/IVRI/SPFc-6/2015	0.133	0.178	0.178	0.185	0.173	0.185	0.184	0.180	

Fig.55: Estimates of Evolutionary Divergence between PSV and related Sequences based on polyprotein gene. The number of base substitutions per site from between sequences are shown. Standard error estimate(s) are shown above the diagonal. Analyses were conducted using the Maximum Composite Likelihood model.

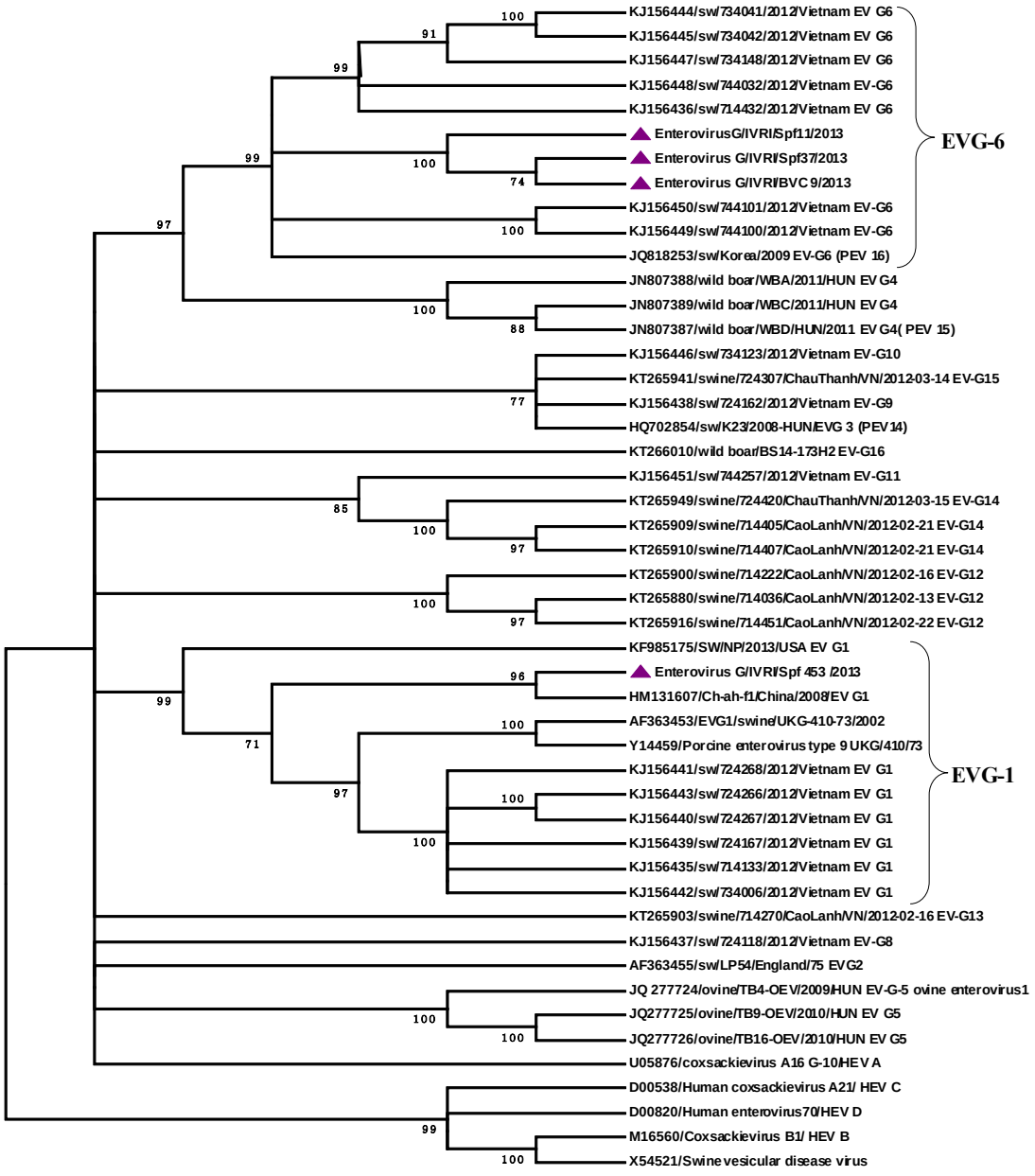


Fig.56: Phylogenetic tree based on sequence of VP1 gene (partial) region of the isolates, using the neighbor-joining method with MEGA 6. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The isolates from this study is marked with a solid triangle.

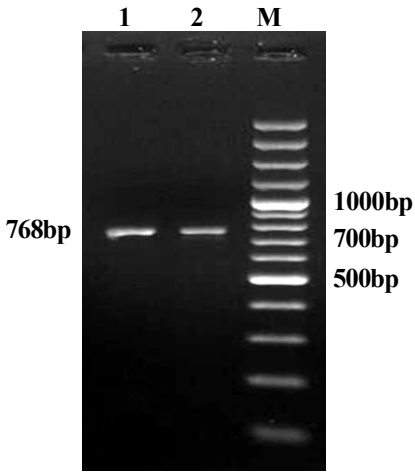


Fig.57: Ethidium bromide stained 1.5% agarose gel showing amplification of 768 bp fragment of EV G
Lane 1 : SPF 11
Lane 2 : SPF 453
Lane M : Marker

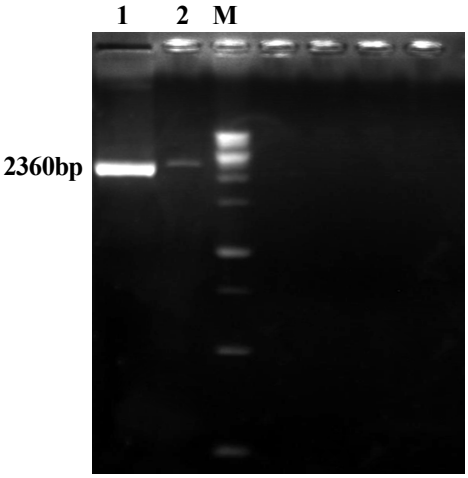


Fig.58: Ethidium bromide stained 1.5% agarose gel showing amplification of 2360 bp fragment of EV G
Lanes 1-2 : SPF 11
Lane M : Marker

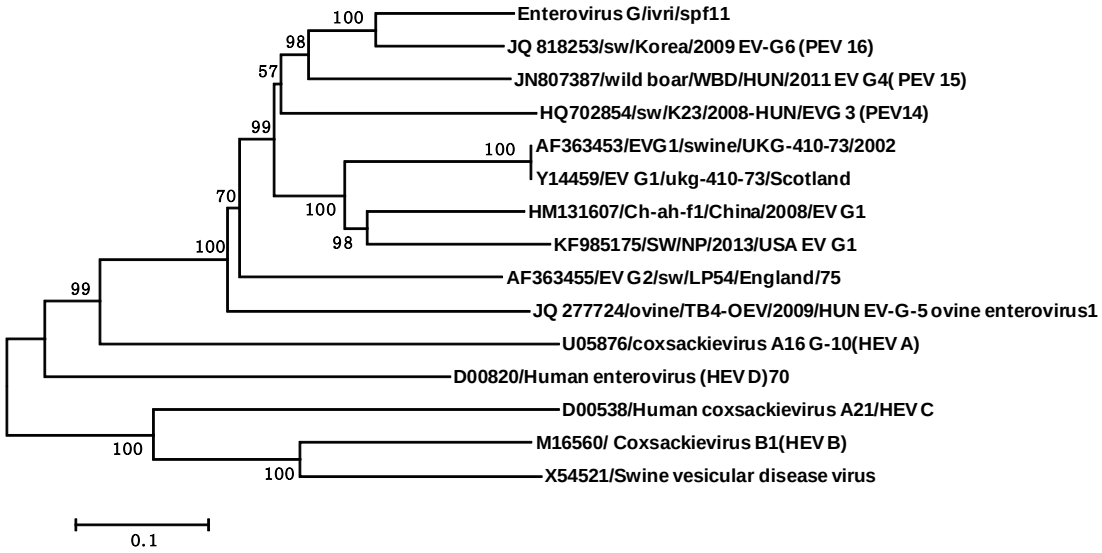
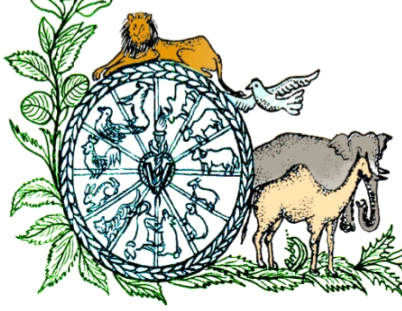


Fig.59: Phylogenetic tree based on sequence of P1 region, using the neighbor-joining method with MEGA 6. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The sequence from this study is named as Enterovirus G/ivri/spf11.

Discussion



5 DISCUSSION

Properties such as growth in cell cultures, nature of their cytopathic effect and genomic analysis led to their reclassification into three distinct genera: Teschovirus, Sapelovirus and Enterovirus (Kaku *et al.,* 2001; Krumbholz *et al*., 2002). Enteroviruses of porcine origin have been classified as enterovirus G. They primarily infect the gastrointestinal tract, replicating in its epithelia and being shed in faeces, often remaining in the environment for long duration. Porcine enteric picornaviruses are ubiquitous in distribution in swine species reported all over the world. These are common swine pathogens which cause a wide range of illnesses in swine ranging from asymptomatic infection to acute fatal encephalomyelitis (Trefny, 1930; Harding *et al.*, 1957; Yamada *et al.,* 2004), reproductive disorders (Dunne *et al.*, 1965), Diarrhoea(Izawa, 1962; Honda *et al.,* 1990) and dermal lesions (Knowles, 2006). Cytopathogenic porcine enteroviruses have been isolated from pigs with clinical signs of Diarrhoea(Kresse *et al.,* 1977). Clinical signs observed in pigs from such herds are diarrhea, debilitation and gauntness. Porcine enteroviruses have been isolated from healthy as well as diarrhoeal domestic pigs (Honda *et al.*, 1990). Porcine sapelovirus and porcine teschovirus have been isolated from organs of pigs showing signs of acute diarrhea, respiratory distress, and death in China (Lan *et al.,* 2011 and Zhang *et al.,* 2010). In recent outbreaks the morbidity and mortality has been reported upto 60% and 40%, respectively (Deng *et al.,* 2012).

Enteroviruses are important pathogens for human beings also. Although encephalitis is a rare presentation of EV infection, many EV serotypes (Coxsackievirus A9, A10, and B5,

echovirus 4, 5, 9, 11, 19, and 30 and EV 71, 75, 76 and 89) have been reported in encephalitis cases from different parts of the world including India (Dalwai *et al.,* 2009; Kumar *et al.,* 2011; Lewthwaite *et al.,* 2010; Lin *et al.*, 2003; Sapkal, *et al.*, 2009). Encephalitis is a significant cause of morbidity and mortality in children each year in Uttar Pradesh and Bihar and enteroviruses have been implicated in many studies as etiological agents in cases of encephalitis (Joshi *et al.* 2012; Kumar *et al.,* 2012).

PTV, PSV and EVG infections are currently diagnosed by PCR (Krumbholz *et al.,* 2003; Palmquist *et al.,* 2002; Zell *et al.,* 2000) which replaced time consuming methods such as virus isolation in cell cultures and differentiation of serotypes by serological assays (Auerbach *et al.,* 1994; Knowles and Buckley, 1980).

In present study RT-PCR testing of faecal specimens indicated the prevalence of PTV infection to be 6.84% (13 out of 190 cases). It was found in animals of ages group of 4 weeks to adulthood. Sapelovirus was present in 5 out of 70 animals examined. Prevalence rate of Sapelovirus was 7.14. The infected animals were from 5-9 weeks of age. Enterovirus G was identified in 21 out of 190 animals with prevalence rate of 11 in 5-9 weeks old piglets. PTV was found in only one of the farms examined (SPF, IVRI) whereas PSV and EV G were detected in two farms each (Sapelovirus in U.P. and EV G in Bihar and U.P. both) . These viruses were detectected in apparently healthy animals as well as animals showing signs of diarrhoea. Similar results have been reported in previous studies in different countries of the world. In Spain, 206 cytopathic viruses were isolated from 600 porcine fecal samples between 2004 and 2005 out of which 97 (47%) were identified as teschoviruses and 18 (9%) as sapeloviruses using RT-PCR methods whereas EV G or Swine vesicular disease virus were not found among the isolates (Buitrago *et al.*, 2010). In Italy, out of 40 viral strains isolated on cell culture from gut contents, rectal swabs and faeces from apparently healthy pigs, 21 were found positive to teschovirus RNA, whereas one and three were positive to Porcine Sapelovirus (PSV) and PEV-B RNA, respectively (Sozzi *et al.,* 2010). Similarly Wang *et al.* (2010) reported the isolation of PTV 2 strain JF613 from pigs showing symptoms of pyrexia, diarrhea, respiratory distress and nervous disorders (difficulty in standing, recumbent and hindlimb paralysis) in a commercial pig farm in Heilongjiang Province in China. Yeou-Liang *et al.* (2011)

reported identification of PTV in 58 fecal samples out of 525 faecal samples collected from each county and city in Taiwan. Infected pigs did not show any clinical symptoms. PEV genome was identified in 5 of 10 (50%) faecal samples collected from apparently healthy wild boar piglets in Hungary (Boros *et al.*, 2012a).

Enterovirus B was the most prevalent virus detected in both domestic pigs and wild boars (50.2% and 69.4%, respectively), followed by Porcine teschovirus and Porcine sapelovirus in Czech republic as reported by Prodelalova, (2012). The majority of positive domestic pigs (69.4%) and wild boars (64.3%) were infected with two or three tested viruses. There was no significant difference in prevalences of teschoviruses, sapeloviruses, and enteroviruses among healthy and diarrhoeic pigs which supports our finding of these viruses in sick and apparently healthy animals. PEV (EV G) have been detected from diarrhoic pigs in USA (Anbalagan *et al.*, 2014), healthy pigs in middle and eastern China (Yang *et al.*, 2013), healthy as well as diarrhoeic pigs in Brazil and Vietnam (Donin *et al.*, 2014; Van Dung *et al.*, 2014).

Comparatively lower level of infection was found for all the three viruses detected in this study. Apart from lower sample size, elimination of nested step in RT-PCR which was done to avoid the contamination during PCR could be a reason for lower level of detection of these viruses. Also our rearing practices in the form of small herd size and backyard system of farming may be responsible for lower prevalence rates.

Mixed infection of enteric picornaviruses was also detected in the study population. Co-infection of PTV with EV G was detected in 7 cases (3.68%) whereas co-infection of PSV with EV G was found in 2 out of 70 (2.85%) cases. Mixed infection has been reported in earlier studies of Sozzi *et al.*(2010) in Italy, Prodelalova (2012) in Czech Republic, Cano-Gomez *et al.* (2013) in Spain and Donin *et al.* (2014) in Brazil.

Virus isolation is considered as one of the most reliable methods to establish an infection of viral etiology (Storch, 2000). PTV was isolated in IB-RS-2 cells from PTV positive faecal samples. The CPE was observed first in 2nd or 3rd passage in IB-RS-2 cells depending on sample inoculated. The Type I CPE was observed for PTV inoculated samples which is

characterized by round, refractile cells grouped in foci, followed by detachment in 3-5 days. PSV was isolated from PCR positive faecal samples in IB-RS-2 cells. The CPE was noticed in 1st or 2nd passage depending on the sample inoculated. For sapelovirus, type II CPE was observed which was characterized by refractile cells, enlargement, rounding, increased cytoplasmic granularity, rosette-like appearance due to cytoplasmic protrusions through the cell membrane and detachment of the cells. Clear CPE was first observed on 3rd day PI in 2nd passage, and at day 4, the cells completely detached from the surface of the culture flask. In the subsequent passages, complete detachment of cells was seen within 24 hours. These observations are in agreement with earlier reports (Zoletto, 1965; Dunne *et al.,* 1971). Zoletto (1965) classified the enteroviruses into three groups based on the type of CPE observed named as Type I, Type II and type III CPE. Type I CPE was characterized by round refractile cells grouped in foci, that of type II as refracting cells with evident and characteristic cytoplamic prominences and of third group with round refracting cells with little tendency to group and appear disseminated. Viruses of type II group require very short time to detach the cells so that the monolayer may be detached within 24 hours in the 1st passage while the time required by viruses of type 1st and 3rd groups is 5-6 and 2-3 days, respectively. According to current classification the CPE type I, II and III porcine enteroviruses are known as PTV, PSV and EVG, respectively. Similar pattern of CPE and time for detachment was observed for PTV and PSV in our study. Though PCR positivity for EV G was observed in the study virus could not be isolated. Inactivation of virus during post collection transport and storage may have been the reason for failure in EV G isolation.

The growth of the viruses in cell culture was confirmed by RT- PCR and nucleotide sequencing for both group of the viruses. Similar approach was adopted by Wang *et al.* (2010), Zhang *et al.* (2010) and Lan *et al.* (2011). Based on the VP1 gene sequence of the virus isolates, two of the isolates designated as IVRI/SPF-15-2/2014 and IVRI/SPF 63/CC/ 2015 were determined as PTV- 5 whereas the third isolate designated as IVRI/ SPF 11-2/ 2015 was determined as PTV-8.

Real-time RT-PCR (rRT-PCR) assay is a rapid and reliable method for detection of pathogens. The TaqMan based rRT-PCR assay is considered more specific, so in present

study, real-time TaqMan assays were developed for rapid diagnosis of porcine teschovirus and enterovirus G. TaqMan probe and primers were designed targeting the 5' UTR region sequences of PTV and EVG obtained during the study along with 5'UTR sequences available in the public domain.

The specificity of the fluorogenic rRT-PCR assay for PTV detection was analyzed by testing viral RNAs obtained from cell-grown PTV serotypes and other related porcine viruses such as PSV and EVG. The assay detected all three PTV strains analyzed while it did not react with nucleic acids from any of the non-PTV porcine viruses tested. Nucleic acids isolated from the non-PTV porcine viruses were detected by their respective specific RT-PCR methods, thus confirming the specificity of the developed assay. The sensitivity of the assay was tested by 10 fold serial dilutions of PTV genome inserted plasmid. The real-time RT-PCR was able to quantify the standard plasmid down to 100 copies/µl. Several rRT-PCR assays (Jiménez-Clavero *et al.,* 2003; Krumbholz *et al.*, 2003, Cano-Gómez, *et al.,* 2011; Zhang *et al.,* 2013) have been developed for detection of PTV genome. While assays described by Jiménez-Clavero *et al.* (2003), Cano-Gómez *et al.* (2011) and Zhang *et al.* (2013) were TaqMan based assays, themethod described by Krumbholz *et al.* (2003) used energy transfer Light cycler method. TaqMan based assays are being more commonly used than energy transfer based methods in laboratory diagnosis of pathogens (Hoffmann *et al.,* 2009). Openness of the TaqMan method makes it more suitable because it can be performed in any real-time PCR platform, whereas the energy transfer method is optimized for the LightCycler platform, which is quite different from other real-time thermal-cycler equipment which can't be transferred to other platforms.

TaqMan based rRT-PCR assay for enterovirus G detected specifically only the porcine enteroviruses currently available with the laboratory, while it did not react with nucleic acids from any of the non-EVG porcine viruses tested such as PTV and Porcine sapelovirus. The sensitivity of the assay was tested by testing the 10 fold serial dilutions of standard plasmid inserted with 5'utr region of the EV genome. The assay was able to identify upto 10 copies/il of standard plasmid. Standard curve was created with r^2 value of 0.979 to quantify the viral load in clinical specimens and tissue samples. This is the first report of rRT-PCR assay for EVG detection.

Enteric picornaviruses viruses were detected in ailing animals with diarrhoea as well as in apparently healthy animals. The piglets with Diarrhoea had whitish to grayish yellow colored faeces. These viruses were identified in six necropsied cases. Of these cases in only one case PTV could be identified in tissue samples. On necropsy examination the small intestine was sometimes thin walled and filled with yellowish liquid contents, frequently congested mucosa and sometimes with whitish semi-solid or mucoid contents. Patchy areas of petechial haemorrhages were also found in ileum mucosa. Gross lesions were visible mostly in small intestine portion of the GI tract whereas caecum, colon and rectum didn't reveal significant changes. Jejunum was the most affected portion. Mesenteric lymph nodes were congested. In the CNS only gross lesions of note were congestion of meningeal blood vessels. Similar gross lesions have been reported in experimental studies with PTV 8 where the liver and intestine showed congestions, and the intestine was filled with yellow liquid. In addition, a gaseous distention of the stomach was observed (Zhang *et al.,* 2010). Similar gross lesions of GIT has been recorded in experimental studies with PSV in China (Lan *et al.,* 2011). The small intestinal mucosa showed extensive congestion. Punctate hemorrhage and hyperemia were found in the duramater of brain. Gross lesions of GIT or CNS associated with EV G has not been described.

Some animals from a herd with history of nervous symptoms grossly showing vesicular lesions in skin of ventral abdomen, ears and around hooves of feet, interdigital space were positive for porcine sapelovirus. Such lesions have not been associated with PSV but has been described with EVG (Knowles, 1988). But this sample was negative for EVG, Foot and mouth disease virus and SVDV.

The major histo-pathological lesions with gastro-intestinal organs in PTV or EV G positive cases were inflammation of intestines along with desquamation of villous epithelium mainly in jejunum and ileum. Lymphoid cells depletion in follicles of cortical region and eosinophilic infiltration with mild vascular congestion of was seen in mesenteric lymph nodes. Loss of villous structures in jejunum and submucosal vascular engorgement, lymphoid depletion in Payer's patches in ileum was also present. In a previous study reported by Zhang *et al*.

(2010) experimentally infected pigs with PTV showed a pronounced loss of villi in small intestine combined with a hemorrhagic infiltration and a degeneration of mucosa cells of the lamina propria mucosae. In the caecum, normal arrangement in layers was dissolved and some solitary follicles of the submucosa exhibited a disintegrated structure. The colonic mucosa was damaged severely as the lamina propria and the crypts were eroded. Some lymphocytic and hemorrhagic infiltration was visible. This experimental study of PTV 8 inoculation in pigs confirms the pathogenic potential of PTV- 8 and its causative role in diarrhoea. Experimental study in piglets with Indian isolate of PTV-8 is required to know its pathogenicity in pigs.

CNS lesions were present in only two necropsy cases positive for PTV. The CNS lesions were characterized by perivascular cuffing with mononuclear cells and eosinophils, neuronal degeneration in spinal ganglion, meningeal and parenchymal blood vessel congestion and gliosis. The lesions were present mainly in cerebrum, hippocampus and to a lesser extent in pons and medulla. Similar histological changes of non-suppurative encephalomyelitis, characterized by perivascular cuffing of the mononuclear cells, focal gliosis, neuronal necrosis and neuronophagia were observed in pigs naturally infected with PTV by Pogranichniy *et al.*, (2003), Yamada *et al.*, (2004), and Takahashi *et al.,* (2008). In addition Yamada *et al.* (2004) described severe lesions in the spinal cord. In the ventral horns, nerve cells were degenerated to severe degrees upto and including necrosis accompanied by neuronophagia, inflammatory or glial nodules, occasional hemorrhages and a rather diffuse infiltration of mononuclear cells. In the white matter of the spinal cord, perivascular cuffing and infiltration of mononuclear cells and focal gliosis were also observed. In addition to the infiltrative changes, severe vacuolar changes and axonal swelling were observed in the white matter of the spinal cord. In general lesions were most severe in midbrain, brainstem, and cerebellar peduncles and occasionally in cerebral cortex (Pogranichniy *et al.*, 2003, Yamada *et al.*, 2004) contrary to our study where lesions were more common in cerebral hemisphere. Clearly, experimental studies with the Indian isolates of PTV are required to assess their neuro-pathogenic potential.

The enteric picornaviruses virus could not be demonstrated in tissues by *In situ* hybridization. The reason for this could be the low viral load present in the tissue at time of collection. These viruses could be demonstrated by immunohistochemistry in future studies. In present study IHC was not done because of unavailability of antibodies against these viruses.

For molecular characterization 783 bp sequence of VP1 gene of PTVs was amplified. In phylogenetic analysis of VP1 gene sequence of two isolates named as IVRI/SPF-15-2/ 2014 and IVRI/SPF 63/CC/2015 showed maximum identity (81-84%) with PTV 5 with highest identity with PTV-5 strain F 26 (Accession No. AF296090). The third isolate designated in the study as SPF 11-2/2015 clustered with PTV- 8. The nucleotide identity of this isolate with PTV 8 ranged from 85-91% with highest identity of 91% with PTV 8 strain 25-&-VII (Accession No. AF296118). The fourth sequence designated as IVRI/SPF 63/ 2013 shared 72% sequence identity with PTV-13 strain wild boar/WB2C-TV/2011/HUN (Accession No. JQ425405). Other serotypes shared 70-72% identity with this strain indicating the possibility of a new serotype of PTV. Phylogenetic analysis of the 4 VP1 region sequences obtained in this study, analyzed with sequences retrieved from NCBI database showed 13 clades corresponding to 13 genotypes of the PTV. Two of the isolates (SPF-15-2/2014 and SPF 63/ CC/2015) clustered with PTV-5. The third isolate designated in the study as SPF 11-2/2015 clustered with PTV- 8.

According to current knowledge, there is no sequence-based PTV "serotyping" method accepted by the ICTV Picornaviridae Study Group, and therefore genome based methods which are confirmed by serological experiments can be applied to investigate the taxonomic position of the study sequences (Boros *et al.*, 2012b). Molecular characterization of picornaviruses generally relies on structural proteins. It has been previously pointed out that epitopes responsible for serotype differentiation are located mostly in exposed areas within outer capsid proteins (VP1–3) (Usherwood and Nash, 1995). Therefore, the optimal strategy for molecular typing of these viruses should involve one or more of these proteins. In this context, the capsid proteins 1A to 1D (VP4 to VP1) are the most divergent proteins of the PTV serotypes. Extents of pairwise amino acid identity range from 79 to 87% for VP4 –VP2, 76 to 91% for VP3, and 66 to 82% for VP1(Zell *et al.*, 2001). VP1 is often the region where the most genetic variability is concentrated, given that it contains immunodominant epitopes which make serotype correlation more likely (Borrego *et al.,* 2002; Usherwood and Nash, 1995). In the particular case of PTVs, neutralizing epitopes have been described involving

both VP1 and VP2 (Kaku *et al.,* 2007), and methods for molecular typing based on partial sequences of both VP1 (La Rosa *et al.,* 2006) and VP2 (Kaku *et al.,* 2007) have been proposed. A new approach based on the analysis of the complete VP1 gene including the C-terminal region was presented by Cano-Gomez *et. al.* (2011) which has been followed in this study for all three picornaviruses identified in this study. Based on polyprotein gene also this isolate clusterd with PTV5 strain F 26. Recently PTV serotypes 1, 2, and PTV 8has been identified in pigs in China and Haiti with association with disease symptoms ranging from encephalomyelitis, pneumonia and diarrhoea (Feng *et al.,* 2007, Wang *et al.,* 2010, Zhang *et al.,* 2010, Deng *et al.,* 2012 and Qiu *et al.,* 2013). Feng *et al.* (2007) reported isolation of PTV from brain tissues of diseased piglets showing encephalomyelitis in a farm in Peoples Republic of China. Comparison of the sequences of the amino acid and nucleotides and phylogenetic analysis of the polyprotein showed that isolated virus is PTV-1. The isolated virus had closest relationship with Talfan strain. Zhang *et al.* (2010) reported the isolation of PTV-8 in pig farms in the Jilin province from organ samples and it was identified as the causal agent of acute diarrhea, respiratory distress and death of pigs in the swine farm. It was grown in swine primary kidney cells(PK-15) and in a swine testicular cell line (ST cells). When inoculated into healthy pigs, PTV-8 Jilin/2003 caused the same symptoms as those observed in the affected herd (Zhang et al., 2010). PTV was also isolated from a dead piglet from a herd of 200 sows showing reproductive failure in Fuyu, Heilongjiang Province, China (Lin et al., 2012).

In Spain, PTV2, 4, and 6 were the most common serotypes, while serotypes 1, 5, 7, and 12 were also common (Cano-Gomez *et al.,* 2011). PTV serotypes 1, 2, 4, 5, 7, 8, 10, and 11 were detected in Italy, but PTV1, 2, 8, and 11 were the most common serotypes (Sozzi *et al.,* 2010). PTV-5 which was one of the serotypes identified in this study can cause mild form of teschovirus encephalomyelitis (OIE, 2009). PTV -8, another isolate identified in this study has been implicated as causal agent of acute diarrhea, respiratory distress, and death of pigs on a swine farm in Jilin Province, northern China (Zhang *et al.,* 2010). The sequence designated as IVRI/SPF 63/ 2013 formed a clade with PTV-13 having nucleotide 72% sequence identity isolated from wild boars in hungary (Boros *et al*., 2012 b). The pairwise amino acid sequence identity ranged from 69-77% with highest identity of 77% with PTV-13 followed by

75% identity with serotypes PTV- 5 and PTV-7 which suggests the possible circulation of a new serotype which can be confirmed in further studies. Sun et al. (2015) reported circulation of a possible new serotype of PTV based on the partial VP1 sequence. This isolate designated as SH8 did not correspond to any known serotype. PTV4 and PTV6 showed similar levels of sequence identity (82-83%) to PTV SH8. They suggested that PTV SH8 is a new serotype, distinct from the new serotype PTV wild boar/WB2C-TV/2011/HUN.

As teschoviruses are commonly present in the intestinal tract of healthy animals also, so the pathogenicity of the identified viruses can only be determined with certainty by inoculation in specific pathogen free pigs. Further, examination of historical samples and study of teschoviruses in bigger sample sizes will reveal other aspects of epidemiology such as genetic diversity and interaction with other pathogens in causation of disease in pigs.

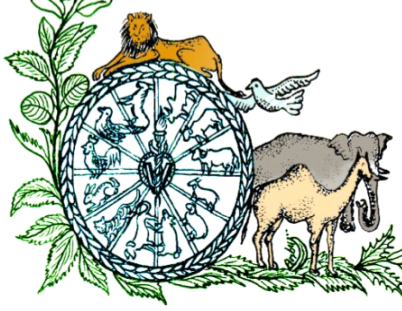
For molecular characterization of PSVs 383 bp sequence of 3D polymerase gene was amplified. In phylogenetic tree the three isolates from this study cultured together and were closely related to PSV strain V13/ England/57 and PSV strain V13/ England/2000. The phylogenetic tree based on 6969 bp long polyprotein gene of one isolate showed it to be most closely related to PSV strain V13/ England/2000. The role of porcine sapeloviruses as a pathogen particularly as a cause of diarrhoea, has been unclear given the fact that the entero-like viruses have frequently been isolated from faeces of healthy piglets also (Lamont and Betts, 1960; Buitrago *et al.,* 2010; Cano-Gomez *et al.,* 2013*)*. Although PSV, formerly porcine enterovirus 8 (PEV-8), infections are frequently asymptomatic they have been associated with diarrhoea, pneumonia, polioencephalomyelitis and reproductive disorders (Huang *et. al.,* 1980; Honda *et al.,*1990; Knowles, 2006; Lan *et al.,* 2011; Schock *et al.,* 2014). PSV-induced acute diarrhea, respiratory distress and polioencephalomyelitis in pigs was reported recently in an experimental study in China (Lan *et al.,* 2011). Enteric picornaviruses including sapeloviruses are commonly present in the intestinal tract of healthy animals. However lack of maternal antibody, treatment with immunosuppressive drugs and co-infections with other pathogens, have been shown to play a major role in the severity of entero-like virus infections in pigs (Takahashi *et al*., 2008, Chiu *et al*., 2014).

In Phylogenetic analysis based on 672 bp sequence of VP1 gene(partial) of EV G the three isolates from this study EVG/IVRI/SPF 11/2013, EVG/IVRI/SPF 37/2013 and EVG/IVRI/BVC 9/2013 clustered together and were closely related to EV G6 isolated from Vietnam and Korea. The fourth isolate showed highest similarity with EV G1 strain *Ch-ah-f1* isolated from china which was earlier classified as PEV9. In Blast analysis it showed 83% identity with strain *Ch-ah-f1* reported from China. In phylogenetic analysis of 2505 bp long P1 region of isolate EVG/IVRI/SPF 11/2013 clustered with Enterovirus G6 isolated from Korea earlier named as PEV 16. Recently EV G1 has been reported from USA (Anbalagan *et al.,* 2014) from a diarrhoic pig. New serotypes of EVG have been reported from various parts of the world and have been formally assigned as as EVG-3 to EVG-11 by the Picornavirus Study Group (Boros *et al*., 2011; Boros *et al*., 2012a; Moon *et al*., 2012 and Van Dung *et al.* 2014).

None of the identified porcine enteric picornaviruses had any significant nucleotide similarity with the human enteroviruses genome sequences available in public domain.

It is difficult to determine whether our finding of PTV or PSV in Indian pigs represents a recent introduction of this virus or it has been circulating in Indian herds undetected for many years. Examination of historical samples can clarify this and other aspects of epidemiology of porcine enteric Picornaviruses in India. But given the occurrence of diseases such as diarrhoea respiratory distress and encephalomyelitis with unexplained etiology in many cases the isolation of PTV and PSV cannot be ignored and continuous monitoring for these viruses should be done in pigs in bigger population.

Summary and Conclusions



6 SUMMARY AND CONCLUSIONS

Study was conducted to find out prevalence and pathology of porcine enteric picornaviruses that have been traditionally associated with CNS pathology and to explore their possible relationship to human enteroviruses which cause acute encephalitis syndrome in children. Random faecal samples/ rectal contents from 190 pigs and tissue samples from 52 dead animals were collected from different farms with history of diarrhoea. Based on RT-PCR study of faecal specimens PTV was prevalent in 13 out of 190 cases (6.84 %) in this study. It was found in animals of aged 4 weeks to adults. Sapelovirus was present in 5 out of 70 animals (7.14%) of the animals examined. The infected animals were from 5-9 weeks of age. Enterovirus G was identified in 21 out of 190 animals with overall prevalence of 11.05 which were in age range of 5-9 weeks. PTV was identified in intestinal tissue and mesenteric lymph nodes in only one necropsy sample. Enteric picornaviruses were detected in pigs with diarrhoea as well as in apparently healthy animals.

Three isolates of PTV were isolated in IB-RS-2 cells from PTV positive faecal samples. The Type I CPE was observed for PTV inoculated samples, characterized by round, refractile cells grouped in foci, followed by detachment in 3-5 days. Two of the isolates were determined as PTV-5 whereas the third isolate was determined as PTV-8 based on sequence analysis of the isolates.

Three isolates of PSV were isolated from PCR positive faecal samples in IB-RS-2 cells. The CPE was noticed in 1st or 2nd passage where cells detached completely from surface within 24 hours . For sapelovirus type II CPE was observed which is characterized by

refractile cells, enlargement, cell rounding, increased cytoplasmic granularity, rosette-like appearance due to cytoplasmic protrusions through the cell membrane and detachment of the cells.

The piglets with diarrhoea had whitish to grayish yellow colored faeces. Grossly the intestine was sometimes thin walled and filled with yellowish liquid contents, frequently congested and sometimes with whitish semi-solid or mucoid contents. Patchy areas of petechial haemorrhoges were also found in ileal mucosa. Gross lesions were visible mostly in small intestine portion of the GI tract whereas caecum, colon and rectum were free of gross lesions. Jejunum was the most affected portion. Mesenteric lymph nodes were congested. Some animals from a herd with history of nervous symptoms grossly showing vesicular lesions in skin of ventral stomach, ears and around hooves of feet, inter-digital space were positive for porcine sapelovirus.

The major gastro-intestinal histo-pathological lesions associated with PTV or EV G positive cases were inflammation of intestines along with desquamation of villi epithelium mainly in jejunum and ileum portion. There was severe congestion of intestinal blood vessels, destruction and desquamation of villous epithelium in small intestine, goblet cell hyperplasia, infiltration of mononuclear cells and lymphoid cell depletion in Payer's patches in ileum. Congestion of blood vessels and infiltration of mononuclear cells were also seen in duodenal mucosa. Mononuclear cells infiltration in lamina propria and sub-mucosal blood vessel congestion was present in cardiac region of stomach. Lymphoid cells depletion in follicles of cortical region and eosinophilic infiltration with mild vascular congestion was seen in mesenteric lymph nodes. The CNS lesions were characterized by perivascular cuffing with mononuclear cells and eosinophils, neuronal degeneration in spinal ganglion, meningeal and parenchymal blood vessel congestion and gliosis. The lesions were present mainly in cerebrum, hippocampus and to a lessor extent in pons and medulla.

TaqMan probe and primers were designed targeting 5' UTR region and validated in an attempt to develop TaqMan based real-time RT-PCR assays for specific detection of PTV and EVG. The rRT-PCR assay for PTV detected all three PTV strains analyzed, currently available with the laboratory, while it did not react with nucleic acids from any of the related

but non-PTV porcine viruses tested such as PSV and PEV. The sensitivity of the assay was tested by testing the 10 fold serial dilutions of PTV genome inserted plasmid. The real-time RT-PCR was able to quantify the standard plasmid down to 100 copies/?l.

TaqMan based rRT-PCR assay for enterovirus G detected specifically only the porcine enteroviruses currently available with the laboratory, while it did not react with nucleic acids from any of the non-EVG porcine viruses tested such as PTV and Porcine sapelovirus. The sensitivity of the assay was tested by testing the 10 fold serial dilutions of standard plasmid inserted with 5'UTR region of the EV genome. The assay was able to identify upto 10 copies/ ?l of standard plasmid.

Molecular characterization of PTVs was done by comparing 783 nucleotide sequence of VP1 gene or polyprotein gene sequence obtained from next generation sequencing. Two isolates, IVRI/SPF-15-2/2014 and IVRI/SPF 63/CC/2015 showed maximum identity (81-84%) with PTV 5 with highest identity with PTV-5 strain F 26 (Accession No. AF296090). Third isolate, SPF 11-2/2015 clustered with PTV- 8. The nucleotide identity of this isolate with PTV 8 ranged from 85-91% with highest identity of 91% with PTV 8 strain 25-T-VII from UK (Accession No. AF296118). The fourth sequence, IVRI/SPF 63/ 2013 shared 72% sequence identity with PTV-13 strain wild boar/WB2C-TV/2011/HUN from Hungary (Accession No. JQ425405). Other serotypes shared 70-72% nucleotide identity with this strain. Pairwise amino acid sequence identity ranged from 69-77% with highest identity of 77% with wild boar/WB2C-TV/2011/HUN strain followed by 75% identity with serotypes PTV- 5 and PTV-7 indicating the possible circulation of a new serotype of PTV in Indian pig population. In phylogenetic tree this virus clustered with PTV-13. All three types of PTV described in this study were udentified from same farm so more than one serotype of PTV can circulate in a herd.

For molecular characterization of PSVs comparison of 383 bp sequence of 3D polymerase gene was done. The three isolates from this study clustered together and were closely related to PSV strain V13/ England/57 and PSV strain V13/ England/2000. PSV isolate IVRI/SPF C6/2015 was sequenced by Next generation sequencing (NGS). This isolate

contained 6969 bp long polyprotein gene. The phylogenetic tree based on 6969 bp long polyprotein gene of this isolate showed it to be most closely related to PSV strain V13/ England/2000.

In phylogenetic analysis based on partial VP1 gene of EV G the three isolates from this study EVG/IVRI/SPF 11/2013, EVG/IVRI/SPF 37/2013 and EVG/IVRI/BVC 9/2013 clustered together and were closely related to EV G6 isolated from Vietnam (Accession No. KJ156436, KJ156449, KJ156450) and Korea (Accession No. JQ818253). The fourth isolate showed highest similarity (83% identity) with EV G1 strain Ch-ah-f1 ((Accession No. HM 131607) isolated from china. In phylogenetic analysis of 2505 bp long P1 region of isolate EVG/IVRI/SPF 11/2013 clustered with Enterovirus G6 isolated from Korea earlier named as PEV 16.

None of the identified porcine enteric picornaviruses had any significant nucleotide similarity with the human enteroviruses genome sequences available in public domain.

Conclusion

The result of this study indicates that Porcine Teschovirus, Porcine Sapelovirus and Enterovirus G are prevalent in local swine population of Uttar Pradesh and Bihar. Based on RT-PCR study of faecal specimens prevalence of PTV, PSV and EVG was 6.84 %, 7.14% and 11.05%, respectively. Co-infection by more than one enteric Picornaviruses was also found. Porcine Teschovirus and porcine sapelovirus was isolated in IB-RS-2 cell culture for the first time from Indian pig population which confirms the circulation of these viruses in India.

TaqMan based real-time RT-PCR assays were developed and validated for rapid and specific detection of PTV and EV G in clinical or morbid tissue.

For the first time in India the complete genome sequencing and genetic characterization of PTV was done. Three serotypes of PTV (PTV5, PTV 8 and one putative new serotype) were identified.

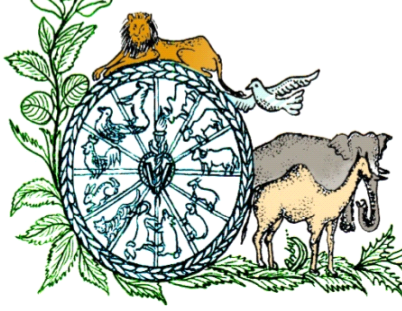
The complete genome sequencing of Porcine sapelovirus and genetic characterization revealed close similarity of this virus to Porcine sapelovirus isolated from England.

Enterovirus G identified in this study showed highest similarity with EV G reported from Korea and Vietnam and were grouped as EV G6. The second type of EVG identified showed highest similarity with EV G reported from China and it was grouped as EV G1.

None of the identified porcine enteric picornaviruses had any significant nucleotide similarity with the human enteroviruses.

Given the reports of diseases such as diarrhoea and encephalomyelitis caused by PTV and PSV the isolation of these viruses from local pigs cannot be ignored and continuous monitoring of virus should be done in pigs in bigger population.

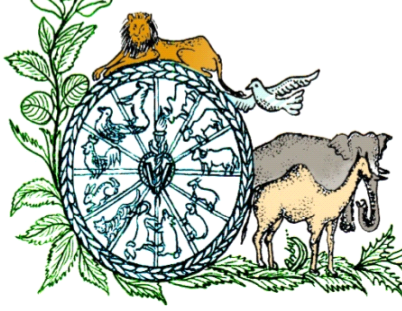
Mini Abstract



7 MINI ABSTRACT

Study was conducted to find out prevalence and pathology of porcine enteric picornaviruses that have been traditionally associated with CNS pathology and to explore their possible relationship to human enteroviruses which cause acute encephalitis syndrome in children. Random faecal samples/ rectal contents from 190 animals and tissue samples from 52 necropsy cases were collected from different farms with history of diarrhoea. Based on RT-PCR study of faecal specimens prevalence of PTV, PSV and EVG was 6.84 %, 7.14% and 11.05%, respectively. Co-infection by more than one enteric Picornaviruses was also found. Enteric picornaviruses were detected in animals with diarrhoea as well as in apparently healthy animals. Three isolates of PTV were isolated in IB-RS-2 cells from PTV positive faecal samples. The CPE was characterized by round, refractile cells grouped in foci, followed by detachment in 3-5 days. Two of the isolates were determined as PTV-5 whereas the third isolate was determined as PTV-8 based on sequence analysis of the isolates. Three isolates of PSV were isolated from PCR positive faecal samples in IB-RS-2 cells. CPE was characterized by refractile cells, enlargement, cell rounding, increased cytoplasmic granularity, rosette-like appearance due to cytoplasmic protrusions through the cell membrane and detachment of the cells. Grossly the intestine was sometimes thin walled and filled with yellowish liquid contents, frequently congested and sometimes with whitish semi-solid or mucoid contents. Patchy areas of petechial haemorrhoges were also found in ileal mucosa. Jejunum was the most affected portion. Mesenteric lymph nodes were congested. The major histo-pathological lesions associated with PTV or EV G positive cases were inflammation of intestines along with desquamation of villous epithelium mainly in jejunum and ileum portion. There was severe congestion of intestinal blood vessels, destruction and desquamation of villous epithelium in small intestine, goblet cell hyperplasia, infiltration of mononuclear cells and lymphoid cell depletion in payer's patches in ileum. Lymphoid cell depletion in follicles of cortical region and eosinophilic infiltration with mild vascular congestion was seen in mesenteric lymph nodes. The CNS lesions were characterized by perivascular cuffing with mononuclear cells and eosinophils, neuronal degeneration in spinal ganglion, meningeal and parenchymal blood vessel congestion and gliosis mainly in cerebrum, hippocampus and to a lessor extent in pons and medulla. The rRT-PCR assay for PTV was developed which detected all three PTV strains analyzed, while it did not react with nucleic acids from any of the related but non-PTV porcine viruses tested such as PSV and PEV. The real-time RT-PCR was able to quantify the standard plasmid down to 100 copies/?l. TaqMan based rRT-PCR assay for enterovirus G detected specifically only the porcine enteroviruses, while it did not react with nucleic acids from any of the non-EVG porcine viruses tested such as PTV and Porcine sapelovirus. The assay was able to identify upto 10 copies/µl of standard plasmid. Two isolates of PTV namely IVRI/SPF-15-2/ 2014 and IVRI/SPF 63/CC/2015 showed maximum identity with PTV 5. Third isolate, IVRI/SPF 11-2/2015 showed maximum similarity with PTV 8 strain 25-T-VII. Based on nucleotide and amino acid sequence, existence of a new serotype of PTV was also indicated. The complete genome sequencing of Porcine sapelovirus was done by Next generation sequencing. Genetic characterization was done on the basis of polyprotein gene or VP1 gene which showed close similarity of this virus to Porcine sapelovirus isolated from England. One group of Enterovirus G identified in this study showed highest similarity with EV G reported from Korea and Vietnam and were grouped as EV G6. The second type of EVG identified showed highest similarity with EV G reported from China and it was grouped as EV G1.

लघु सारांश



8 लघु सारांश

शूकर आंत्र पिकॉरना वायरस जो कि परंपरागत रूप से केन्द्रीय तंत्रिाकातंत्र की विकृतियिों से सम्बन्धित किए गये हैं, उनकी व्यापकता एवं विकृति महत्ता को उजागर करने तथा बच्चों में तीव्र मस्तिष्कोप परिलक्षणों को पैदा करने वाले इंटेरोवायरस से संभावित सम्बन्ध का पता लगाने के लिए अध्ययन किए गये। विभिन्न शुकर प्रक्षेत्रों से 190 मूल नमूने क्रम रहित रूप से तथा दस्त्र प्रभावित 52 शूकरों के उत्तक नमूने एकत्रित किए गये। मल के आर.टी.–पी.सी.आर. अध्ययन के द्वारा पोर्साइन टेस्को वायरस, पोर्साइन सैपिलो वायरस तथा इंटेरोवायरस जी. का प्रसार प्रतिशत क्रमशः 6.84%, 7. 14% तथा 11.05% पाया गया। एक से ज्यादा आंत्र पिकॉरना वायरस का सह संक्रमण भी पाया गया। इन विषाणुओं का संक्रमण दस्त प्रभावित तथा उपरी तौर से स्वस्थ जानवरों दोनों में पाया गया। पी.टी.वी. के तीन तरह के नमूनों को पी.टी.वी. पूर्ण मल नमूनों से कोशिका संर्वधन के द्वारा आइ.बी. आर.एस.–2 कोशिकाओं में पृथक किया गया। पी.टी.वी. संक्रमित आई.बी.आर. एस–2 कोशिका बड़ी आकार की, स्थूल संक्रमित चमकीले एवं समूहों में एकत्रित पाई गयी एवं 3–5 दिनों में सतह से हट गयी। पी.टी.वी. के दो नमूने पी.टी.वी.–5 के रूप में निर्धारित की गयी तथा एक नमूना पी.टी.वी.–8 के रूप में निर्धारित किया गया। तीन पोर्साइन सैपिलोवायरस भी शूकर मल से कोशिका संवर्धन विधि ा से आई.बी.आर.एस.–2 कोशिकाओं में संबर्धित किए गये। पी.एस.वी. का साइटोपैथिक प्रभाव भी कोशिकाओं के स्थूलीकरण, आकार वृधि, चमतकार होना तथा सतह से अलग होने के रूप में थी। लेकिन कोशिकाओं का सतह से पूर्ण रूप से अलग होने का समय 24 घंटे से कम था।वाह्य रूप से आंते कभी–कभी पतली दीवारों के साथ तथा पीले तरल सामग्री से भरी हुई, अक्सर रक्त संकुलित एवं उजली अर्द्ध ठोस म्यूकस मिश्रित सामग्री से भरी हुई थी। उत्तकीय विकृतियों में आँत का सूजन तथा आँत की रोयेदार संरचना का अवक्षण, मोनोन्यूक्लियर कोशिकाओं का अंतःस्पदन मुख्य रूप से पायी गई। केन्द्रीय तंत्रिकातंत्र की उत्तक विकृतियोंमें पेरिवैसकुलर कफींग तंत्रिकाओं का अपकर्ष, मेनिनजियल रक्त नलिकाओं में संकुलन प्रमुख रूप से शामिल है। पी.टी.वी. तथा इंटेरोवायरस जी के लिए निदान के टेकमैन्आधारित रियल टाइम आर.टी.पी.सी.आर. विधि विकसित की गयी। इस विकसित विधि के द्वारा पी.टी.वी. एवं इंटेरोवायरस को विशिष्ट रूप से उत्तको तथा क्लिनिकल नमूनों में बहुत कम समय में पहचाना जा सकता हे। पी.टी.वी. एवं इंटेरोवायरस जी के जिलए संवेदनशीलता सीमा क्रमशः 100 विषाणु/ माईक्रोलीटर तथा 10 विषाणु/माईक्रोलीटर थी। इस अध्ययन से पहचाने गये दो पी.टी.वी सबसे ज्यादा पी.टी.वी.–5 के न्युक्लियोटाइड समानता दर्शाते है। जबकि तीसरा आइसोलेट पी.टी.वी.–8 से सबसे करीबी संबंध दर्शाता है।एक नये सीरोटाइप के हाने की भी संभावना दर्ज की गई। सैपिलोवायरस के पॉलीप्रोटिन जीन के आधार पर सबसे अधिक समानता इंगलैण्ड में पहचाने गये सैपिलो वायरस से थी। दो तरह के इंटेरोवारयरस इस अध्ययन में पहचाने गये एवं इनकी समानता इंटेरोवायरस जी–6 एवं जी–1 से सबसे ज्यादा थी। इस अध्ययन में पहचाने गये किसी भी विषाणु की समानता मानव इंटेरोवायरस से नहीं पायी गयी।

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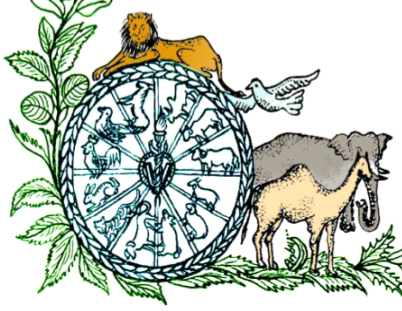

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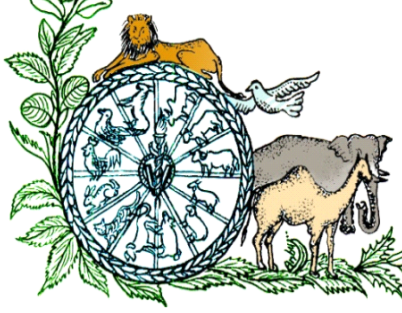
Appendix



APPENDIX

Dulbecco's minimum essential medium (DMEM) with 10% Fetal calf serum (FCS) (100 ml)

DMEM (Gibco)	90.0 ml
FCS (Gibco)	10.0 ml
Antibiotic antimycotic solution (Sigma)	5.0 ml

Prepare aseptically. Store at $4^{O}C$

Antibiotic solution (100 ml)

Na Benzyl Penicillin	10^7 I.U.
Streptomycin sulphate	10 g
PBS	To make 100 ml

Filter sterilize and store in aliquots at -20°C.

Phosphate buffered saline (PBS) (1000 ml)

NaCl	8.0 g
KCl	0.2 g
Na2HPO4 x 12 H2O	2.37 g
KH2PO4	0.2 g
Aqua bidest.	To make 1000 ml

Autoclave at 15 psi at 121°C for 15 min. Store at room temperature.

4% Paraformaldehyde (100 ml)

Paraformaldehyde	4 gm
PBS	To make 100 ml

Add 1-2 NaOH pellets and heat to dissolve. Prepare fresh just before use.

Acetone: Methanol (1:1) (100 ml)

Acetone	50 ml
Methanol	50 ml

PBS containing 1% Triton-X-100 (100 ml)

Triton-X-100	1 ml
PBS (0.01 M, pH 7.4)	To make 100 ml

PBS-T (1000 ml)

Tween 20	0.5 ml
PBS (0.01 M, pH 7.4)	To make 1000 ml

Tri sodium citrate buffer (pH-6)

Tri-sodium citrate	2.69 gm
Aqua bidest	1000 ml

2% APTES (3-Aminopropyltriethoxysilan)

APTES	2 ml
Acetone	98 ml

0.2 % Glycine in PBS (1000 ml)

Glycine	20 g
PBS (0.01 M, pH 7.4)	To make 1000 ml

0.1 M Triethanolamine buffer (pH 8.0) (100 ml)

1 M Triethanolamine buffer	18.565 g
DEPC water	1000 ml

20 x SSC (50 mM NaCl and 15 mM sodium citrate pH 7.0) (1000 ml)

NaCl	175.3 g (150 mM)
Sodium Citrate	88.2 g (300 mM)

Add to 800 ml DEPC water. Adjust pH to 7.0 with NaOH. Make up to 1000 ml.

DEPC H2O (1000 ml)

DEPC	500 µl
Aqua bidest.	1000 ml

Shake, let stand at room temperature for 12 hrs. and autoclave at 15 psi at 121o C for 20 min

2 x Sodium Saline Citrate (SSC) with 0.1% SDS (1000 ml)

20 x SSC	100 ml
10 x SDS	10 ml
DEPC water	make up to1000 ml

1 x Sodium Saline Citrate (SSC) with 0.1% SDS (1000 ml)

20 x SSC	50 ml
10 x SDS	10 ml
DEPC water	make up to 1000 ml

0.5 x Sodium Saline Citrate (SSC) with 0.1% SDS (1000 ml)

20 x SSC	25 ml
10 x SDS	10 ml
DEPC water	make up to 1000 ml

0.1% SDS solution (100 ml)

SDS	0.10 g
Aqua bidest.	To make 100 ml

Heat at 68°C to dissolve. Autoclave at 15 psi for 15 min. Adjust pH to 7.2.

Proteinase K stock (10 mg/ml)

Proteinase K	10 mg
Aqua bidest.	1 ml

Store at -20°C.

0.1M Tris -HCl EDTA buffer (pH 8.0)

Tris-HCl (100 mM)	12.114 g
EDTA (50 mM)	18.612 g
Distilled water (DEPC Rx) to make final volume	1000 ml
Store at room temp.	

EDTA (0.5 M) (pH 8.0) (100 ml)

EDTA	18.612 g
Aqua bidest.	80 ml

Stir on magnetic stirrer vigorously and mix. Adjust pH to 8.0 with 4 M NaOH. Make volume to 100 ml. Auto clave at 15 psi for 15 min. Store at room temperature

4 M NaOH (100 ml)

NaOH	16 g
Nuclease free H2O	To make 100 ml
Autoclave at 20 psi for 15 min	

Tris-Borate EDTA (TBE) Buffer (5X)

Tris base	54.00 g
Boric acid	27.50 g
0.5M EDTA (pH 8.0)	20.00 mL
Distilled water (upto)	1000 mL

Tris and EDTA were mixed in 800 mL of autoclaved water, stirred until dissolved completely. Then boric acid was added and stirred, final volume was adjusted to 1000mL with autoclaved distilled water. Stored at room temperature.

0.5X Working Solution of TBE (0.045 M Tris-borate, 0.001 M EDTA)

TBE Buffer (5X)	100 mL
Double distilled water	900 mL

Sterilized by autoclaving at 15 lb pressure, 120?C for 20 min. Stored at room temperature.

Ethidium Bromide stock solution (10 mg/mL)

Ethidium Bromide	1 g
Double distilled water	100 mL

Solution stirred on magnetic stirrer for several hours to ensure that the dye dissolved properly. The solution was transferred to aluminium foil wrapped amber colored bottle and stored at 4°C.

6 x Gel loading buffer (10 ml)

Bromophenol blue	250 mg
Xylene cyanol FF	250 mg
Sucrose	4.0 g
Aqua bidest.	To make 10 ml

Ethidium bromide staining solution for gels (1000µl)

Ethidium bromide	500 mg
Double distilled water	100 ml

X-gal (25 mg/ml)

X-gal	25 mL
Dimethyl formamide	1.0 mL

0.25% Acetic Anhydride

Acetic anhydride	0.25ml
Distilled water (DEPC Rx) to make final volume	100ml

IPTG (25 mg/ ml)

IPTG	25 mg
Double distilled water	25 mL

1x TE buffer (pH 8.0)

Tris-HCl	10 mM
EDTA	1 mM

Store at room temp.

LB agar

LB agar	35 gm
Double distilled water	1000 ml

LB (Luria- Bertani) broth

LB broth	2.5gm
Double distilled water	1000ml

VITAE

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