DEVELOPMENT OF A DNA VACCINE AGAINST PORCINE CIRCOVIRUS TYPE 2 AND EVALUATION OF ITS IMMUNO-POTENTIAL IN MICE MODEL

Thesis

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IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Doctor of Philosophy
(Animal Biotechnology)

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Dedicated to....

My Beloved Parents, Wife,
Family and
Covid Warriors

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ABBREVIATIONS

%	:	Percent/Percentage
<	:	Less than
±	:	Plus-minus
µg	:	Microgram(s)
µl	:	Microlitre(s)
µM	:	Micromolar
ANOVA	:	Analysis of variance
ASFV	:	African swine fever virus
bp	:	Base pair
$CaCl_2$	:	Calcium Chloride
cDNA	:	Complementary deoxy-ribonucleic acid
CO_2	:	Carbon dioxide
Con A	:	Concanavalin A
CSFV	:	Classical swine fever virus
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxy-ribonucleic acid
dNTP's	:	Deoxyribonucleotide tri phosphate
EDTA	:	Ethylenediamine tetra-acetic acid
ELISA	:	Enzyme-linked immunosorbent assay
Etbr	:	Ethidium bromide
Fig	:	Figure
g	:	Relative centrifugal force
gm	:	Gram
hr/hrs	:	Hour(s)
HRPO	:	Horse radish peroxidase
IAEC	:	Institutional Animal Ethics Committee
IFN-γ	:	Interferon gamma
IL4	:	Interleukin 4
IM	:	Intramuscular
IU	:	International Unit
kb	:	Kilobase
kDa	:	Kilodaltons
LB	:	Luria Bertini
Log	:	Logarithm
LTT	:	Lymphocyte Transformation Test

M	:	Molar
min	:	Minute(s)
ml	:	Millilitre(s)
mm	:	Millimeter(s)
mM	:	Millimolar
MTT	:	3-(4, 5 dimethyl thiazolyl-2)-2, 5- diphenyltetrazolium
NER	:	North-eastern region
NFW	:	Nuclease-free water
ng	:	Nanogram(s)
No.	:	Number
nts	:	Nucleotides
°C	:	Degree Celsius
OD	:	Optical density
ORF	:	Open reading frame
PAGE	:	Polyacrylamide gel electrophoresis
PBMCs	:	Peripheral blood mononuclear cells
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
PCV	:	Porcine Circovirus
PCVAD	:	Porcine circovirus-associated disease
PDNS	:	Porcine dermatitis and nephropathy syndrome
pH	:	Log hydrogen ion concentration
PMWS	:	Postweaning multisystemic wasting syndrome
PRRSV	:	Porcine reproductive and respiratory syndrome virus
PVDF	:	Polyvinylidene fluoride
RBCs	:	Red blood cells
Rep	:	Replicase
RIPA	:	Radioimmunoprecipitation assay buffer
RNA	:	Ribonucleic acid
rpm	:	Revolutions per minute
RPMI	:	Roswell Park Memorial Institute Medium
RT- PCR	:	Reverse transcriptase polymerase chain reaction
RT	:	Room temperature
SDS	:	Sodium dodecyl sulphate
Sec	:	Second(s)
SI	:	Stimulation Index
ss	:	Single stranded
Taq	:	Thermus aquaticus

TCID	:	Tissue culture infective dose
TE	:	Tris EDTA
TNF-α	:	Tumour Necrosis Factor-alpha
Tris	:	Tris-hydroxy methyl aminoethane
U	:	Unit(s)
UV	:	Ultraviolet
V/cm	:	Volts/centimeter
v/v	:	Volume/Volume
VLP	:	Virus-like particles
w/v	:	Weight/Volume
β	:	Beta
Δ	:	Delta

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Introduction

1 INTRODUCTION

The swine industry has been contributing impressively to the global economy. In India, the swine industry is in a progressive mode, especially in the north-eastern region (NER) and there is tremendous scope to expand this industry to a larger scale. Pig farming is a key source of income for the rural population, especially for the tribal population of the northeast India. Landless and marginal farmers of Assam and other north-eastern states usually rear pigs for their livelihood. Pork is rich in animal protein and other valuable nutrients, and pork preparations have become one of the most favourite dishes for the youths of this generation particularly in the urban areas, besides its popularity among the tribal population of NER. According to the 20th Livestock census 2019, the total pig population in India is 9.06 million, of which Assam alone shares around 23.18 %. NER is considered as the hub of pig farming in the country and it contributes significantly to the national economy. Moreover, pig farming has a tremendous scope to provide livelihood to the youths who lost their jobs or income sources due to the ongoing COVID-19 pandemic and can help reducing the negative economic impact of the pandemic and restoring the economic growth of this region.

Although the swine industry has immense potential to enhance the nation's economy, many emerging and re-emerging viral diseases caused by Classical swine fever virus (CSFV), Porcine circovirus 2 (PCV2), Porcine reproductive and respiratory syndrome virus (PRRSV), and the recently encountered African swine fever virus (ASFV) are acting as prime obstructing factors in development and expansion of this industry in this region. PCV-2 infection is one of the emerging threats to the Indian swine population as several recent studies have documented the circulation of PCV2 in pig herds of India including NER (Anoopraj *et al*., 2015; Karuppannan *et al.,* 2016; Pegu *et al*., 2017; Mukherjee *et al*., 2019; Barman *et al*., 2020; Deka *et al*., 2021).

Porcine Circovirus Type 2 is a common pig virus which is distributed worldwide among pigs. PCV2 belongs to the family *Circoviridae* and the genus Circovirus having an unsegmented, circular, single-stranded DNA genome (Allan *et al*., 2000; Rosario *et al*., 2017). Till date, four types of PCV are identified, designated as PCV1, PCV2, PCV3 and PCV4 (Opriessnig *et al*., 2020). Porcine circovirus type 2 is pathogenic in nature and found to be associated with several other clinical manifestations and pathological lesions, collectively known by 'Porcine circovirus disease' (PCVD) or 'Porcine circovirus-associated disease' (PCVAD) based on the geographical location (Segalés *et al*., 2005; Opriessnig *et al*., 2007; Opriessnig and Langohr, 2013). Clinical manifestations of PCV2 infection includes post-weaning multisystemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), respiratory complications, enteric disorders, and reproductive failure (Opriessnig *et al*., 2020). PCV2 produces immunosuppression in pigs, and it becomes more severe when there is a concomitant infection by other pathogens (Vincent *et al.*, 2007). Co-infection of PCV2 with swine viruses such as CSFV, PRRSV, Swine Influenza Virus, Porcine Epidemic Diarrhoea Virus (PEDV), Pseudorabies Virus (PRV), Porcine Parvovirus (PPV) and PCV3 has been documented in several literatures (Ouyang *et al*., 2019) and such co-infection can lead to more detrimental condition in pigs. Moreover, Huang *et al.* (2011) reported a decrease in the efficacy of lapinized CSF vaccine when pigs are infected with PCV2.

The genome size of PCV2 is between 1766 and 1769 nucleotides with two main and important open reading frames (ORFs), namely ORF1 and ORF2 in opposite directions. The ORF1 (*Rep* gene) of PCV2 encodes protein Rep and Rep' which are involved in replication process (Lv *et al.,* 2014; Opriessnig *et al*., 2020). The only structural protein of PCV2, 'capsid' is encoded by ORF2 or *Cap* gene (Olvera *et al*., 2007; Segalés *et al.*, 2008). The *cap or ORF2* gene is regarded as a potent phylogenetic marker of PCV2 and currently eight genotypes of PCV2 (PCV2a to PCV2h) have been identified by using *ORF2* gene sequences of several isolates (Franzo and Segalés, 2018). The other identified ORFs of PCV2 are ORF3 and ORF4. The ORF3 is translated into an apoptotic protein which induces apoptosis in PCV2 infected cells (Liu *et al*, 2005; Liu et al., 2006a; Lin *et al.,* 2011a). The ORF4 or apoptosis-suppressing gene was described to possess anti-apoptotic function (He *et al*., 2013; Gao *et al*., 2014).

The capsid protein containing 233-234 amino acids was found to be the most immunogenic protein of PCV2 and a potent vaccine candidate (Pogranichnyy *et al*., 2000; Mankertz *et al.*, 2004). Capsid protein induces strong immune response (including neutralizing antibodies) in pigs and can protect pigs from PCV2 infection (Lv *et al.,* 2014). Most of the commercial subunit vaccines available consist of PCV2 capsid protein. Guo *et al.* (2012) reported a mutated strain of PCV 2b, *viz*. mPCV 2b, and observed that the mutation that results in elongation of the capsid protein by one amino acid (Lysine) at the C-terminal (at position 234) increases the virulence of PCV 2 compared to the wild type. However, the mutation in stop codon of cap gene leading to elongation of ORF2 by an extra lysine residue codon at 3' end was initially observed in three PCV2 strains from china which formed a separated clade in phylogenetic analysis and designated as PCV2d genotype (Guo *et al*., 2010). Moreover, mPCV2b mutant is now designated as PCV2d by many researchers due to its rapid global circulation and this variant type has been isolated from vaccine-failure cases (Franzo *et al.,* 2016). This may be due to antigenic variation between the mutant form or PCV2d and the old genotype PCV2a, which constitutes most of the commercial vaccines available so far (Opriessnig *et al.,* 2013b; Kwon *et al.,* 2017). The PCV2d genotype with an additional lysine residue at the C-terminal has recently been reported from pigs of South India (Parthiban *et al*., 2021). Recently, the structure of PCV2d capsid protein has been revealed and structure-based sequence analysis has shown differences with PCV2a and PCV2b genotyped with respect to amino acids that exposed in the surface of capsid protein (Khayat *et al.,* 2019). Earlier studies have confirmed that the last three or four residues of capsid protein contains immuno-dominant epitopes (Lekcharoensuk *et al.,* 2004; Shang *et al.,* 2009). Therefore, it is essential to note the immune response elicited by the capsid protein of PCV2d genotype having an extra lysine at the C-terminal end (at position 234).

Vaccination has the ability to control PCV2 infection effectively, but woefully at present there is no indigenously manufactured commercial vaccine against PCV2 in India. There are commercial vaccines (chimeric, subunit, or inactivated) in the international market which can significantly prevent PCV2 infection in pigs, but the production process is cumbersome and laborious, provide short term immunity and the most limiting factor for the Indian farmers for

their use is the cost of the vaccines. Moreover, the available commercial vaccines sometimes fail to protect the vaccinated pigs due to genotypic shift of PCV2 (Opriessnig *et al*., 2013b; Kwon *et al*., 2017; Opriessnig *et al*., 2020) i.e., initial PCV2a shifted to PCV2b and then PCV2b was replaced by PCV2d (Patterson and Opriessnig, 2010; Xiao *et al*., 2016; Franzo and Segales, 2018; Opriessnig *et al*, 2020). Out of eight genotypes of PCV2, the most dominant and virulent one currently reported worldwide is PCV2d (Guo *et al.*, 2010, Xiao *et al.*, 2015). A few studies conducted in China from 2016 to 2018 have revealed an increase in prevalence of PCV2d as compared to the other genotypes (Hou *et al.,* 2019b). Even a recent study conducted in northeast region of India has predicted the dominancy of PCV2d genotypes (D'silva *et al.,* 2021). Genotypes PCV2a and PCV2b are also virulent and distributed worldwide, while other genotypes are reported sporadically with limited temporal persistence (Franzo and Segalés, 2020).

Many years after the uncovering of PCV2, the initial genotypic shift of PCV2a to PCV2b was ascertained between 2003 and 2006, (Patterson and Opriessnig, 2010; Opriessnig *et al*., 2020), and the second major shift (PCV2b to PCV2d) occurred between 2010 and 2015 (Xiao *et al*., 2015; Xiao *et al.,* 2016; Franzo and Segales, 2018). Most of the commercial vaccines (Circovac®, Porcilis®, IngelvacCircoFLEX®, etc.) available in the international market are based on the old genotype (PCV2a). Vaccine failure episodes were reported to be associated with outbreaks of PCV2d genotype (Seo *et al.*, 2014; Franzo *et al*., 2016). Cross protection by PCV2a genotype-based vaccine against other genotypes is still debatable because different studies have shown contradicting results in this aspect (Franzo and Segalés, 2020). Hence, it is a prerequisite to develop a potential vaccine using new promising vaccine candidates such as vaccines based on the capsid protein of PCV2d genotype so that defensive immune response might be developed against PCV2 infection.

Commercial PCV2 vaccines can reduce PCV2 infection and protect pigs but the duration of protection is short and cross-protection against all PCV2 genotypes is still not obvious. Therefore, the hunt for a better vaccine candidate against PCV2 is still on and development of a DNA vaccine is one potential option. A number of experimental DNA vaccines developed by various workers have shown promising results against PCV2 infection in mice model as well as in the primary host pig (Li *et al.,* 2015; Park *et al.,* 2017; Hou *et al.*, 2019a).

Most of these DNA vaccine constructs contain the *cap* gene which encodes the capsid protein of either PCV2a or PCV2b (Silva Júnior *et al*., 2009; Sylla *et al.,* 2014; Park *et al.,* 2017) except for one that used the *cap* gene of PCV2d (Hou *et al.*, 2019a). DNA vaccine is a promising substitute to orthodox vaccines against animal and human pathogens. DNA vaccines are cost effective, has high thermal stability, the production process is uncomplicated, and can elicit a broad range of immunity, *i.e*. both humoral and cell-mediated immunity (Liu *et al*., 2006b; Guo *et al.,* 2015; Hou *et al.*, 2019a). In this regard, PCV2 DNA vaccine is expected to be economical and suitable for use in NER of India considering the socio-economic status of the farmers, agro-climatic conditions and the limited availability of cold chain infrastructure needed for conventional vaccines.

Molecular epidemiological studies recorded considerable prevalence rate of PCV2 in the NER and other parts of India (Anoopraj *et al*., 2015; Pegu *et al*., 2017; Mukherjee *et al*., 2018; Barman *et al.,* 2020; Deka *et al*., 2021) which suggests its endemic status in the country. The circulating genotypes detected in India are PCV2a, PCV2b, PCV2d and PCV2g (Barman *et al*., 2018; Parthiban *et al.,* 2021; Rajkhowa *et al*., 2021) and genotype PCV2d is likely to be endemic in NER (D'silva *et al.,* 2021). In addition, a few recombinant strains were also reported from India (Anoopraj *et al*., 2015; Mukherjee *et al*., 2019; Rajkhowa *et al.,* 2021). With the increased prevalence of PCV2 in NER (Barman *et al.,* 2020), there is an emergency to inaugurate a potential cost-effective vaccine that not only suits to the prevailing climatic condition and the cold chain infrastructure of NER but also could provide strong and long-lasting immunity.

Keeping the above facts in view, the present study was proposed with the objective to develop a suitable new DNA vaccine encoding the capsid protein of PCV2 belonging to genotype PCV2d with a lysine residue at position 234.

Objectives:

1. **To generate a DNA vaccine construct encoding the mutated capsid gene of PCV2.**
2. **To evaluate the immunogenicity of the DNA vaccine candidate in mice model.**

Review of Literature

2 REVIEW OF LITERATURE

2.1 Circoviruses of porcine

Porcine Circovirus (PCV) is a common virus of both domestic and wild pigs which is considered as the smallest virus autonomously replicating in mammalian cells. PCV is a member of the genus *Circovirus* belonging to the family *Circoviridae* characterized by a non-enveloped virion with icosahedral symmetry having average diameter of 17 to 21 nanometres (Opriessnig *et al*., 2007; Rosario *et al*., 2017; Opriessnig *et al*., 2020). PCV has a single-stranded DNA (ssDNA) genome (~1.75 to 2 kb in size) which is circular and covalently closed (Opriessnig *et al*., 2007; Faurez *et al*., 2009; Rosario *et al*., 2017; Opriessnig *et al*., 2020). The genome of PCV is ambisense in nature with two major open reading frames (ORFs), *viz*. ORF1 and ORF2 (Mankertz *et al*., 2004; Faurez *et al*., 2009). The ORF1, present in the positive strand, encodes the proteins involved in replication of PCV and the ORF2 encodes the only structural protein, the capsid protein, which is located in the negative strand of the replicative form (Mankertz *et al*., 2004; Faurez *et al*., 2009; Rosario *et al*., 2017; Klaumann *et al*., 2018a).

Currently, four types of PCV are identified, *viz*. Porcine Circovirus Types 1, 2, 3 and 4. In 1974, the first PCV was detected as a chronic contaminant of continuous porcine kidney cell line, PK-15 (ATCC-CCL33) and was considered as a picorna virus-like contaminant (Tischer *et al.*, 1974). Later in 1982, it was described as a circular ssDNA virus and now it is designated as PCV 1 which is non-pathogenic in nature, especially to swine, and it fails to produce any disease in pigs under experimental condition (Tischer *et al*., 1982; Tischer *et*

al., 1986; Allan *et al.*, 1999). PCV1 genome is about 1758 to 1760 nucleotides (Opriessnig *et al*., 2020).

In 1991, the primary report of post-weaning multisystemic wasting syndrome (PWMS) was described in a high-health swine herd of Canada (Harding and Clark, 1997; Allan *et al*., 1998; Saikumar and Das, 2019) and the prime contributory agent of PWMS was identified as PCV2 because it exhibited a different pattern of restriction digestion from that of PCV1 (Nayar *et al*., 1997; Allan *et al*., 1999). Subsequently, PWMS was identified in several countries including, USA, Japan, France, Korea and many others (Guo *et al*., 2010). However, several studies conducted on archive tissue and serum samples of pigs documented the circulation of PCV2 since 1969 (Opriessnig *et al*., 2007). The genome of PCV2 is between 1766-1769 nucleotides (Fenaux *et al*., 2000; Guo *et al*., 2010; Klaumann *et al*., 2018a; Saikumar, and Das, 2019; Opriessnig *et al*., 2020).

The third type of PCV designated as PCV3 was reported from pigs of North Carolina, USA in 2015 by a metagenomics-based approach and it was found to be associated with porcine dermatitis and nephropathy syndrome (PDNS) and reproductive failure (Palinski *et al*., 2017). But it was also detected in apparently healthy pigs. Later, it was reported from other countries of Europe, Asia and South America which indicated its widespread prevalence in the world (Klaumann *et al*., 2018a). However, many retrospective investigations have proved its existence since 1993 (Klaumann *et al*., 2018b; Sun *et al*., 2018; Ye *et al*., 2018). The average genome of PCV3 is between 1999 and 2001 nucleotides (Palinski *et al*., 2017; Fux *et al*., 2018).

Recently (2019), PCV4 the fourth type of PCV was reported from pigs of Hunan province of China suffering from PDNS, enteric complications and respiratory distress (Zhang *et al*., 2020). Like PCV3, it was also noticed in apparently healthy pigs (Zhang *et al*., 2020). Eventually, it was detected in Korea (Nguyen *et al*., 2021) and certain region of China (Sun *et al*., 2021; Tian *et al*., 2021). However, a retrospective surveillance done in China indicated its existence at least from 2016 (Ha *et al*., 2021). The circular genome of PCV4 contains 1770 nucleotides (Zhang *et al*., 2020; Ha *et al*., 2021).

2.2 Molecular organization of Porcine Circovirus 2

Like other members of *Circovirus*, PCV2 also possesses a closed, circular and ssDNA genome. The genome of PCV2 is 1767-1769 nucleotides (nts) in size which is ambisense in nature because the initially identified ORF1 and ORF2 are oriented in opposite directions (Faurez *et al*., 2009; Lv *et al*., 2014; Opriessnig *et al*., 2020). The genome of PCV2 was predicted to contain 11 overlapping ORFs from ORF1 to ORF11 (Hamel *et al*., 1998). However, only four of the ORFs (ORFs 1, 2, 3 and 4) are well characterized (Lv *et al*., 2014; Klaumann *et al*., 2018a). The ORFs 1, 5, 7 and 10 are present in the positive-strand of PCV2 genome whereas the ORFs 2, 3, 4, 6, 8, 9 and 11 are present in the opposite strand (Hamel *et al*., 1998).

The opposite orientation of ORF1 and ORF2 makes the genome of PCV2 ambisense in nature. Moreover, this arrangement has created two inter-genic regions in the PCV2 genome (Fig 2.1). One of the inter-genic regions is present between 3' ends of these ORFs which is shorter in length, whereas the other inter-genic region is larger and it is located between 5' ends of ORF1 and ORF2 (Lv *et al*, 2014). The PCV2 genome is replicated *via* rolling-circle mechanism and the larger inter-genic region contains the origin of replication which is constituted by a putative stem-loop (SL) and four hexamer repeats (Fig 2.1) (Faurez *et al*., 2009; Finsterbusch and Mankertz, 2009). The stem-loop structure contains a conserved nanomer in its loop. The replicases bind to the hexamer motifs present close to the stem-loop (Finsterbusch and Mankertz, 2009).

The ORF1, also known as *rep* gene, present in the plus-strand of PCV2 is the largest ORF with 945 nucleotides, from position 51- 995 (Lv *et al*., 2014). It encodes two major proteins, Rep and Rep' essential for viral replication (Lv *et al*., 2014). The Rep protein is encoded by the full-length *rep* gene, which is 314 amino acids long or approximately 35.8 kDa (Finsterbusch and Mankertz, 2009). The Rep' protein contains 178 amino acids which is encoded by a differentially spliced frameshift version of *rep* gene transcript (Finsterbusch and Mankertz, 2009). However, transcript mapping of *rep* gene revealed expression of six other minor RNAs produced by alternate splicing which are either members of Rep-associated

RNAs (Rep3a, Rep3b and Rep3c) or NS-associated RNAs (NS0, NS515 and NS672) (Cheung, 2003).

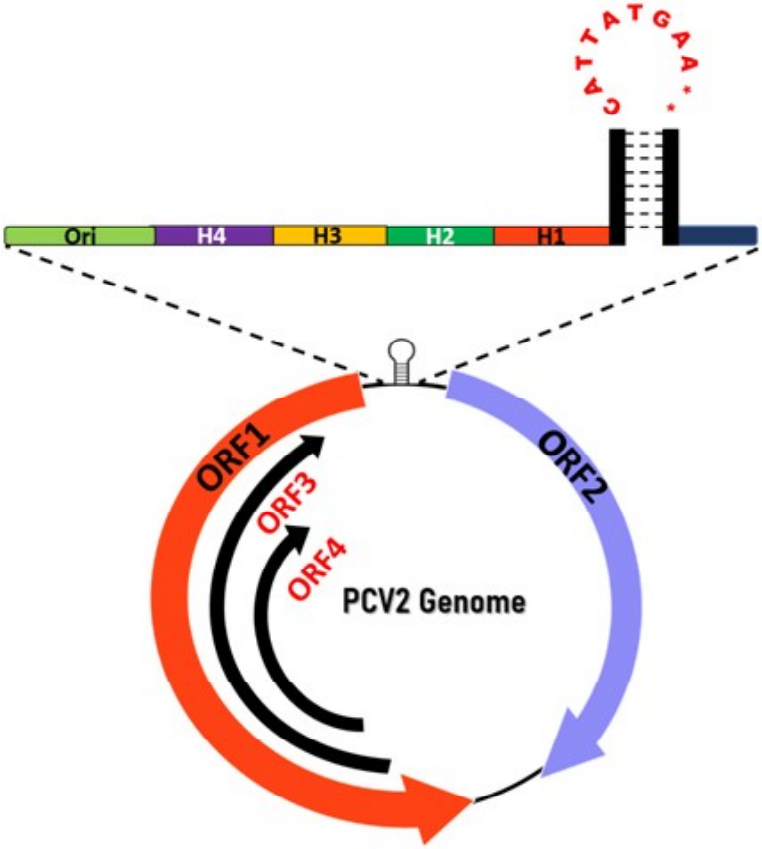

Fig 2.1: Genome organization of PCV2. The orange arrow indicates the ORF1 present in the positive strand. The light purple arrow indicates the ORF2 located in the opposite direction. The ORF3 and ORF4 are indicated with black arrow. The origin of replication, which is situated in inter-genic region between 5' end of ORF1 and ORF2, consists of a stem-loop with conserved nanomer and hexamer motifs (H2, H2, H3 and H4).

The cap gene or ORF2 encodes the major immunogenic protein, the capsid protein, which is about 27 kDa with 233 to 236 amino acids (Lv *et al*., 2014; Opriessnig *et al*., 2020). Its length is about 702-705 nucleotides which is present on the complementary strand from position 1735-1034. The capsid protein is the sole structural protein of PCV2 and considered to be the most variable protein of PCV (Klaumann *et al*., 2018a) due to which it has been used as a phylogenetic marker. The capsid protein participates actively in cellular entry of the virus via receptors located on the host cells.

The third ORF, *i.e*. ORF3 is located within ORF1 but in the anti-clockwise direction. It is also known as the apoptosis-inducing gene (Liu *et al*., 2005). It is approximately 315 nucleotides in length which spans from position 671–357 and encodes a non-structural protein (11. kDa approximately) with 104 amino acids. After the identification of ORF3 in 2005 (Liu *et al*., 2005), several studies were conducted to specify its function. Later, it was established that protein encoded by ORF3 induces apoptosis in the virus infected cells (PK15 and PBMC) in *in vitro* condition (Lin *et al*., 2011a), and by virtue of this apoptotic property, it takes part in viral pathogenesis both *in vitro* as well as *in vivo* conditions (Liu *et al*., 2005; Liu *et al*., 2006a).

The ORF4 is nested within ORF3 and it is transcribed in the similar orientation like ORF3 but opposite to ORF1 (Lv *et al*., 2014). It is the smallest ORF compared to other three which is only 180 nucleotides in length located from 565- 386 position of PCV2 genome (Lv *et al*., 2014). The protein encoded by ORF4 contains only 59 amino acids having 6.5 kDa molecular mass approximately (He *et al*., 2013). The ORF4 is known as the apoptosis-suppressing gene because it exhibited to have anti-apoptotic function (He *et al*., 2013; Gao *et al*., 2014). However, its active role in viral pathogenies is still not obvious (Lv *et al*., 2014).

2.3 Genotypes of Porcine Circovirus 2

Porcine Circovirus 2 exhibits high rate of mutation like other ssDNA viruses, which is about 10^{-3}-10^{-4} substitutions/year/site leading to emergence of several variants over time (Franzo and Segalés, 2020). Currently, eight genotypes of PCV2 (PCV2a-2h) are well established (Fig. 2.2) which were reported from different swine rearing countries (Franzo and Segale´s, 2018). Initially, PCV2 was sub-grouped into PCV2a and PCV2b genotypes based on cut-off of 2% at nucleotide level for complete genome and 3.5% for ORF2 (Grau-Roma *et al*., 2008). Subsequently, based on the same cut-off, a third genotype, PCV2c was reported from Denmark which was detected in archived samples (Dupont *et al*., 2008). Although data indicates its circulation in Brazil and China (Franzo *et al*., 2015a; Liu *et al*., 2016), but it has not been detected for a long time. The fourth genotype, PCV2d, was first reported from China (Guo *et al*., 2010), however its existence in Switzerland could be traced back retrospectively to 1998

(Xiao *et al*., 2015). Later, a new genotype of PCV2 (PCV2e) was reported from Mexico and USA (Harmon *et al*., 2015; Davies *et al*., 2016). Again, based on a retrospective investigation led in China a novel genotype, 'PCV2f', was proposed (Bao *et al*., 2018). Recently, in addition to the existing six genotypes, two other novel genotypes, PCV2g and PCV2h have been proposed by Franzo and Segale´s (2018). They have classified PCV2 into eight genotypes (PCV2a-h) based on intra-genotype p-distance of ORF2 (maximum of 13%) and bootstrap value of more than 70% at the respective internal node having minimum of 15 sequences. Currently, this is the most accepted classification of PCV2 genotypes, although recently another novel genotype (PCV2i) has been detected in USA (Wang *et al*., 2020).

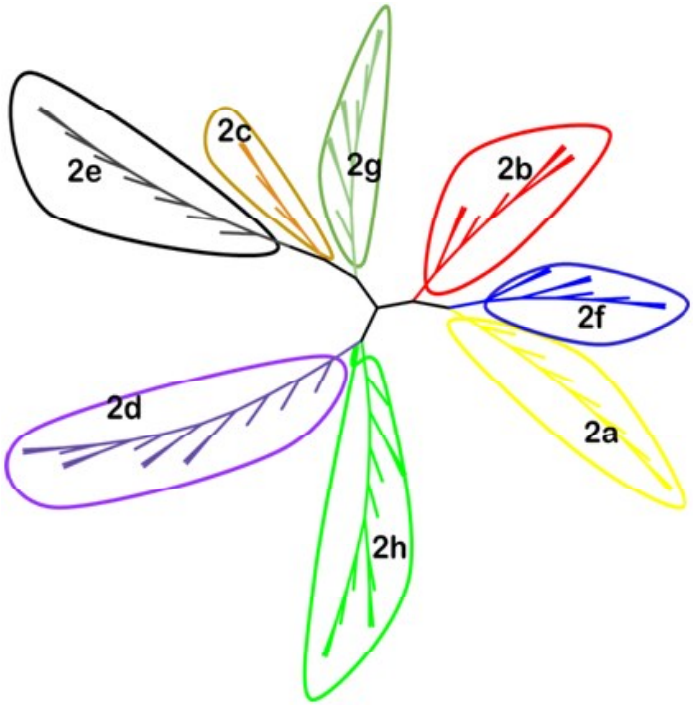

Fig 2.2: Genotypes of Porcine Circovirus 2

Besides the continuous evolutionary character of PCV2, another important characteristic of PCV2 is the genotype shift. Till date, two major episodes of genotype shift was observed among the prevalent genotypes of PCV2. From 1996 to 2000, the major genotype circulating in global pig population was PCV2a, but thereafter it was replaced by the PCV2b genotype. Thus, the first genotypic shift of PCV2 from PCV2a to PCV2b occurred between 2003 and 2006 (Patterson and Opriessnig, 2010; Franzo and Segales, 2018; Opriessnig *et al.*, 2020). The second episode of genotype shift was noticed in between 2010

and 2015 where PCV2b was shifted to PCV2d (Xiao *et al*., 2015; Xiao *et al*., 2016; Franzo and Segales, 2018). The genotypic shift was noticed not only in domestic pigs but also the same pattern was observed in wild pigs which might be a spill over from the domesticated swine (Opriessnig *et al.*, 2020).

In the current scenario, out of all the genotypes of PCV2, the most prevalent and the worldwide distributed genotypes are PCV2a, PCV2b as well as PCV2d (Franzo and Segales, 2018). The other genotypes are detected sporadically and many are still undetected for a long time or considered to be extinguished (Franzo and Segales, 2018; Franzo and Segalés, 2020).

2.3.1 Porcine Circovirus 2d (PCV2d) Genotype

Currently, one of the most prevalent genotypes of PCV2 is 'PCV2d', which is distributed globally and it is virulent like PCV2a and PCV2b. The genotype PCV2d was initially identified in 2010 (Guo *et al*., 2010), but a retrospective analysis reveals its existence in Switzerland since 1998 (Xiao *et al*., 2015). Initial phylogenetic analysis designated the current PCV2d genotype as PCV2b-1C (Olvera *et al*., 2007). Subsequently, it was identified as a mutant of PCV2b and designated as mPCV-2b (Guo *et al*., 2012; Xiao *et al*., 2012; Opriessnig *et al*., 2013b; Salgado *et al*., 2014;). It was also linked with vaccine failure cases (Xiao *et al*., 2012; Salgado *et al*., 2014). The mutant variant of PCV2b consist of a specific mutation in the stop codon of ORF2 which changes the stop codon to a lysine codon (Guo *et al*., 2012). This results in elongation of the capsid protein amino acid sequence by an additional amino acid (lysine residue) at position 234. Moreover, pathogenicity of this mutated variant was established to be higher than PCV2a or PCV2b (Guo *et al*., 2012). However, eventually this new genotype was reclassified as PCV2d (Franzo *et al*., 2015b) and the recent classification has further established it as the PCV2d genotype (Franzo and Segales, 2018).

Initially, the PCV2d genotype was predominant in Asia but due to the genotypic shift, it has eventually spread to Europe and America. Currently, it has spread to the global pig population and its prevalence is now at higher level than that of the other genotypes. Like many other countries, PCV2 is considered to be endemic in pig herds of India and found to be associated with the clinically affected pigs. Most of the molecular investigations conducted in

India has recorded the circulation of the PCV2d genotype along with other genotypes (Anoopraj *et al*., 2015; Barman *et al.,* 2018; Mukherjee *et al*., 2018; Parthiban *et al*., 2021). Moreover, the recent reports indicates that genotype PCV2d is in phase of endemic in NER of India (D'silva *et al,* 2021; Rajkhowa *et al*., 2021)

2.4 Capsid protein of Porcine Circovirus 2

There is only one structural protein of PCV2 encoded by ORF2 or *cap* gene known as the capsid protein. The approximate molecular weight of the capsid protein is 27.8 kDa which is composed of 233-234 amino acids. Host immune response is primarily stimulated by capsid protein of PCV2 (Nawagitgul *et al.*, 2000) and considered as the most immunogenic protein of PCV2 (Mankertz *et al*., 2004). It acts as a virulence determinant and helps in attachment of PCV2 to the host cells *via* chondroitin sulphate B or heparin sulphate (glycosaminoglycan) receptors present on the target cells of host (Misinzo *et al*., 2006). The capsid protein is a leading candidate to design new generation vaccines because it is the only immunogenic protein associated with the production of specific neutralizing antibodies against PCV2 (Pogranichnyy *et al.*, 2000). The capsid protein expressed in prokaryotic or in baculovirus expression system can self-assembly into virus-like particles (VLPs) and can mimic the icosahedral symmetry of the native PCV2 virions (Mo *et al*., 2019) which is a promising subunit vaccine candidate.

The N-terminus of Cap protein contains the Nuclear Localization Signal (NLS) which is also essential for stable VLP formation (Mo *et al*., 2019). The carboxyl terminus of capsid protein is critical for self-assembling of Cap into VLPs. The C-terminal is also essential for entry of PCV2 into the cells and its propagation in host cells (Zhan *et al*., 2020). Again, capsid protein C-terminal also contains immune-dominate epitopes, particularly consisting of the last three or four amino acids (Lekcharoensuk *et al.,* 2004; Shang *et al.,* 2009). It has been established that mutation in the capsid protein, *i.e*. insertion of an extra lysine residue at 234 position increases its pathogenicity (Guo *et al.,* 2012) and the PCV2 strains with this mutation are designated as genotype 'PCV 2d'. This new PCV 2 sub-type (PCV2d) has evolved recently; therefore, data related to the 3D structure of this particular protein with 234 amino acids is scanty.

2.5 Disease associated with Porcine Circovirus 2 infection

Porcine Circovirus 2 targets primarily the young pigs (5-16 weeks old) but mature pigs are also susceptible. Earlier, disease associated with PCV2 was designated as PMWS (Harding and Clark, 1997). Later it was observed that besides PMWS, PCV2 was also associated with several other clinical manifestations such as enteric disease, reproductive disease, respiratory disease, and porcine dermatitis and nephropathy syndrome (Opriessnig *et al*., 2020). Therefore, in Europe infections caused by PCV2 are collectively known as porcine circovirus diseases (PCVD) and in North America as porcine circovirus-associated diseases (PCVAD) (Segalés *et al*., 2012). Common clinical manifestations associated with PCV2 are as follows:

2.5.1 Postweaning multisystemic wasting syndrome (PMWS)

The term postweaning multisystemic wasting syndrome was described in 1991 but its association with PCV2 was reported in 1997 (Segalés and Domingo, 2002). Typical clinical manifestation of PMWS is progressive weight loss, wasting, respiratory distress, enlarged subcutaneous lymph nodes, lethargy, lymphadenopathy, jaundice, pale skin diarrhoea and many other non-specific clinical signs (Gillespie *et al*., 2009; Opriessnig and Langohr, 2013). The most striking microscopic lesions are lymphocyte depletion replaced by histiocyte in lymphoid tissues and presence of basophilic intracytoplasmic inclusion bodies in histiocytic cells (Segalés and Domingo, 2002). Mortality and morbidity rates exhibited in pigs suffering from PMWS are 4-20% and 4-30%, respectively, however mortality may rise to 50-80% in certain adverse conditions (Segalés and Domingo, 2002; Gillespie *et al*., 2009; Saikumar and Das, 2019).

2.5.2 Respiratory disease (Pneumonia) associated with PCV2

The respiratory complications associated with PCV2 is a part of the porcine respiratory disease complex (PRDC) which is a multi-factorial disease (Kim *et al*., 2003). The primary clinical signs include respiratory distress, dyspnea, coughing, nasal discharge, fever, anorexia and decreased growth, and reduced feed efficiency (Kim *et al*. 2003; Gillespie *et al*., 2009; Anoopraj *et al*. 2015). The clinical signs many a time overlap with PMWS. The microscopic

lesions encountered are granulomatous broncho-interstitial pneumonia with necrosis, bronchiolar fibrosis and ulcerative bronchiolitis (Gillespie *et al*., 2009).

2.5.3 Reproductive failure associated with PCV2

The first case of reproductive failure due to PCV2 infection was reported from Canada in 1999 (West *et al*., 1999). Dams, particularly gilts, are prone to reproductive disease associated with PCV2. The typical clinical signs are abortion, foetal mummification, still births, high rate of preweaning mortalities and birth of weak piglets (West *et al*., 1999). Non-suppurative or necrotizing and/or fibrosing myocarditis are the histopathological lesions observed in neonatal and still born pigs (Mikami *et al*., 2005). However, the PCV2 associated reproductive disease is rarely observed in field condition which might be due to its high sero-prevalence in breeding herds (Pensaert *et al*. 2004; Gillespie *et al*., 2009).

2.5.4 Porcine dermatitis and nephropathy syndrome (PDNS)

PDNS was initially reported in 1993 in UK (Smith *et al*., 1993) but its association with PCV2 was established in 2000 (Rosell *et al*., 2000). It is fatal in nature and affects all age groups of pigs; however, mortality is as high as 100% in grower pigs and in case of younger pigs it can rise up to 50% (Gillespie *et al*., 2009; Segalés, 2012; Saikumar and Das, 2019). The clinical sign includes skin lesions in hind legs characterized by red to purple scabs with blackish centre along with fever and lathery (Gillespie *et al*., 2009; Opriessnig and Langohr, 2013). The kidney appears to be waxy, tan, and enlarged with petechial haemorrhages. Histological lesions are necroses and fibrinous glomerulo-nephritis in kidney along with deposition of antigen immune complexes in the glomerular capillary walls as well as dermal and epidermal necrosis is observed in skin (Opriessnig *et al*., 2007; Gillespie *et al.,* 2009).

2.5.5 Enteric disease associated with PCV2

Enteritis in pigs caused by PCV2 is clinically similar to chronic ileitis caused by *Lawsonia intracellularis* or enteritis observed in salmonellosis (Jensen *et al*., 2006; Opriessnig *et al*., 2011). Enteritis due to PCV2 is encountered in 8-16 weeks old pigs (Gillespie *et al*., 2009). The affected pigs exhibit diarrhoea, decrease in growth rate and increased mortality

(Gillespie *et al*., 2009). Necropsy lesions include thick intestinal mucosa and enlarged mesenteric lymph nodes, while microscopic lesions are granulomatous enteritis and lymphoid depletion with histiocytosis in Peyer's patches (Jensen *et al*., 2006; Gillespie *et al*., 2009).

2.5.6 Neuropathy associated with PCV2

PCV2 associated with nervine disorder was reported in 2001 when piglets born with congenital tremors were found to be positive for PCV2 in the brain tissue and spinal cord (Stevenson *et al*., 2001). It affects 6-8 weeks old pigs. Like other neurological diseases, it is also characterized by common clinical signs like nystagmus, opisthotonus and convulsions (Gillespie *et al*., 2009). Microscopically, cerebellar lymphohistiocytic vasculitis along with lympho-histiocytic meningitis or haemorrhage is observed (Corrêa *et al*., 2007).

2.6 Diagnosis of Porcine Circovirus infection

Most commonly, the diagnosis of PCVAD is done based on clinical signs and detection of PCV2 antigens and nucleic acids in different tissues like lymphoid organs, liver, lungs, kidney or blood. The gold standard tests for diagnosis of PCVAD are detection of PCV2 nucleic acid or antigen by *in situ* hybridization (ISH) or polymerase chain reaction (PCR), and immuno-histochemistry (IHC) (Opriessnig *et al*., 2007).

Besides conventional PCR, other platforms of PCR such as Nested PCR, Multiplex PCR, Real-Time PCR (qPCR), genotype-specific Multiplex qPCR and duplex nanoparticle-based PCR have been optimized and developed for detection and differentiation of PCV2 infection in pigs (Larochelle *et al*. 1999; Kim and Chae 2003; Gagnon *et al*. 2008; Xu *et al*. 2012; Zhang *et al*. 2018). Other assays developed for diagnosis of PCVAD include immunoperoxidase assay, ELISA, immunofluorescence assay, virus isolation, virus neutralization assays and electron microscopy (Gillespie *et al*., 2009).

2.7 Porcine Circovirus 2 Vaccines

The best possible way to prevent PCV2 infection is to vaccinate pig herds at proper time. There are no less than five commercial PCV2 vaccines available in the market. The introduction of PCV2 vaccine has remarkably reduced the detrimental impact of PCV2 both

in clinical and subclinical infection (Segalés, 2015). PCV2 vaccination has improved the reproductive failure rate, reduced the viral excretion among infected pigs, and there is an overall decrease in PCV2 circulation in the pig population (Dvorak *et al*., 2016; Eddicks *et al*., 2016; Rose *et al*., 2016; Franzo and Segalés, 2020)

The commercial PCV2 vaccines were initially launched in Europe in 2004 and USA in 2006 (Segalés, 2015). The first commercial PCV2 vaccine was marketed in Europe as CIRCOVAC® (Merial) which is an inactivated-whole virus vaccine fortified with oil adjuvant (Opriessnig *et al*., 2007). It was initially intended for breeding stocks particularly sows and gilts but it is recommended for piglets older than 3 weeks. The first US-FDA approved vaccine introduced in America was Suvaxyn®PCV2One Dose™ (marketed by Fort Dodge Animal Health). Currently, it is marketed by Pûzer under the brand name Fostera™. It is an inactivated chimeric vaccine with PCV2 immunogenic capsid protein which was generated by ligating ORF2 to PCV1 genomic backbone (Fenaux *et al*., 2003; Fenaux *et al*., 2004). This vaccine is intended for single shot which is recommended for 3 weeks and above age piglets. There are other three commercial vaccines Ingelvac CircoFLEX® (Boehringer Ingelheim), Porcilis®PCV (Schering-Plough/Merck) and Circumvent® (Intervet/Merck); these are capsid protein-based subunit vaccines expressed in baculovirus vector system (Beach and Meng, 2012; Chae, 2012; Afghah *et al*., 2017). These subunits vaccines were also recommended for piglets above three weeks of age (Afghah *et al*., 2017). The detailed features of the commercial PCV2 vaccines are shown in Table 2.1.

Table 2.1: List of PCV2 commercial vaccines

Sl No.	Vaccine Name	Vaccine Type & antigen	Genotype	Recommended Dosage, Schedule &Route
1.	CIRCOVAC®	Inactivated whole virus	PCV2a	One 0.5 ml in piglets of 3 weeks or older by IMOne 2 ml dose in gilts and sow by IM followed by 2nd dose 3-4 weeks later but 2 weeks before mating. One compulsory dose prior to 2 weeks of farrowing.
2.	Fostera™	Inactivated Chimeric PCV1/2	PCV2a	One 2 ml dose in piglets of 3-4 weeks old by IM

3.	Ingelvac CircoFLEX®	Subunit of Capsid	PCV2a	One 1 ml dose in piglets of > 3 weeks age, IM
4.	Porcilis®PCV	Subunit of Capsid	PCV2a	One 2 ml dose in piglets of >3 weeks age, IM
5.	Circumvent®	Subunit of Capsid	PCV2a	One 2 ml dose in piglets of >3 weeks age followed by 2nd dose after 3 weeks, IM

Although available commercial vaccines are showing promising results in preventing PCV2 infection, it cannot completely eradicate the disease because the commercial vaccines are either inactivated or subunit vaccines, thereby they cannot induce immunity persistent for a longer period. Moreover, due to the global genotypic shift of PCV2, there are cases of vaccine failures because most of the commercial vaccines developed from the old genotype PCV2a (Opriessning *et al*., 2013b; Kwon *et al*., 2017). The PCV2 vaccines are considered as 'leaky-vaccines' because the available PCV2 vaccine can smartly prevent PCVAD by electing strong immune response but cannot completely shut the transmission process and influence the evolution of new strains or genotypes (Afghah *et al*., 2017; Franzo and Segalés, 2020). Further, the frequent introduction of new genotypes always opens the debate against cross-protection conferred by homologous and heterologous strains. Different investigators have provided different data and conclusions. Therefore, the race for development of promising and effective vaccine to impart long term immunity against available virulent genotypes is on and several vaccines have been designed which are either in the experimental stage or in field trials. Yang *et al.* (2012) reported that a single dose of formalin-inactivated PCV2 vaccine with oil adjuvant could protect pigs against challenge infection. In another experimental trial, it was observed that an upgraded version of PCV1-2b chimeric vaccine could completely clear the challenge viruses belonging to the genotypes PCV2a and PCV2b (Beach *et al.,* 2010). However, in another investigation led by Opriessnig *et al*. (2013a) with similar vaccine candidate (chimeric PCV1-2b/PCV1-2a), it was observed that the PCV1-2b vaccine was more promising than PCV1-2a against PCV2b strains. Opriessnig *et al.* (2014) had shown that a subunit vaccine produced from the mutant PCV2b genotype is equally effective like commercial vaccine produced from PCV2a against PCV2 infection in pigs. Seo *et al.* (2014) described that a PCV2 commercial vaccine (Fostera PCV, Zoetis, Madison, NJ, USA) was safe and effective

in case of 3 weeks old piglets having maternally derived antibodies and PCV2 viraemia. It was also proved that PCV2a based commercial vaccine has the ability to protect pigs when challenged with the mutant PCV2b (mPCV2b) having two extra amino acids in the capsid protein (Guo *et al*., 2015). However, it was also observed that mPCV2b alone could induce PCVAD in pigs. In another study, three commercial vaccines could completely wash out the PCV2b viraemia but not PCV2d, although there was reduction in PCV2d viral load (Jeong *et al*., 2015). In 2016, there was a report on production of recombinant PCV2 VLPs in *E. coli* which was successful in stimulating significant immune response in mice model (Wu *et al*., 2016). Liu *et al*. (2018) evaluated the immune response of PCV2 inactivated and purified subunit proteins (Capsid, Rep and ORF3) in mice model. The induction of PCV2 specific antibody as well as neutralizing antibody were significantly higher in mice inoculated with inactivated PCV2 vaccine than in the mice injected with subunit purified proteins. Unlike other dominant genotypes of PCV2, protection and cross-protection studies in vaccinated animals conferred by the PCV 2d genotype are very limited. However, recently it was observed that vaccination of pigs with a DNA vaccine encoding the capsid protein of PCV2d strain could provide protection to both PCV 2b and 2d genotypes (Hou *et al.,* 2019a).

2.8 Porcine Circovirus 2 DNA Vaccines

The concept of genetic/DNA immunization has evolved since 1950s, but there was rapid development in 1990s. The DNA vaccines are composed of bacterial plasmids that encode and express antigenic protein(s) of pathogen(s) and upon administration to animal stimulates both humoral and cellular immune responses against the antigenic protein(s). Immunization with DNA vaccine have been shown to be a promising approach for protecting human and animals against various pathogens because of low production cost, thermal stability and ability to generate a broad range of long-lasting cellular and humoral immune responses (Liu *et al*., 2006b; Saade *et al*., 2008). The use of DNA vaccine is getting its momentum not only in other species but also in pigs, and there are many successful experimental stories regarding DNA vaccination against several swine pathogens (Gerdts *et al*., 1997; Benvenisti *et al*., 2001).

Even though available commercial PCV2 vaccines can effectively protect pigs from PCV2 infection and restricts the spread of the virus but, sometimes it fails to give an umbrella protection against all genotypes of PCV2. Hence, it is essential to formulate alternate vaccines that are cheap and more promising than the currently available PCV2 vaccines. Like records of DNA vaccines for other swine pathogens, there are also a few experimental reports on PCV2 DNA vaccine but similar studies are yet to be reported from India. Kamstrup *et al*. (2004) evaluated the immune response of DNA vaccine against PCV2 in mice model. The vaccine was designed against capsid protein of PCV2 and when it was gun fired to mice, there was production of antibodies against PCV2 suggesting it to be a suitable vaccine candidate. Immunization of BALB/c mice with plasmids expressing structural protein of PCV2 showed remarkable seroconversion and lymphocytes proliferation (Silva Júnior *et al*., 2009). This indicates that DNA vaccine encoding capsid protein of PCV2 has the potential to induce both cell-mediated as well as humoral immune response. In 2011, a bicistronic DNA vaccine was demonstrated against PCV2 and Porcine Parvovirus (Lin *et al*., 2011b). The experimental vaccine was composed of PCV2 *cap* gene and *VP2* gene of porcine parvovirus and it was successful in developing anti-capsid antibody, anti-VP2 antibody, virus neutralizing antibody, and cellular immune responses against PCV2 and PPV when immunized in mice model.

Sylla *et al*. (2014) designed and evaluated a DNA vaccine that expresses capsid protein of PCV2 in mice model. The vaccine candidate (*cap* gene) was cloned in pEGFP-N1 vector which was named as pEGFP-Cap. This DNA vaccine induced solid immunity against PCV2 infection in mice by inducing high level of anti-cap antibodies and cytokines in serum. In the challenge study, the PCV2 viral load was found to be very low in mice vaccinated with pEGFP-Cap plasmids compared to the other control groups vaccinated with PBS or empty vector (pEGFP-N1). Moreover, no microscopic lesion was observed in the inguinal lymph nodes of challenged mice vaccinated with the DNA vaccine and a few cells of lymph nodes were positive for PCV2 antigens in immuno-histochemistry in the same vaccinated mice group.

Guo *et al*. (2015) constructed a PCV2 DNA vaccine that consisted of a plasmid co-expressing the capsid protein of PCV2 as well as porcine interlukine-6 (IL-6) and compared its efficacy with other plasmids that expressed only the capsid protein. The vaccine was

successful in producing specific anti-PCV2 antibodies and T-cells proliferation in mice model and upon challenge, the vaccinated mice showed 86% protection and significantly low viral load. Comparison of the results revealed that the plasmid co-expressing capsid protein and IL-6 was superior in inducing protective immunity than the plasmid expressing only capsid protein.

Li *et al*. (2016) evaluated the immune response of mice to a PCV2 nucleic acid or DNA vaccine that contains a specific CpG motif which acts as an adjuvant. They constructed two different plasmids, one containing CpG motif with capsid protein gene of PCV2 (18CpG-pVAX1-ORF2), whereas the other plasmid (pVAX1-ORF2) was devoid of CpG motif and contained only the *cap* gene of PCV2. The mice group inoculated with pVAX1-ORF2 and 18CpG-pVAX1-ORF2 induced higher anti-PCV2 capsid antibody compared to the control group and the mice vaccinated with 18CpG-pVAX1-ORF2 plasmid had significantly higher PCV2-specific antibody. Challenge study showed that the mice group vaccinated with CpG motif containing plasmid had smaller PCV2 load in different organs than the mice in the other groups.

In another study, Park *et al*. (2017) evaluated the efficacy of PCV2 DNA vaccine with different adjuvant formulations. The experimental DNA vaccine was based on ORF2 of PCV2 along with three different adjuvants, *viz*. aluminium hydroxide, cobalt oxide and liposome in single combination. The DNA vaccine with liposome adjuvant conferred better immunity than the other two adjuvants when injected to pig. In the challenge study, the pigs immunized with the ORF2 based DNA vaccine complexed with liposome had exhibited significantly lower levels of PCV2 viral load. Moreover, the same group of pigs had higher level of interferon-γ-secreting cells and neutralizing antibodies compared to other groups which was statistically significant.

Recently, a novel DNA vaccine was generated by Hou *et al*. (2019a) that expressed a fusion protein consisting of PCV2 ORF2 and three copies of C3d-P28 (the minimum-binding domain of complement C3 cascade terminal component) which was designated as pVOC3. The immune response conferred by this DNA vaccine was evaluated in pigs and the

results were compared with other groups of pigs injected with plasmid DNA expressing only ORF2 of PCV2d (pVOC), empty vector (pV) and negative control (PBS). The pigs vaccinated with pVOC3 DNA vaccine construct exhibited better protection in terms of reduced viral load and viraemia after infection. This DNA vaccine candidate produced specific antibodies against PCV2 and also stimulated the production of interferon-γ secreting cells specific to PCV2. Moreover, the vaccine construct also successfully conferred cross-protection in pigs when challenged with PCV2b or PCV2d.

The above cited literatures support that DNA vaccine could be effective in prevention and control of PCV2 infection in swine population and it has the prospect of commercialization.

Materials and Methods

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Virus and PK-15 cell line

Porcine Circovirus 2 strain Assam-01 with GenBank accession number MN266483 having $10^{6.5}$ $TCID_{50}$/ml in PK-15 cells and Porcine Circovirus free PK-15 cell line were obtained for the present study from Department of Veterinary Microbiology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, Assam, India.

3.1.2 Oligonucleotides

The primer sets were either designed in-house for the present study using Primer 3 software or certain published primer sets were used as shown in Table (3.1).

Table 3.1. List of primers used in this study

Sl. No.	Name	Primer Sequence (5'→3')	Annealing Temperature (°C)	Product Size (bp)	Reference
1	mCap-1	GA***CTCGAG***ATGACGTATCCA AGGAGG	54	717	In-house designed
	mCap-2	CT***ACGCGT***TCA**CTT**AGGGTT AAGTGGGG			
2	PCV-N1	GGCCCGCAGTATTCTGATTAC	60	167	In-house designed
	PCV-N2	GGGAAAGGGTGACGAACTG			
3	RepF	AAGTGAGCGGGAAAATGC	50	463	In-house designed
	RepR	GGAAATTCAGGGCATGGG			
4	IL4-F	GAGACTCTTTCGGGCTTTTCG	60	88	Chen *et al*., 2012
	IL4-R	CAGGAAGTCTTTCAGTGATGTGG			
5	IFN-γF	GCTTTGCAGCTCTTCCTCATG	60	112	Chen *et al*., 2012
	IFN-γR	CTTCCACATCTATGCCACTTGAG			
6	TNF-αF	CCACCACGCTCTTCTGTCTAC	60	103	Baluk *et al*., 2009
	TNF-αR	AGGGTCTGGGCCATAGAACT			
7	β-actinF	AGCGGTTCCGATGCCCT	60	123	Chen *et al*., 2012
	β-actinR	AGAGGTCTTTACGGATGTCAACG			

3.1.3 Vector and bacterial host

The cloning vector pJET1.2/blunt (CloneJET PCR Cloning Kit, Thermo Scientific) was used for cloning of the amplified PCR product. The mammalian expression vector pCI neo (Promega, USA) was used to design the DNA vaccine construct and DH5α competent cells (New England Biolabs, UK) were used for transformation.

3.1.4 List of scientific instruments used in the research work

- Class II Type A2 Biological Safety Cabinet (ESCO, Lifesciences group, Singapore)
- Thermocycler Machine (Applied Biosystems, USA)
- Spinix Vortex Speed 3000 rpm (Tarson)
- Agarose Gel electrophoresis system (Hoefer, US)
- High speed refrigerated centrifuge Z32 HK (Hermle Benchmark, Germany)
- BioSpectrometer®Basic (Eppendorf, Germany)
- Shaker Incubator (Narang scientific, India)
- Gel Documentation system (Bio-Rad, USA)
- Mini-PROTEAN vertical gel electrophoresis system (Bio-Rad, USA)
- Cell culture CO_2 Incubator (ThermoFisher Scientific, USA)
- Inverted microscope (Nikon, Tokyo)
- ELISA plate reader (BioRad, USA)
- One Step Real Time PCR (Applied Biosystems, USA)
- Thermomixer (Eppendorf, Germany)
- Dry Bath Incubator (Genexy, India)
- Ice flaking machine (Simag)
- Laminar air flow (Macro scientific, India)
- Trans-Blot SD semi-dry transfer cell (Bio-Rad, USA)
- UV spectrophotometer (Analytik Jena, Germany)
- Water purification system (Millipore, USA)

3.2 Methodology

3.2.1 DNA extraction from PK-15 cells:

Extraction of total DNA from PK-15 cells infected with the PCV2 was done by using DNeasy Blood & Tissue Kits (QIAGEN) with minor modification as follows:

a. Cultured cells were spinned at 550x g for 5 min. Supernatant was discarded and pellet was re-suspended in 200µl PBS.

b. Twenty microlitre of Proteinase K was added to the above sample and another 200 µl of buffer AL provided in the kit was added and mixed by vortexing, followed by incubation of the mixture at 56 °C for 20 min.

c. Then 200 µl of ethanol was added into the sample followed by mixing thoroughly by vortexing to obtain a homogenous solution.

d. The above mixture was transferred to DNeasy Mini spin column that was placed on the collection tube provided in the kit. The mixture was centrifuged for one minute at 7500x *g* min and the flow through along with the collection tube was discarded.

e. The spin column was again placed in a fresh collection tube and 500 µl of buffer AW1 was pipetted into the column followed by centrifugation for one min at 7500x *g*. The flow through along with the collection tube was discarded.

f. Another fresh 2 ml collection tube was placed under the used DNeasy Mini spin column and 500 µl AW2 buffer was added and centrifuged for three minutes at 17,500x *g*. Once again, the flow through along with the collection tube were discarded.

g. The column was again set on 1.5 ml tube and buffer AE (50 µl) was pipetted in the DNeasy membrane of the spin column. The sample in the column was incubated for two minutes at room temperature, and then centrifuged for 2 minutes at 7500 x *g*.

h. The DNeasy Mini spin column was discarded and the elute present in the 1.5 ml micro-centrifuge tube was properly labelled and stored at -20 °C for downstream processing.

3.2.2 Spectrophotometry:

The concentration and purity of the DNA sample was assessed by measuring the optical densities (OD) at 260 nm and OD 260:280 ratio, respectively using BioSpectrometer Basic (Eppendorf, Germany). DNA sample having an OD ratio 260:280 of 1.7 to 1.8 were considered as of good quality.

3.2.3 Amplification of mutated Cap (*mCap*) Gene of PCV2

3.2.3.1 Primer designing

The primer sets (mCap-1 and mCap-2) were designed in-house for amplification of *cap* gene of PCV2 having *Xho*I restriction site in the 5' end and *Mlu*I restriction site in the 3' end. The primer also had a lysine residue codon (AAG) just before the stop codon in the mCap-2 primer (Table 3.1).

3.2.3.2 Protocol for Polymerase chain reaction (PCR)

The mutated *cap* gene (designated hereinafter as *mCap*) was amplified by conventional PCR. Reaction mixture was prepared in 0.2 ml PCR tubes by adding 12.50 µl of DreamTaq Master Mix (ThermoFisher Scientific, USA) containing Taq DNA Polymerase, optimized buffer, $MgCl_2$, and dNTPs, 9.50 µl of nuclease-free water (NFW) and 0.5 µl (10 µM) of each primer. The above mixture was properly mixed by vortex and 2µl (550 ng/µl) of template DNA was added to make the total reaction volume 25 µl. All the steps were carried out in ice. The reaction was carried out in Thermocycler Machine (Applied Biosystems, USA) set with PCR cycling conditions as shown in Table 3.2. The PCR products were further stored at -20 °C for future analysis.

Table 3.2: PCR cycling conditions used for amplification of mutated *cap* gene

Step No.	Steps	Temperature	Time
I	Initial denaturation	94 °C	10 min
II	Denaturation	94 °C	30 sec
III	Annealing	54 ° C	45 sec
IV	Extension	72 °C	1 min
	40 cycles from Step II to Step IV		
V	Final Extension	72 °C	10 min
VI	Hold	4 °C	

3.2.3.3 Detection of PCR amplified products by agarose gel electrophoresis

The amplified PCR products were analysed using 1.5% agarose having 0.5 µg/ml ethidium bromide. The size of the amplicons was estimated using molecular marker (ThermoFisher Scientific, USA) and was run parallelly along with the amplified products. Four microlitre (µl) of amplified product was mixed with 2 µl of 6X loading dye (ThermoFisher Scientific, USA) and was loaded in the wells. Electrophoresis was conducted using constant voltage of 80V in 1X TAE buffer for 60 min. The amplified products were visualized for expected size of the bands under UV light in a Gel Doc system (Bio-Rad, USA) and documented for further analysis.

3.2.3.4 Gel extraction of PCR Products

The PCR amplicons of designated size was excised from agarose gel using a clean, sharp scalpel and put in a 2 ml tube. The PCR amplicons present in the excised gel were extracted and purified using NucleoSpin® extraction kit (MACHEREY-NAGEL, Germany) as per the instructions provided in the kit. The concentration of the eluted PCR products was determined by BioSpectrometer Basic.

3.2.4 Cloning and sequencing of PCR amplified mutated *cap* gene

3.2.4.1 Cloning of PCR-amplified mutated *cap* gene

The cloning of PCR-amplified *mCap* gene was done with CloneJET PCR Cloning Kit (ThermoFisher Scientific, USA) as per the manufacturer's instructions. Since the PCR

amplification was done with DreamTaq master mix, therefore the PCR products contained 3'-dA overhangs which was removed by following the sticky end cloning method as described by the manufacturer with minor modifications. At first, the blunting reaction was set-up in ice with the following components in a 1.5 ml tube.

Component	**Volume (µl)**
Reaction Buffer (2X)	10
PCR Product	1
Nuclease-free water	6
DNA Blunting Enzyme	1
Total Volume	**18**

The above mixture was vortexed and briefly centrifuged for 10-15 sec. Then the mixture was incubated in a Thermomixture (Eppendorf, Germany) at 70°C for 5 min and immediately chilled on ice. Finally, the ligation mixture was prepared by adding the following components to the blunting reaction mixture.

Component	**Volume (µl)**
Cloning Vector pJET1.2/blunt (50 ng/µL)	1
T4 DNA Ligase	1
Total Volume	**20**

The mixture was vortexed and centrifuged for a short period (10-15 sec) and incubated at 22 °C for 15 min. This ligation mixture was directly used for further transformation in DH5α cells.

3.2.4.2 Competent cell preparation by calcium chloride method

For transformation of recombinant plasmid, DH5α *E. coli* cells were made competent by calcium chloride method as described by Sambrook and Russel (2001) with minor

modifications. Single bacterial colony was picked from a plate with a sterile loop and inoculated into fresh 5 ml Luria-Bertani (LB) broth that was incubated in a shaking incubator at 37°C for overnight. Then 2 ml of overnight grown culture was inoculated in 100 ml LB broth and incubated at 37°C in a shaking incubator until the culture attained an O.D. of 0.4-0.6 at 600 nm (3-4 hours incubation). The culture was chilled on ice for about 30 min and 100 ml of the culture was transferred to two 50 ml tubes. Then the cells were harvested by centrifugation at 3500x *g* for 15 min at 4°C. The harvested cell pellets were re-suspension with 5ml of chilled 100mM calcium chloride ($CaCl_2$) and incubated for 30 min on ice. This step was followed by centrifugation of the cell suspensions for 15 min at 3500x *g* at 4 °C. Supernatant was discarded, followed by re-suspension of the cell pellets with 2.5 ml of ice-cold 100 mM $CaCl_2$ and 0.5 ml of 80% sterile glycerol. The cell suspension was mixed gently and stored at -80 °C as aliquots of 100 µl in a 1.5 ml tube.

3.2.4.3 Transformation by heat shock

Frozen aliquots of DH5α competent cells (100 µl) were thawed on ice for 15 min and then 10 µl of above ligation mixture was added to it, followed by incubation on ice for 30 min. Then heat shock was given to the cells at 42°C for 45 sec in a dry bath which was followed by immediate snap cooling on ice for 5 min. After that 1ml pre-warmed (37 °C) Super Optimal Broth (Sigma, US) was added to the mixture and kept in a shaker incubator at 37 °C for 90 min. The cells were centrifuged at 8500× *g* for 3 min and the supernatant was discarded. The cell pellet was re-suspended in 100 µl of LB broth and plated on LB agar plates added with ampicillin (100 µg/ml) followed by incubation for overnight at 37 °C. The pJET1.2/blunt has a unique property that it possesses a lethal gene in the cloning site which is disrupted when an external gene is inserted. Otherwise, the self-ligation of the vector expresses the lethal gene and the bacterial cells do not survive. Therefore, colonies that survive carry the recombinant pJET1.2 plasmid having a DNA fragment insert in its cloning site. The survived colonies were further streaked on LB agar plate and then inoculated into 5 ml LB broth added with ampicillin (100 µg/ml) which was incubated at 37 °C for 12-16 hours in a shaking incubator followed by plasmid DNA isolation.

3.2.4.4 Plasmid DNA Isolation

Plasmid extraction from DH5α *E.coli* cells was done by QIAprep Miniprep Kit (Qiagen, Germany). The protocol followed was as per the manufacturer's instructions with minor modifications. The overnight grown bacterial culture was centrifuged for 5 min at 7500x *g*. The supernatant was discarded and the cells were re-suspended completely with 250 µl of P1 buffer fortified with RNase-A (20 µg/ml) in a 2 ml tube. Then another 250 µl of P2 buffer was added to the cell suspension and mixed gently by inverting tube 5-6 times. After that 350 µl of N3 buffer was added followed by centrifugation at 17,500x *g* for 15 min at 4 °C. The clear supernatant about 700 µl was pipetted carefully to the spin columns with the collection tube below it and centrifuged for one min at 17,500x g. The flow through was discarded and the spin column was washed with 500 µl of buffer PB by spinning at 17,500 x g for one min. After discarding the flow through, the column was washed again with 750 µl buffer PE by spinning at 17,500x g for one min. The residual wash buffer was removed from the spin column by spinning once again for one min. Finally, plasmid DNA was eluted in a 1.5 tube by adding 30 µl elution buffer EB to the spin column and incubating for 3 min at room temperature followed by 2 min centrifugation at 17,500 x g. The tube was properly labelled and kept at -20 °C for future use. The quality and quantity of the plasmid DNA was checked by using a BioSpectrometer Basic (Eppendorf, Germany).

3.2.4.5 Sequencing of the recombinant plasmid

The insertion of *mCap* gene in pJET 1.2/blunt vector was confirmed by using the PCR protocol as described above in section 3.2.3.2. The positive plasmids were subjected to sequencing (Sanger's method) which was outsourced at 1st BASE, Malaysia. The recombinant pJET/1.2 plasmid containing *mCap* gene was designated as pJET-C.

3.2.5. Designing of DNA Vaccine construct

The mutated *cap* gene (*mCap*) was amplified from the recombinant plasmid pJET-C and then it was cloned in the pCI-neo mammalian expression vector.

3.2.5.1 Restriction endonuclease digestion

The mutated capsid gene was re-amplified in PCR from pJET-C plasmid with the pre-designed primers for *mCap* gene (mCap-1 and mCap-2) by using the protocol as described above in section 3.2.3.2. Both the amplified *mCap* gene and the pCI-neo mammalian expression vector were double digested with restriction enzymes *Xho*I and *Mlu*I (NEB, USA). The composition of RE digestion reaction mixture is described in Table 3.3. The reaction mixture was incubated at 37 °C for 3 hour and subjected to agarose gel electrophoresis. The desired bands of *mCap* gene and pCI-neo vector were excised from agarose gel and purified by NucleoSpin® extraction kit (MACHEREY-NAGEL, Germany). Then the concentration along with purity was determined.

Table 3.3: Reaction mixture of restriction digestion

Sl. No.	Reagents	Quantity
1.	10X NE Buffer 3.1	2.5 µl
2.	PCR product/plasmid	500 ng
3.	*Xho*I	1 µl
4.	*Mlu*I	1 µl
5.	Nuclease-free water	Up to 25
	Total reaction volume	**25 µl**

3.2.5.2 Ligation of purified amplicon of mCap gene and pCI-neo vector

The gel purified PCR amplicons of *mCap* gene and pCI-neo vector were ligated in molar ratio of 3:1 (insert: vector). The components of the ligation mixture are described in Table 3.4. The optimum quantity of PCR amplicon required for ligation with the vector was estimated using the following formula:

$$\text{Mass of insert (ng) required} = \frac{\text{(Vector mass (ng) x size of insert in bp)}}{\text{vector length in bp}} \text{x (molar ratio of insert/vector)}$$

Table 3.4: Preparation of ligation reaction mixture

Sl. No.	Component	Volume (µl)
1.	2X Reaction Buffer	10
2.	Purified PCR product (20 ng/µl)	1
3.	pCI-neo vector (50 ng/µl)	1
4.	T4 DNA Ligase	1
5.	Nuclease free water	7
	Total volume	**20 µl**

The tube containing the above ligation reaction mixture was briefly vortexed and centrifuged for 10 to 15 sec and incubated at 22 °C for 45 min.

3.2.5.3 Transformation and confirmation of the recombinant clones

The ligation mixture was further transformed in DH5α *E. coli* cells by following the same protocol as described in section 3.2.4.3. Screening of the recombinant clones for the presence of the desired insert was done by colony PCR using specific *mCap* gene primers. Details of the reaction mixture for colony PCR are given in Table 3.5.

Table 3.5: Components of Colony PCR

Sl. No.	Component	Volume (µl)
1.	Template (Bacterial colony)	Single Colony
2.	mCap-1 (10 µM)	1 µl
3.	mCap-2 (10 µM)	1 µl
4.	DreamTaq Master Mix	12.5 µl
5.	Nuclease Free Water	10.5 µl
	Total volume	**25 µl**

The same PCR conditions were applied as described in Table 3.2 for colony PCR to screen the positive colonies. The PCR positive colonies were inoculated in 5 ml of LB broth and incubated in a shaker incubator at 37 °C for 14-16 hours. The plasmid was isolated and insertion of *mCap* gene was further confirmed by double digestion of the recombinant pCI-neo vector with *Xho*I and *Mlu*I for release of *mCap* gene from the pCI-neo vector. Sequencing was done for one of the recombinant pCI-neo plasmids by outsourcing (1st Base, Malaysia). Once again, the sequence was analysed to cross-check certain features of the *mCap* gene such as insertion of the lysine residue codon at 3' end, presence of any form of error in the coding segment as well as the orientation of the *mCap* gene in the pCI-neo vector. The recombinant pCI-neo plasmid carrying the *mCap* gene was named as pCI-Cap (Fig 3.1). Glycerol stock was prepared from the positive colonies and stored at -80°C for further downstream processing.

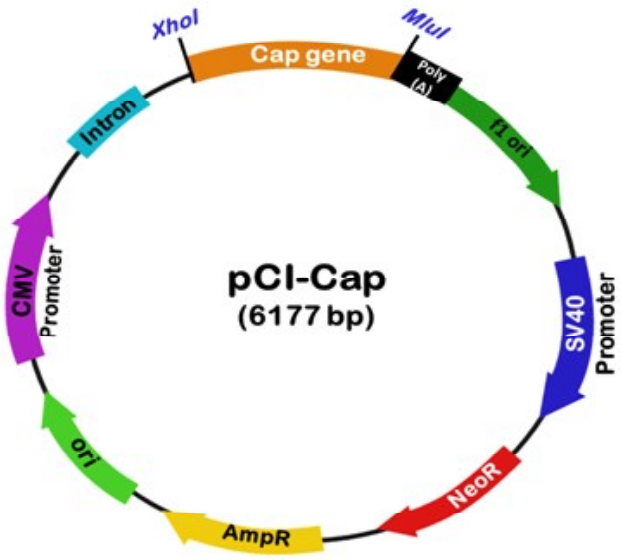

Fig 3.1: DNA vaccine construct (pCI-Cap) with mutated *Cap* gene

3.2.6 *In vitro* expression analysis of the mutant capsid protein by the DNA vaccine construct

3.2.6.1 Transfection

The pCI-Cap plasmid was transfected in PK-15 cells by means of lipofection, which was done by using Lipofectamine™ 2000 Transfection Reagent (Invitrogen, USA). For transfection, a 6-well cell culture plate (Nunc, USA) was used and four wells of the plate were

seeded with PK-15 cells along with growth medium. The cells were grown upto 70-80% confluency by incubating at 37 °C with 5% CO_2. After attainment of the desired confluency, the growth medium was removed and the cells were washed with 2.5 ml sterile PBS before transfection. After washing, 2.5 ml of reduced Opti-MEM medium was added in the wells and incubated for 1 hour. Two tubes (A & B) were prepared as described in Table 3.6. The content of tube A was gently mixed with that of tube B and incubated at room temperature for 15 mins. Then 250 µl of the A-B mixture containing DNA-lipid complex was dispensed into two experimental wells of the cell culture plate and in two other wells, 250 µl of only Opti-MEM medium was added that served as the control. The plate was incubated for 5 hrs at 37 °C with 5% CO_2. After incubation, the medium containing the mixture was removed and fresh growth medium was added to all the four wells and the cells were allowed to attain 100% confluency. The cells were harvested to check the expression of the desired protein by western blotting.

Table 3.6: Preparation of Lipofectamine-pCI-Cap DNA Complex

Components	Tube A	Tube B
Opti-MEM	300 µl	300 µl
Lipofectamine 2000	20 µl	-
pCI-Cap	-	20 µg

3.2.6.2 Western Blotting to detect the *in-vitro* expressed capsid protein

3.2.6.2.1 Preparation of protein sample from cell culture

The culture medium was aspirated from the wells without disturbing the transfected cells. The cells were washed twice with DPBS to remove the residual medium. Chilled RIPA buffer (500 µl) was added to each well and the plate was incubated at 4 °C for 10 min. Then by using a cell scraper all the cells were scraped from the each well and transferred to a 1.5 ml tube. The cell lysate was pelleted by spinning at 8500x g for 15 min at 4 °C to remove the debris. The supernatant was carefully transferred to a fresh 1.5 ml tube for further analysis.

3.2.6.2.2 Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

Before western blotting, the prepared protein sample was resolved in SDS-PAGE (Laemmli, 1970). SDS-PAGE was performed in Mini-PROTEAN Tetra cell electrophoresis system (Bio-Rad, USA). The clean glass plates were placed appropriately in the gel casting assembly and 12% resolving gel was poured between the glass plates which covers 2/3 volume of the gel. The resolving gel was allowed to solidify, after that 5% stacking gel was poured over it and 1 mm comb was placed immediately. Once the gel was solidified, assembly was overlaid with 1X Tris-glycine running buffer. The protein sample was mixed with 5X sample buffer and boiled at 95 ºC for 10 minutes along with a pre-stained protein molecular weight marker. Then 25 µl sample and 10 µl protein marker was loaded in the wells. The electrophoresis was carried out at constant 90V until the tracking dye reached the bottom of the gel.

3.2.6.2.3 Transfer of proteins to Polyvinylidene fluoride (PVDF) membrane

After completion of SDS-PAGE, the gel was washed twice with distilled water and then kept in transfer buffer for equilibration. The PVDF membrane was activated with 20% methanol and equilibrated in transfer buffer. Then the gel was placed over the PVDF member which was sandwiched between thick blotting pads in the assembly of Trans-Blot SD semi-dry transfer cell (Bio-Rad). Air bubble, if any, was removed using a roller. The electrophoresis was carried out in a semi-dry condition at 18 V for 1 hour.

3.2.6.2.4 Detection of capsid protein in blotting membrane

Following transfer of protein, the PVDF membrane was incubated overnight at 4 °C with blocking buffer (5% BSA in TBST). The membrane was washed thrice with TBST for 5 min each. Then the membrane was incubated with primary antibody (1: 1000 dilution of Porcine Circovirus Type 2 Capsid polyclonal Antibody, Invitrogen) for 1 hour at 37 °C. Again, the membrane was washed three times with TBST for 5 min each. Then it was incubated with goat anti-rabbit HRPO conjugate (Sigma-Aldrich, USA) with 1:3000 dilution at 37°C for 1 hour. After washing three times, it was incubated in DAB solution prepared from SIGMAFAST™ 3,32 -Diaminobenzidine tablets for development of coloured (brown) protein bands.

3.2.7 Animal experiment and immunogenicity evaluation of DNA vaccine construct

3.2.7.2 Immunization protocol

Swiss albino mice were procured from the Department of Veterinary Pharmacology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati-22. The mice were of similar age group (about 5 weeks of age) and body weight (20-30 gm each). They were maintained for 14 weeks in Department of Animal Biotechnology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati-22 during the experiment. The mice were kept in a separate room, provided with premixed feed, proper ventilation and clean drinking water, and entry was restricted to the room during the entire experimental period. Before starting the experiment, the mice were reared for one week for acclimatization. A total of 24 mice were divided randomly into four groups with six mice in each group (Table 3.7). The animal experiment protocols were approved by the Institutional Animal Ethics Committee (IAEC) of College of Veterinary Science, AAU, Khanapara, Guwahati vide No. 770/GO/Re/S/03/CPCSEA/FVSc/AAU/IAEC/19-20/797 dtd 23.12.219. Immunization trial was carried out in four groups of mice: (I) control group injected with PBS, (II) vector control group (pCI-Vec) injected with empty pCI-neo vector, (III) DNA vaccine (pCI-Cap) group injected pCI-neo vector containing mutated capsid gene and (IV) commercial vaccine group (Circoflex) injected with Ingelvac CircoFLEX® vaccine. The pCI-Cap and the pCI-Vec plasmids were extracted in bulk using QIAGEN® Plasmid Midi kit from the overnight grown culture revived from glycerol stock. The plasmids were dissolved in nuclease-free water for preparation of the inoculum. Before immunological trial, the safety of the DNA vaccine was evaluated in a few mice by injecting double dose (200 µg). Till two weeks post injection no clinical sign or adverse rection was observed. The groups, dose, route and vaccination schedule are shown in Table 3.7

Table 3.7: Immunization Schedule in mice

Groups	No. of mice	Age of mice (week)		Dose Primary/ Booster	Route Primary/ Booster
		Primary	Booster		
Control (PBS)	6	6th	8th	100 µl	IM
pCI-Vec	6	6th	8th	100 µg	IM
pCI-Cap	6	6th	8th	100 µg	IM
Circoflex	6	6th	8th	100 µl	IM

3.2.7.3 Collection of blood samples

Whole blood was collected from the tail vein of the experimental mice of all the groups on 0,7th, 14th, 21st, 28th and 35th days post immunization, considering the day of primary immunization as '0' day. From each animal, 200 µl of blood was collected separately in 1.5 ml micro-centrifuge tubes. Immediately after collection of blood, the tubes were placed in ice and kept in slanting position for 1 hour. After 1 hour, the tubes were centrifuged at 4500 rpm for 15 minutes. The supernatant (straw coloured) was collected from each tube as serum. Approximately 30-40 µl of serum was obtained from each tube and kept at -20 °C.

3.2.7.4 Humoral immune response

3.2.7.4.1 Detection of capsid protein antibodies by Indirect ELISA

The coating antigen for indirect ELISA to evaluate antibody titre in sera of experimental mice was prepared from PK-15 cell infected with PCV2 as described by Nawagitgul *et al.* (2002) with minor modifications. Briefly, PK-15 cells having $10^{6.5}$ $TCID_{50}$/ml of PCV2 were harvested and lysed by RIPA. By centrifugation for 15 min at 11000x g, the supernatant was clarified and used as the coating antigen. The optimum dilution for antigen, test serum and conjugate were estimated by checker board titration. The suitable cut-off value was determined based on mean OD of six negative sera + 3 times Standard Deviation as reported by Sun *et al.* (2010). The protocol for indirect ELISA was as follows:

a) ELISA plates (NUNC) were coated with 100 µl PCV2 virus antigen (1:200) diluted in carbonate/bicarbonate buffer having pH 9.6 and was incubated at 37 °C for one hour and kept overnight at 4°C.

b) The coated plates were incubated with blocking solution for 1 hour at 37 °C and washed three times with PBST (Phosphate buffer saline containing 0.05 % Tween 20) and 100 µl test serum samples collected from mice, diluted in blocking buffer containing PBST with 5 % lactalbumin hydrolysate and incubated at 37 °C for 1 hr.

c) The plates were washed again for three times and 100µl anti-mouse IgG Peroxidase conjugate at 1:3000 dilution in blocking buffer was added and incubated at 37 °C for 1 hr.

d) The plates were again washed with PBST and 100 µl TMB substrate solution (ThermoFisher Scientific, USA) was finally added for colour development and the colour reaction was observed within 15 min by incubating the plates at 37 °C.

e) The reaction was stopped by adding 100 µl 1M H_2SO_4.

f) Absorbance was measured in ELISA reader at OD 450 nm

g) For each plate, positive and negative controls, and blanks were kept.

3.2.7.5 Cell-mediated Immune Response

Cell-mediated immune response was evaluated for the DNA vaccine designed by lymphocyte transformation test and relative expression of cytokine gene transcripts in peripheral blood mononuclear cells (PBMCs).

3.2.7.5.1 Lymphocyte Transformation Test (LTT)

The LTT assay was performed on peripheral blood mononuclear cells (PBMCs) as reported by Guo *et al.* (2015). For that, heparinized whole blood was collected from each group of mice on 10th day after the booster injection. For separation of PBMCs, one volume (0.5 ml) of whole blood was layered slowly over a volume of Ficoll-Hypaque and then it was centrifuged at 1300 rpm for 35 min at 15-25 °C. Following centrifugation, the white layer was carefully separated and washed with RPMI 1640 medium fortified with 10% FBS, 1X antibiotic-antimycotics solution and 2 mM L-glutamine. The cells were again re-suspended with the same RPMI-1640 medium. The cell concentration was adjusted to 1x10^6/ml of medium after determining the cell viability by trypan blue exclusion method. Then 100 µl of cell suspension from each group was seeded in a 96-wells plate. The cells of each group were added in triplicates with 5µg/mL concanavalinA as the positive control, 15 µl of IngelvacCircoFLEX® (1.5 µg) as the capsid antigen and the medium alone as the negative control. Followed by incubation of the plate for 48 hr at 37 °C under 5 % CO_2. After the incubation period, 20 µl (5mg/ml) of MTT dye [3-(4, 5-dimethylthiazol-2-yl-2, 5-diphenyl-tetrazoliumbromide], (Sigma, USA) was added to each of the wells. Again, for another 4 hrs the plates were incubated at 37 °C. Then 100 µl of DMSO was pipetted to each well and incubated for another 10 min at 37

°C to dissolved the formazan crystals. Finally, the optical density of each well was recorded at 570 nm and the blastogenic response was estimated. The lymphocyte proliferation was expressed as stimulation index (SI) by using the following formula:

Stimulation Index (SI) = (mean OD of the stimulated cultures)/(mean OD of unstimulated control cultures).

3.2.7.5.2 Relative expression of the cytokine gene transcripts

Relative expression of the cytokine gene transcripts was analysed in the peripheral blood mononuclear cells (PBMCs) of all the mice groups in Real Time PCR by delta-delta Ct or $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Relative expression of three cytokine genes, *viz*., Interleukin 4, interferon-γ (IFN-γ) and Tumour Necrosis Factor-α (TNF-α) was evaluated by SYBR Green Real Time PCR. Beta-actin gene of mice was used as the endogenous control and the values obtained for the mice group injected with PBS were considered as the calibrator. The relative gene expression analysis was carried out in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, US). For cytokine gene expression, total RNA was extracted from the collected PBMCs by TRizol reagent (Invitrogen) and cDNA was synthesized by RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, USA).

3.2.7.5.2.1 RNA extraction from PBMCs

a) PBMCs re-suspended in 300 µl of PBS was taken in a 1.5 ml micro-centrifuge tube and 700 µl TRIzol reagent was added and vortexed vigorously.

b) Then 200 µl of chloroform was added to the homogenized cell lysate and it was vortexed, followed by incubation at room temperature for 15 minutes.

c) Subsequently, it was centrifuged at 11500x *g* for 15 min and the aqueous phase on the top was transferred to a fresh 1.5 ml micro-centrifuge tube.

d) An equal volume of isopropanol was added and it was vortexed again. Then it was incubated at room temperature for 10 min to precipitate the RNA.

e) The above mixture was centrifuged at 11500x *g* for 10 min at 4°C to pellet the precipitated RNA.

f) The supernatant was carefully discarded without disturbing the pellet.

g) Then washing of RNA pellet was done with 70% ethanol.

h) Finally, the air-dried pellet was dissolved in 30 µl nuclease-free water and stored at -80°C untilled used.

3.2.7.5.2.2 Synthesis of cDNA

The RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, USA) was used for synthesis of cDNA from the extracted RNA. The reaction mixture for reverse-transcriptase PCR (RT-PCR) was prepared in a total volume of 20 µl which consisted of equal quantity of RNA template (300 ng) from each sample, Random Hexamer (0.5 µl), Oligo (dT)18 primer (0.5 µl), 5X RT buffer (4 µl), 1 µl of RiboLock RNase Inhibitor (20 U/µL), 10 mM dNTP Mix (2 µl), 1 µl of RevertAid M-MuLV RT (200 U/µL) and NFW up to 20 µl. Initially, template RNA, random primers and oligo (dT)18 primers and the required volume of NFW were mixed and heated at 65 °C for 5 min to prevent formation of hair pins and secondary structures in the template RNA. The reaction mixture was immediately cooled on ice and the other reagents were added. Then RT-PCR was carried out in a programmable thermocycler at 25 °C for 10 min for annealing of primers followed by reverse transcription at 42 °C for 60 min. The reverse transcriptase was then heat-inactivated at 70 °C for 5 min. The cDNA so synthesised was used to carry out qPCR or was stored at -20 °C till further use.

The primer pairs used for amplification of the respective cytokine genes of mice (including the β-actin housekeeping gene) are shown in Table 3.1. The total reaction volume was 20 µl with the following components:

Table 3.8: Reaction mixture of Real Time PCR for cytokine gene expression

Reagents	Quantity
SYBR™ Green PCR Master Mix	10
Forward Primer (5µM)	0.5
Reverse Primer(5µM)	0.5
Nuclease-fFree Water	7
cDNA Template	2
Total Volume	**20**

The Real Time PCR was programmed with following cyclic conditions including the steps for melt curve analysis.

Table: 3.9: Real Time PCR programme for amplification of cytokine genes

Step No.	Step	Temperature	Time
I	Initial denaturation	95 °C	10 min
II	Denaturation	95 °C	15 sec
III	Annealing and Extension	60 °C	1 min
	Step II-III is repeated for 40 cycles		
IV	Dissociation step	95 °C	15 sec
		60 °C	1 min
		95 °C	15 sec
		60 °C	15 sec

Calculations for the ΔΔCT method are given by the formulae mentioned below:

Calculation of ΔCT value:

ΔCT = CT target - CT control

Calculations of the ΔΔCT value:

ΔΔCT = ΔCT test sample - ΔCT calibrator sample

Incorporation of ΔΔCT value into the fold difference:

The fold difference of the target gene transcript, relative to the calibrator was calculated by the $2^{-\Delta\Delta Ct}$ value (Livak and Schmittgen, 2001).

3.2.8 Challenge Study

All the experimental mice were challenged with 0.5 ml of cell culture fluid of PCV2 strain Assam-01 having $10^{6.5}$ $TCID_{50}$/ml virus concentration. The challenge was done on 35 days post-vaccination (3 weeks after booster dose). The mice were sacrificed at two weeks after challenge and susceptible organs like liver, spleen, lymph node and lung were collected to check the viral load in qPCR by standard method curve. The DNA was extracted from the tissues by using QIAGEN DNeasy Blood & Tissue Kits as per manufacturer's protocol. Two sets of in-house primers were designed corresponding to the conserved region of PCV2 ORF1. The primer pair, PCV2-N1 and PCV2-N2, were used to detect the 167 bp of PCV2 ORF1 gene and the other primer set (RepF and RepR) spans 463 bp region of the ORF1 gene. This later primer set was used in conventional PCR to amplify a 463 bp segment of ORF1 which also encompasses the 167 bp of ORF1. Thus, the 463 bp segment was gel purified and used as known standard to create the standard curve. The concentration of gel purified 463 bp was determined and its initial copy number was calculated based on the following calculation:

$$\text{PCR molecules } \mu L^{-1} = \frac{X \text{ g } \mu L^{-1} \text{ DNA}}{\text{length of PCR product in base pairs x } 660} \text{ x } 6.022 \text{ x } 1023$$

The standard curve was prepared by using ten-fold dilution of PCR products which started from 10^9 copies to 10^1 copies per µl. The efficiency of the qPCR reaction was determined using the equation: $E = 10^{(-1/slope)} - 1$.

The reaction mixture for absolute quantification of PCV2 loads in tissue samples consisted of the following components:

Table 3.10: Reaction mixture of Real Time PCR for detection of PCV2 genome

Reagents	Volume (µl)
SYBR™ Green PCR Master Mix	10
Forward Primer (5µM)	0.5
Reverse Primer (5µM)	0.5
Nuclease Free Water	7
DNA Template	2
Total Volume	**20**

The same Real Time PCR programme was used as shown in Table 3.9.

3.2.9 Statistical analysis

Statistical analyses were done for determining the significant difference in antibody titre, stimulation index, cytokine gene expression and viral load between control and immunized mice groups. The analyses were done by one-way and two-way ANOVA using the statistical software SPSS, version 22 and significant difference was estimated at $p < 0.05$ level.

Results

4 RESULTS

4.1: DNA Extraction, Quantity and Quality Analysis

DNA was extracted from the PCV2 positive PK-15 cells (received from Department of Microbiology, College of Veterinary Science, AAU, Khanapara) by using DNeasy Blood & Tissue Kits (QIAGEN). The quality and the concentration of DNA were checked in BioSpectrometer Basic (Eppendorf). Absorbance at 260 nm gives the concentration of DNA and the purity is given by absorbance ratio $OD_{260:280}$.

The DNA concentration was found to be 550 ng/µl and the $OD_{260:280}$ ratio was 1.75 which falls within the normal range of purity ratio. The DNA sample was stored at -20°C for further downstream procedures.

4.2: Amplification of the mutated *cap* gene by PCR

The in-house designed primer set (mCap-1 and mCap-2) shown in Table 3.1 was used to amplify the *cap* gene of PCV2 with an extra lysine residue codon (AAG) at the 3' end. The PCR products were subjected to agarose gel electrophoresis (1.5 % gel, containing ethidium bromide) and a band size of 717 bp (including RE sites) was noticed when observed under UV light in a Gel Documentation System (Fig. 4.1).

4.3. Cloning and sequencing of the mutated *cap* gene

The PCR amplified 717 bp product of the mutated *cap* gene of PCV2d was gel purified and inserted into the pJET1.2/blunt cloning vector as per manufacturer's instruction. DH5α competent cells were transformed with ligation mixture (pJET1.2/blunt vector and *mCap* gene) by heat shock method and cells were plated in Luira-Bertani (LB) agar plate

containing ampicillin (100 µg/ml). The colonies (Fig. 4.2) were screened for the presence of recombinant plasmids containing *cap* gene (*i.e.*. pJET1.2-Cap) by colony PCR. The colony PCR positive colonies were grown overnight and plasmids were isolated using QIAprep Spin Miniprep Kit. The presence of the recombinant plasmids was further confirmed by conventional PCR. The recombinant plasmid showed amplification of 717 bp *mCap* gene (Fig 4.3).

The recombinant plasmid pJET1.2-Cap was sequenced by Sanger's method by outsourcing. Analysis of the sequence using BioEdit software revealed that the *cap* gene of PCV2d contained an extra codon (AAG) at its 3' end (Fig 4.4) and it was devoid of any error in the coding region.

4.4 Designing of DNA vaccine construct

The DNA vaccine was constructed by subcloning the mutated *cap* gene in mammalian expression vector, pCI neo. The pCI neo vector ligated with *cap* gene was transformed into DH5α competent cells by calcium chloride method and plated in LB agar plate containing ampicillin. The colonies grown on the LB plate (Fig. 4.5) were screened for recombinant vectors by colony PCR using mCap-1 and mCap-2 primers (Fig. 4.6). The *cap* gene was released from the recombinant plasmid when it was subjected to double RE digestion with *Xho*I and *Mlu*I (Fig.4.7) and then it was designated as pCI-Cap. Further, sequencing results confirmed the insertion of *cap* gene in the correct orientation without any error (particularly without a stop codon in the middle of the coding region) and contained the extra lysine codon (AAG) at the 3' end.

4.5 Expression analysis of the mutant capsid protein

The PK-15 cells transfected with pCI-Cap DNA vaccine construct were harvested, lysed and subjected to western blotting. The expressed capsid protein with a molecular mass of 27 kDa was detected in western blotting (Fig. 4.8) using the PCV2 capsid protein polyclonal antibody (Invitrogen) as the primary antibody and goat anti-rabbit (HRP-conjugated) as the secondary antibody.

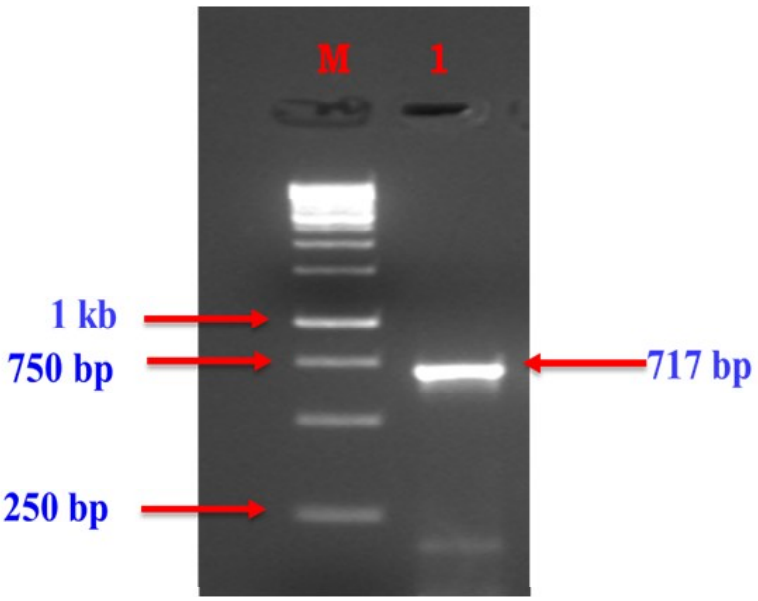

Fig 4.1: PCR amplification of *Cap* gene of PCV2 by specific primers (mCap-1 & mCap-2). Amplification of Cap gene was done to incorporate extra codon AAG at 3' end as well as RE sites on both the flanking region of cap gene.

Lane M : 1 kb DNA Ladder;

Lane 1 : PCR amplicon of cap gene 717 bp with a size of 717 bp.

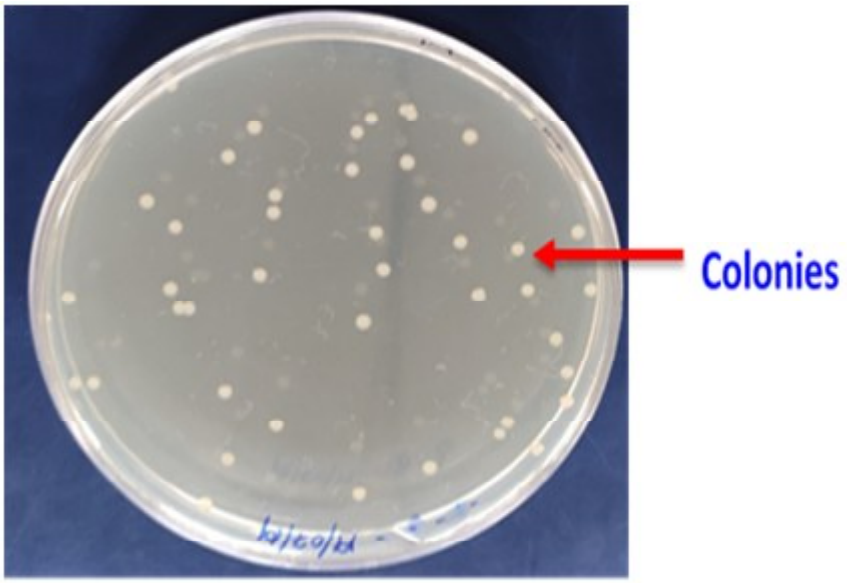

Fig 4.2: Colonies transformed with pJET1.2/Blunt vector ligated with mutated *Cap* gene of PCV2 in LB agar plate containing ampicillin.

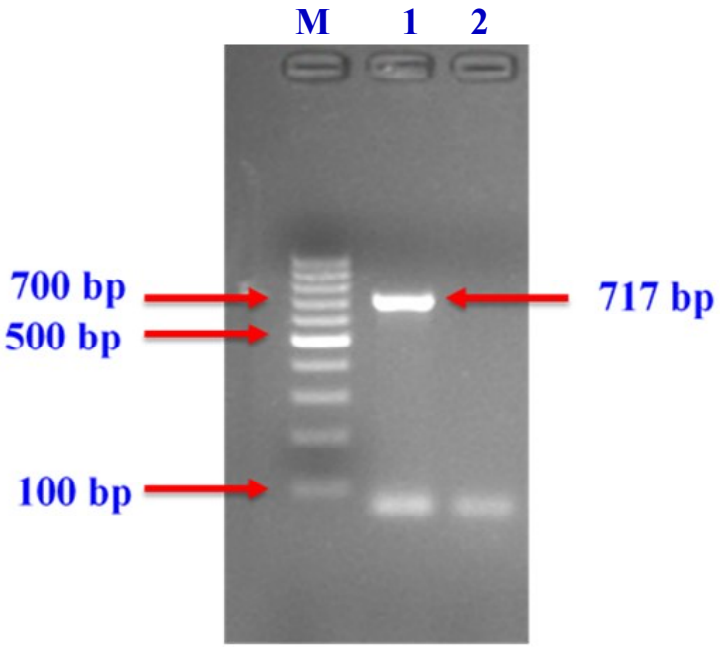

Fig 4.3: PCR amplification of mutated *Cap* gene of PCV2d cloned in pJET1.2/Blunt Vector.

Lane M : 100bp DNA Ladder

Lane 1 : Positive recombinant clone (pJET1.2-Cap)

Lane 2 : Negative clone

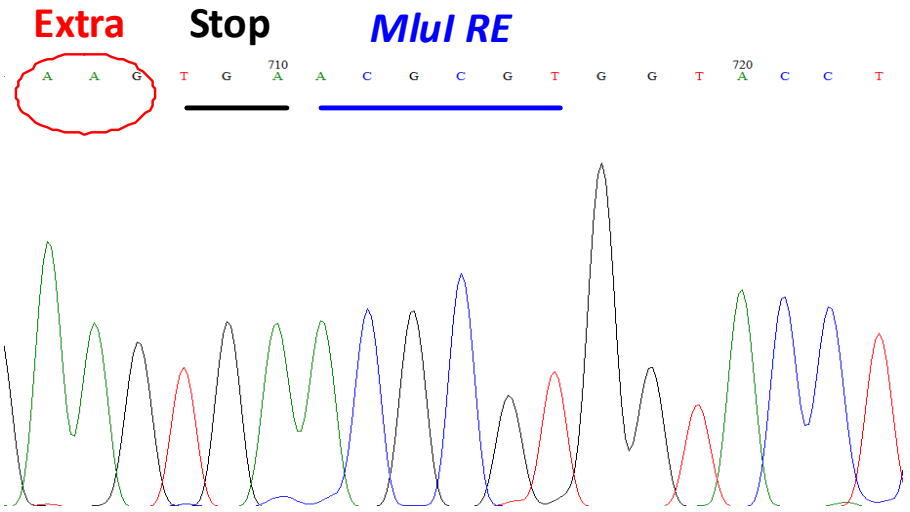

Fig. 4.4: Chromatogram showing the extra lysine residue (AAG) at 3' of *Cap* gene followed by the stop codon (TGA) and *Mlu*I restriction site (ACGCGT)

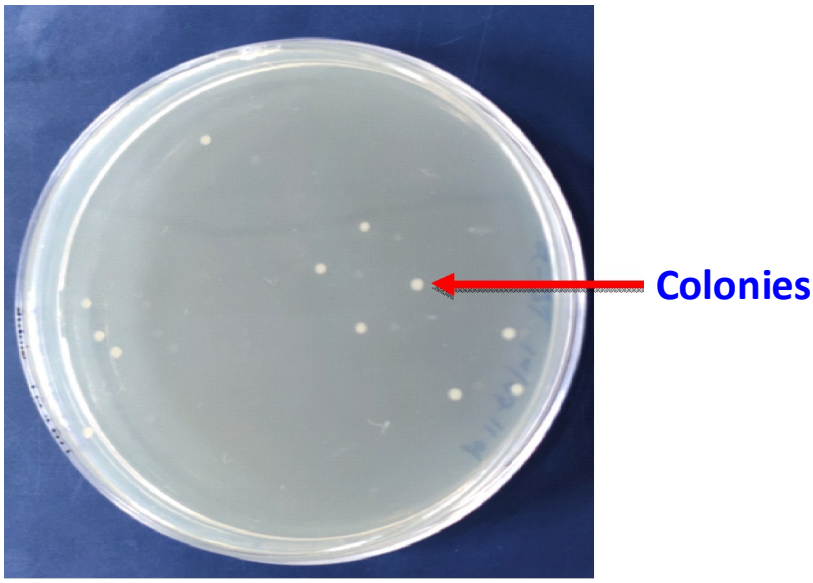

Fig 4.5: Colonies of DH5α cells transformed with DNA Vaccine Construct (pCI-Cap) in LB agar plate containing ampicillin

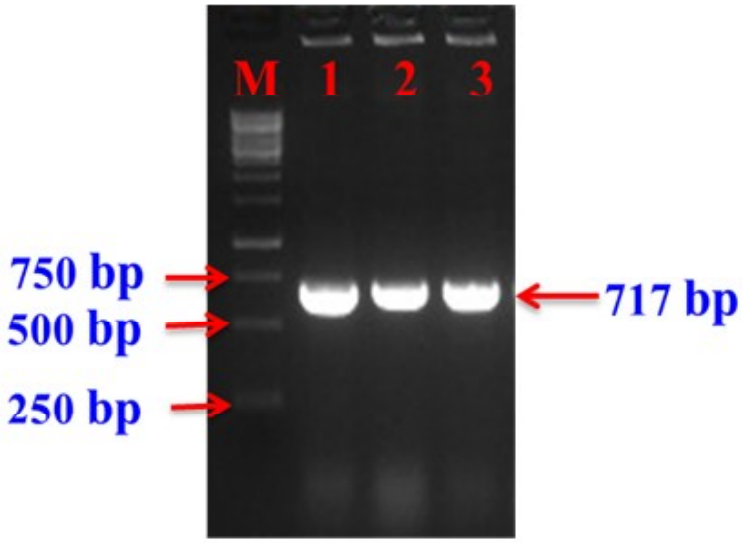

Fig 4.6: Colony PCR for detection of mutated Cap gene of PCV2.

Lane M : 1 Kb DNA Ladder;

Lane 1, 2 & 3 : Positive colonies showing amplification of mCap gene.

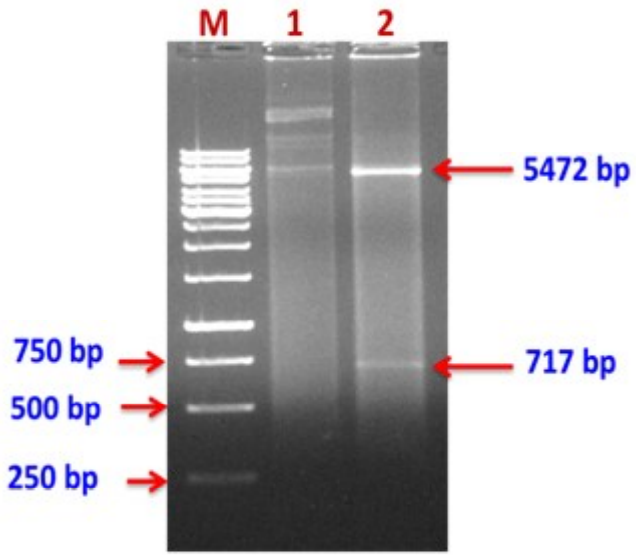

Fig 4.7: RE digestion of recombinant plasmid (pCI-Cap) showing the release of *mCap* gene from pCI-neo vector.

Lane M : 1 Kb DNA ladder

Lane 1 : Undigested Plasmid

Lane 2 : Release of *mCap* gene from pCI-neo vector double digested with *Xho*I and *Mlu*I

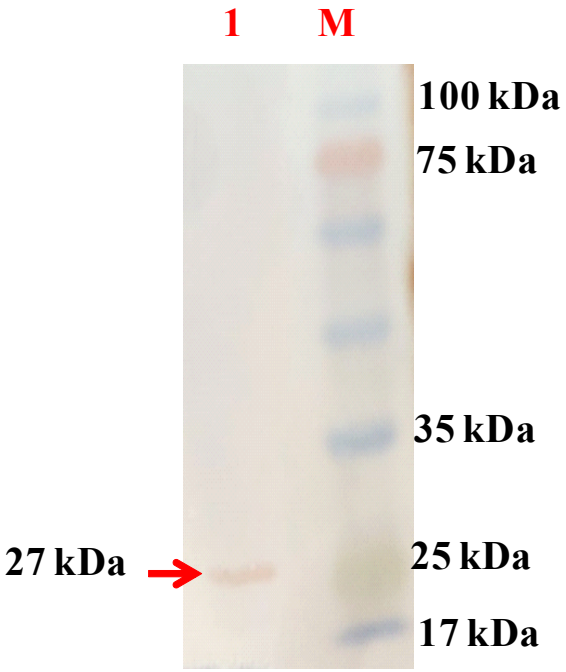

Fig 4.8: Western blot showing the expression of the capsid protein in PK-15 cells transfected with pCI-Cap plasmid.

Lane M : Protein Marker.

Lane 1 : Expressed 27kDa capsid protein in PK-15 cells transfected with pCI-Cap plasmid.

4.6 Evaluation of immunogenicity of the DNA vaccine in mice model

Humoral and cell-mediated immune responses of the designed DNA vaccine candidate were evaluated in the laboratory animal model, *i.e.* mouse. Immunogenicity was evaluated and compared in four groups of mice (Circoplex, pCI-Cap, pCI-Vec and Control) containing six mice in each group. The primary dose was given at 6 weeks of age. The day of inoculation of the first dose was considered as day 0 and a booster dose was given on day 14 post-primary immunization. The mice were challenged with 0.5ml of PCV2 strain Assam-01 having having a virus concentration of $10^{6.5}$ $TCID_{50}$/ml on day 35 post-primary immunization (*i.e.*, 3 weeks after the booster) and were sacrificed after 2 weeks of challenge to check the viral load.

4.6.1 Evaluation of humoral immune response

For evaluation of humoral immune response, sera samples were collected from the four groups of mice at weekly interval (on day 0, 7, 14, 21, 28 and 35) post-primary vaccination. The antibody levels (IgG) against capsid protein were determined by indirect ELISA (based on optical density value at 450 nm). The antibody levels were found to be significantly higher ($p<0,05$) in the pCI-Cap group of mice compared to that of the vector control (pCI-Vec) and the negative control (PBS) group on all the days (except day 0) post-vaccination (Fig. 4.9). However, in comparison to the Circoflex group (commercial vaccine group), the antibody level in pCI-Cap group (DNA vaccine group) was significantly lower on day 21 but there was no significant difference between these two groups on other days. The antibody level reached its peak in the pCI-Cap group on day 21 and there was a slight decline on day 28 as well as on day 35 post-primary vaccination (Fig. 4.9) and a similar trend was noticed in the mice group 'Circoflex' injected with the commercial vaccine (IngelvacCircoFLEX®).

4.6.2. Evaluation of cell-mediated immune response (CMI)

The peripheral blood mononuclear cells (PBMC) were collected from the mice of all groups and used for evaluation of CMI response by means of lymphocyte transformation test and by analysing the relative expression pattern of cytokines gene transcripts by Real-Time PCR.

4.6.2.1 Lymphocyte transformation test (LTT)

The lymphocyte transformation test was done on all four experimental mice groups 10 days after the booster vaccination. The blastogenic response was evaluated by estimating the stimulation index (SI). The SI was significantly higher in DNA (pCI-Cap) and IngelvacCircoFLEX® vaccinated groups compared to that of pCI-Vec empty vector inoculated group and PBS injected control group (Fig. 4.10) when the cells were stimulated with capsid antigen present in the commercial vaccine IngelvacCircoFLEX®. A non-significant but slightly higher SI was noticed in the pCI-Cap vaccinated group compared to the Circoplex group. The SI value for all the groups were in a similar range and no significant difference in SI value was observed among the groups when stimulated with positive control ConA antigen.

4.6.2.2 Relative expression analysis of Cytokine transcripts

The relative expression pattern of mRNA transcripts of three cytokine genes (IL4, INFγ and TNFα) were also analysed on PBMCs of all the mice groups, for which blood was collected from the mice 10 days after the booster dose. The relative expression levels (expressed as fold change) of all the three genes were significantly up-regulated in pCI-Cap and IngelvacCircoFLEX® vaccinated groups compared to the control (pCI-Vec and PBS) groups (Fig. 4.11). The fold change of IL4 was significantly higher ($p<0.05$) in the group inoculated with the commercial vaccine compared to that of the pCI-Cap group. However, the fold change of INF-γ was significantly higher ($p<0.05$) in the pCI-Cap vaccinated group in comparison to the Circoflex and the other groups. The expression level of TNF-α was apparantly higher in the Circoflex group compared to the pCI-Cap group. The expression of TNF-α was also significantly higher ($p<0.05$) in Circoflex and pCI-Cap group compared to the other two groups (pCI-Vec and PBS).

4.7 Challenge study in experimental mice model

All the experimental mice in each group were challenged with the PCV2 strain Assam-01 having a virus concentration of $10^{6.5}$ $TCID_{50}$/mL and the protective efficacy of the DNA vaccine (pCI-Cap) was determined based on the viral load in major organs, particularly in spleen, liver, lymph node and lung. The primers RepF and RepR successfully amplified 463 bp

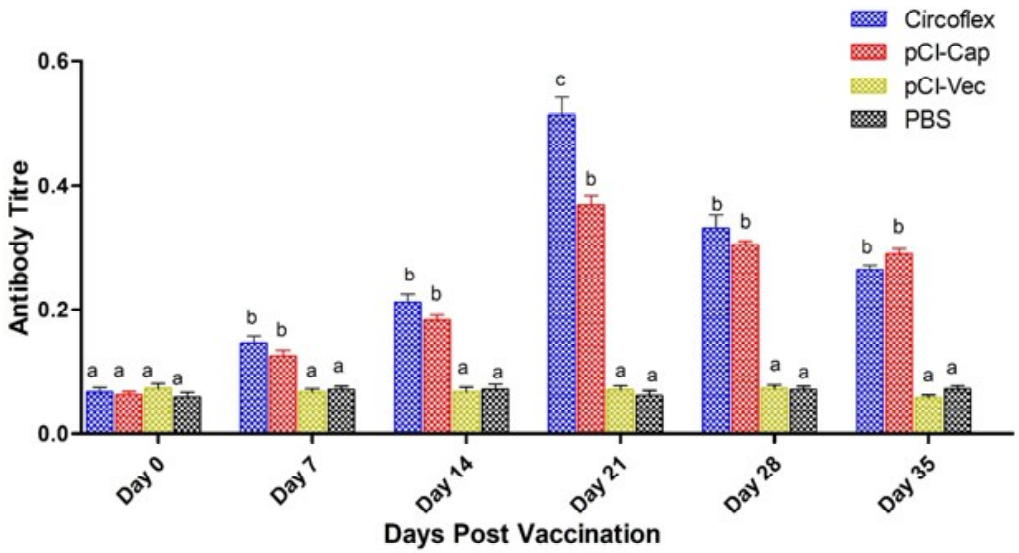

Fig 4.9: Estimation of humoral immune response in different mice groups.

The antibody titre was determined by indirect ELISA (OD_{450nm}) in different groups of mice at 0, 7, 14, 21, 28 and 35 days post vaccination. Values in bar are expressed as Mean± SE. Different superscript small case letters indicates significant difference among groups at particular time point among the groups($p<0.05$).

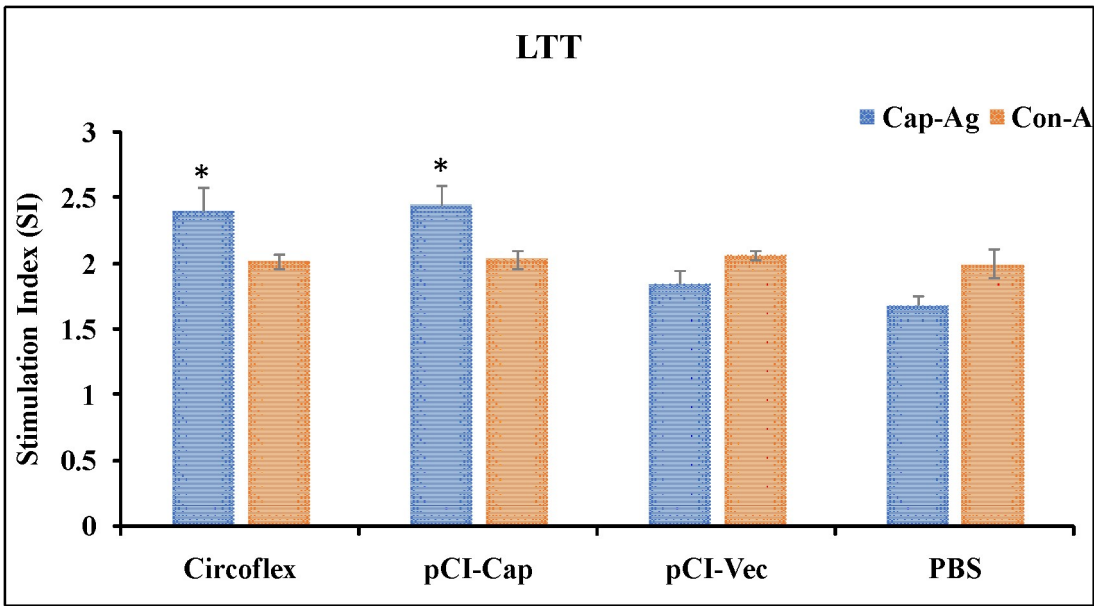

Fig 4.10: Lymphocyte transformation test. Stimulation index values are expressed as Mean±SE.

*Indicates significant difference ($p<0.05$) among groups, particularly of Circoflex and pCI-Cap groups compared to other groups (pCI-Vec or PBS)

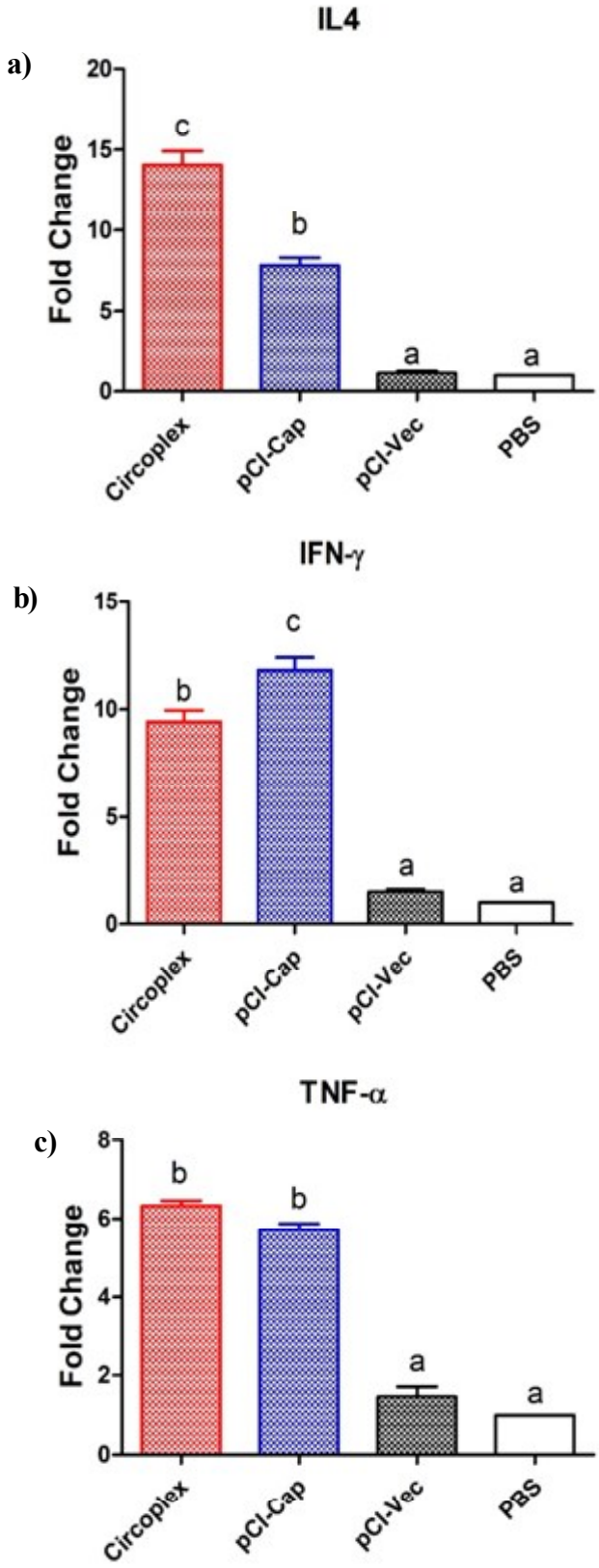

Fig 4.11: Relative expression pattern of cytokine gene transcripts in PBMCs. a) IL4, b) IFN-γ and c) TNF-α. Expression level is represented as fold change ± SE. Different superscript letters represents significant fold change among groups ($p<0.05$).

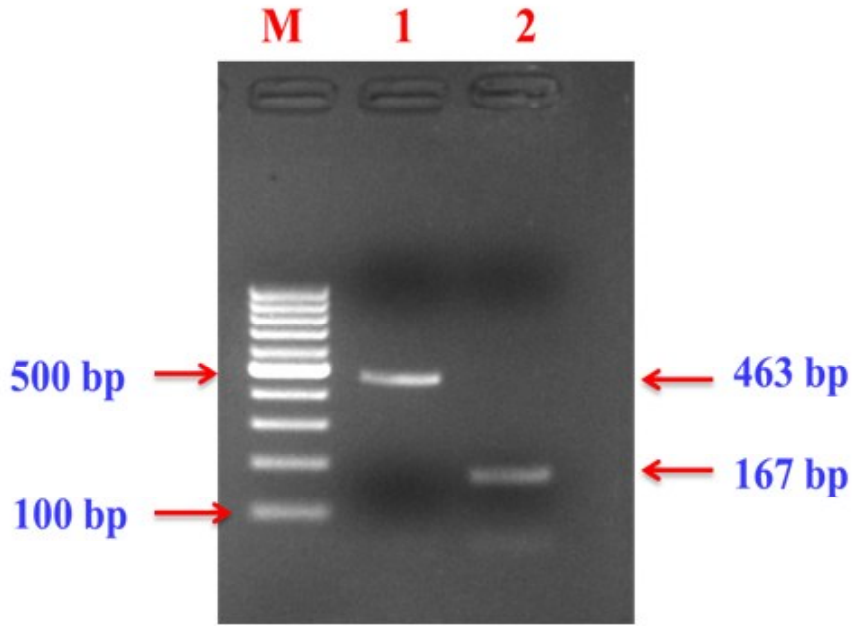

Fig 4.12: PCR amplification of *Rep* gene for qPCR to determine viral load in organs of challenged mice.

Lane LM : 100 bp DNA ladder

Lane L1 : Amplification of *Rep* gene (463 bp) for preparation of standard curve.

Lane L2 : PCR amplification of *Rep* gene (167 bp) by detection primers to be used in qPCR to estimate PCV2 load in tissue samples.

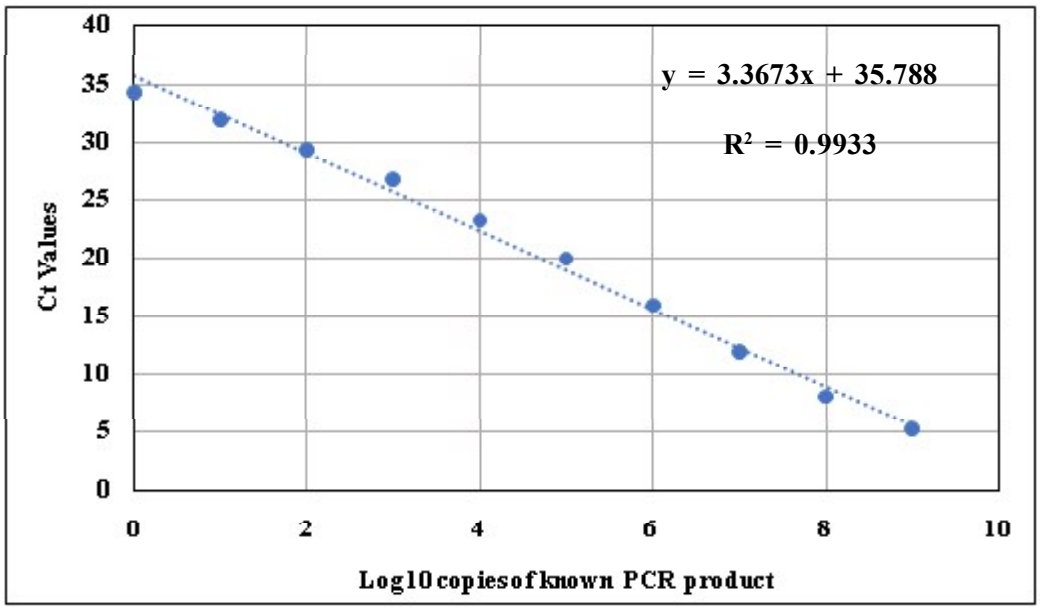

Fig 4.13: Standard curve of qPCR for detection of PCV2 genomic DNA.

C_t values of known PCR product is plotted in y-axis and the $\log_{10}$ concentration (ten-fold dilution) in x-axis.

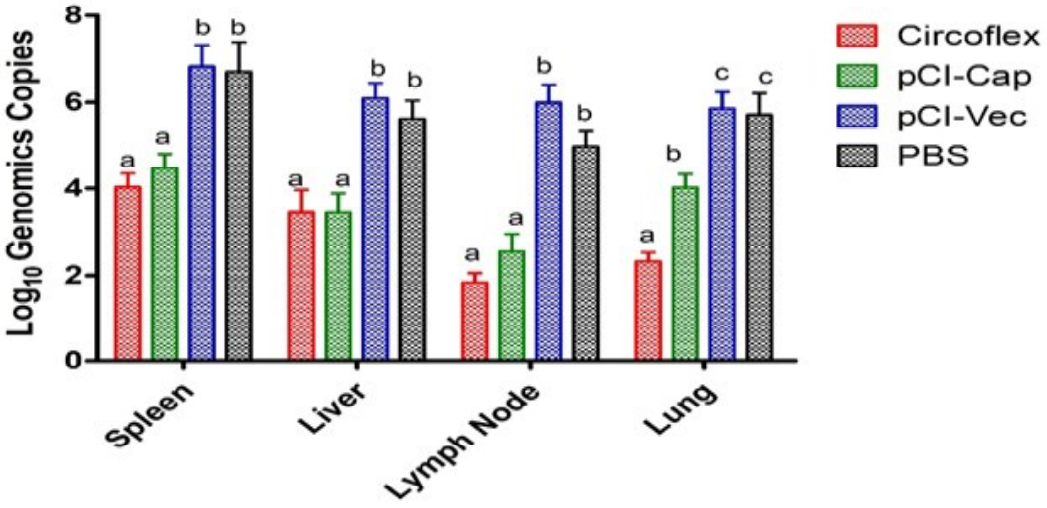

Fig 4.14: PCV2 load in different organs of challenged mice.

Each bar represents the mean $\log_{10}$ genomic copies of PCV2 with ± standard error. Different superscript letters depict significant difference ($p<0.05$) in an organ among the groups.

of PCV2 *ORF1* gene which was gel purified and subsequently used as the known standard to prepare the qPCR standard curve for absolute quantification of the viral load (Fig. 4.12). Similarly, the in-house designed PCV2 detection primers (PCV-N1 and PCV-N2) also amplified 167 bp of PCV2 *ORF1* gene (Fig. 4.12) which were used in qPCR for PCV2 detection in different organs of challenged mice. The standard curve was determined by serial dilution (ten-fold) of the PCR product (463 bp of Rep gene) as the known standard (Fig. 4.13). The efficiency of the qPCR was found to be 98.54% which is in the acceptable range of 90-110 % (Taylor *et al.,* 2019) having a slope of -3.3573 with r^2 of 0.9933. The PCV2 genomic DNA was detected in all the groups of mice; however, the mean $\log_{10}$ genomic copies in four major organs (spleen, liver, lung and lymph node) were significantly lower ($p<0.05$) in the Circoflex and the pCI-Cap vaccinated groups compared to the control group or the pCI-Vec group (Figure 4.14). No significant difference was observed in the mean $\log_{10}$ genomic copies between the Circoflex and the pCI-Cap groups for all the evaluated organs except for lung. However, the load of PCV2 was found to be always lower in all the four organs in the Ciroflex group than the pCI-Cap group but it was found to be significantly lower only in the lung ($p<0.05$).

Discussion

5 DISCUSSION

Porcine circoviruses (PCVs) are non-enveloped single-stranded DNA viruses which are either pathogenic or non-pathogenic in nature. Four types of PCVs (PCV1, PCV2, PCV3, PCV4) have been discovered, out of which PCV2 is of major concern with respect to its pathogenicity in pigs. Porcine circovirus 2 (PCV2)is the primary aetiology of porcine circovirus-associated disease (PCAD), which is distributed in the global pig population. PCV2 is associated with several clinical manifestations such as postweaning multisystemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), reproductive failure, respiratory problems and enteric diseases (Opriessnig*et al*., 2007; Opriessnig and Langohr 2013; Opriessnig*et al*., 2020).

Till date, eight genotypes of PCV-2 (PCV2a-h) have been identified (Franzo and Segalés, 2018) but the major disease-causing genotypes,which are distributed universally,include PCV2a, PCV2b and PCV2d (Franzo and Segalés, 2020). Since discovery of PCV-2, two major genotypic shifts have been observed. The first shift was noticed between 2003 and 2006, in which the most circulating PCV2a genotype was replaced by PCV2b (Patterson and Opriessnig, 2010; Opriessniga*et al*., 2020) and the second important genotype shift was noted between 2010 and2015, where PCV2d replaced PCV2b (Xiao *et al*., 2015; Xiao *et al*., 2016). Like other pig rearing countries, the pig population of India is also susceptible to PCV2 infection, and the most pathogenic genotypes: PCV2a, PCV2b and PCV2d have also been reported from India, particularly from the north-east region (Anoopraj *et al*., 2015; Barman *et al*., 2018; Mukherjee *et al*., 2018; Mukherjee *et al*., 2019).

One of the most potent and effective means to restrict the spread of PCV2 in pigs is through vaccination. It has been documented that the available PCV2 vaccines can decrease viraemia, reduce virus shedding and viral load, limit mortality rate and enhance production traits like average daily weight gain (Wozniak *et al*., 2019). Several commercial PCV-2 vaccines are available in the international market which are either inactivated or subunit vaccines. Although effective, these vaccines are cumbersome to produce, impart short term immunity and are costly in the Indian market. Moreover, sometimes the available commercial vaccines fail to prevent PCVAD in the vaccinated pigs due to the high rate of mutation and occurrence of genotypic shift in PCV2 at periodic intervals (Opriessning *et al*., 2013b; Kwon *et al*., 2017; Opriessnig *et al*., 2020). Therefore, it is necessary to develop an alternative indigenous PCV-2 vaccine for the pig population of India. In this regard, DNA vaccine is one of the choices because the production process is simple, capable of inducing wide range of immunity, and stable, does not interfere with maternal antibodies, and does not require maintenance of cold chain. Most importantly, based on the socio-economic status of majority of Indian farmers and the availability of cold-chain infrastructure, DNA vaccine is a suitable option to control the disease in the Indian condition.

Till date, several experimental DNA vaccines against PCV2 have been evaluated and most of them have shown promising results in terms of their efficacy in inducing humoral and cell-mediated immunity and reducing viraemia and viral load either in the laboratory animal model (mice) or in pigs (Shen *et al*., 2008; Silva Júnior *et al*., 2009; Fu *et al*., 2011; Chen *et al*., 2014; Sylla *et al.,* 2014; Guo *et al.,* 2015; Li *et al.,* 2015; Li *et al*., 2016; Park *et al.,* 2017; Hou *et al.*, 2019a). Most of these experimental DNA vaccines encode ORF2 protein of PCV2 either alone or as a fusion protein in combination with certain other protein domains as adjuvants. The ORF2 encodes the major and only structural protein of PCV2, the capsid protein, which is also the most immunogenic protein that can protect pigs from PCV2 infection (Pogranichnyy *et al*., 2000; Mankertz *et al.*, 2004; Lv *et al.,* 2014). Therefore, the capsid protein is a potent vaccine candidate and the majority of commercial subunit vaccines consist of this protein. Moreover, Guo *et al.* (2012) described that PCV-2 strains having a capsid protein with an extra lysine residue (234 position) at C-terminal are more virulent than the wild

type virus and the last 3 or 4 amino acids of the capsid protein is an immune-dominant epitope (Lekcharoensuk *et al.,* 2004; Shang *et al.,* 2009). Therefore, the capsid protein with an extra lysine residue might be more immunogenic and may provide better protection than the other capsid protein-based vaccine candidates.

Keeping the above facts in view, a PCV2 DNA vaccine construct was designed in the present study which encodes the *cap* gene of PCV2d genotype having an additional lysine residue at 234 position of the capsid protein. The DNA vaccine construct was injected to mice and both the arms of immune response namely, humoral and cell mediated were evaluated. The first experimental DNA vaccine (encoding ORF2) against PCV2 was designed by Kamstrup *et al*. (2004) and injected into mice by a gene gun. The mice vaccinated with PCV2 DNA vaccine produced specific antibodies against ORF2 protein of PCV2. This experiment demonstrated the scope of using a DNA vaccine to counter PCV-2 infection in pigs. Thereafter, several experimental DNA vaccines with different modifications were reported from various corners of the world and most of the experimental PCV2 DNA vaccines have manifested convincing results either in the laboratory animal model or in the primary host.

Designing of DNA vaccine construct

In the present study, the *cap* (ORF2) gene of PCV2d having an extra lysine codon (AAG) was successfully amplified by PCR from the PCV2 strain Assam-01 (MN266483) using a pair of in-house designed primers (mCap-1 & mCap-2). The amplified product was cloned in pJET1.2/Blunt vector and the presence of the lysine codon at 3' end was confirmed by sequencing.The coding region of *cap* gene was found to be error-free. Subsequently, the *cap* gene was sub-cloned in the mammalian expression vector pCI-neo (Fig. 7), which was designated as pCI-Cap and used as a DNA vaccine in this study. Suarez and Schultz-Cherry (2000) reported that pCI-neo vector with immediate early cytomegalovirus (CMV) promoter is a suitable vector for construction of DNA vaccine. Shen *et al.* (2008) constructed a DNA vaccine encoding ORF2 of PCV2 (isolate 'HZ0201' with GenBank Accession no. AY188355) using pCI-neo vector and evaluated its immune response in mice model. In another experiment, Silva Júnior *et al*. (2014) cloned the ORF2 of PCV2 (GenBank Accession No. DQ364650)

in pCI-neo vector and analysed its immunogenic potential in experimental mice. Park *et al*. (2017) designed another PCV2 DNA vaccine construct with ORF of PCV2b (Accession no. KF871068) cloned into pCI-neo vector and evaluated its immune response in piglets. Besides pCI-neo vector, other mammalian expression vectors used for designing DNA vaccine constructs to immunize mice or pigs were pVAX1 (Li *et al*., 2015; Hou *et al*., 2019a), pIRES vector (Lin *et al*., 2011b; Guo *et al*., 2015), pCAGGS vector (Fu *et al*., 2011), pcDNA3.1 (Kamstrup *et al*., 2004) and pEGFP-N1 vector (Sylla *et al*., 2014).

The *in-vitro* expression analysis of pCI-Cap plasmid transfected in PK-15 cells by lipofection shown expression of the capsid protein in western blot. Kamstrup *et al*. (2004) demonstrated the expression of capsid protein by the experimental DNA vaccine construct in PK-15 cells. Likewise, several investigators confirmed by indirect immunofluorescence assay (IFA) the expression of capsid protein in PK-15 cells transfected with DNA vaccine construct that encodes ORF2 gene (Shen *et al*., 2008; Sylla*et al*., 2014; Park *et al*., 2017; Hou *et al*., 2019a). However, some other researchers used different cell lines other than PK-15 to study the *in-vitro* expression of capsid protein by recombinant plasmids which were used as experimental DNA vaccine for PCV2. Silva Júnior *et al.* (2009) reported *in-vitro* expression of capsid protein encoded by the recombinant plasmid 'pCap' in SK6 cells. Lin *et al*. (2011b) reported the expression of ORF2 protein by western blotting of CHO cells transfected with recombinant plasmid pIRES-ORF2. In the same year, Fu *et al.* (2011) used IFA and confocal microscopy to confirm capsid protein expression by the DNA vaccine plasmids transfected in 293T cells.

Evaluation of immune response elicited by the DNA vaccine

Both humoral and cell-mediated immune responses elicited by the designed PCV2 DNA vaccine (pCI-Cap recombinant plasmid) was evaluated and compared with that of the commercial vaccine (IngelvacCircoFLEX®) in the mice model. For estimation of immune response, twenty-four mice were divided randomly into four groups (six in each group), *viz*. pCI-Cap (inoculated with DNA vaccine construct), pCI-Vec (inoculated with empty pCI-neo vector), Circoflex (inoculated with IngelvacCircoFLEX®) and PBS (injected with PBS as negative control) groups.

For estimation of humoral immune response, sera samples from all the groups of mice were collected at weekly intervals on days 0, 7, 14, 21, 28 and 35 post-inoculation. The first day of inoculation was considered as day 0. The level of capsid protein specific antibody was detected in the sera of the experimental mice by means of indirect ELISA and the antibody levels were expressed based on the OD value at 450nm. The mice of pCI-Cap and Circoflex groups started showing detectable levels of anti-capsid antibodies from the day 7 post-inoculation which continued increasing till day 21. Then there was a gradual decline in the level of antibodies on day 28 and it continued further till day 35. However, no antibody was detected in mice immunized with pCI-Vec and PBS. The level of antibodies was at its peak on day 21 post-inoculation which might be due to the inoculation of the booster dose on day 14. In this study, humoral immune response elicited by the DNA vaccine (pCI-Cap) which encodes ORF2 of PCV2d with an extra lysine residue at C-terminal was found to be comparable to that of the commercial vaccine IngelvacCircoFLEX® because there was no significant difference in the antibody level in mice groups vaccinated with these two vaccines except for day 21 post-vaccination. Moreover, the antibody level on day 35 was slightly higher in case of DNA vaccine compared to the commercial vaccine which indicates that immunity conferred by the DNA vaccine was maintained for longer duration. In conformity to the present study, Guo *et al.* (2015) observed the detection of PCV2 specific antibodies in mice at one week after the first injection of experimental DNA vaccine constructs pIRES-ORF2/IL6, pIRES-ORF2 +pIRES-IL6 and pIRES-ORF2 but not in mice inoculated with pIRES (empty vector) and PBS. The amount of antibodies increased in all the groups except the control groups after the booster dose given on the 3rd week from the primary dose. Although the level of antibodies declined slightly on the 4th week, it increased again from 5th to 6th week. However, the mice injected with pIRES-ORF2/IL6 had significantly higher ($p<0.05$) antibody titre than those injected with only pIRES-ORF2. A similar trend in rise of antibody level was also observed by Li *et al.* (2016) when they injected DNA vaccine in experimental mice which consisted of *cap* gene along with a specific CpG motif. They evaluated the immunogenicity of two DNA vaccines namely,18C pG-pVAX1 -ORF2 and pVAX1-ORF2 in mice. Although both the constructs induced humoral immune response, the level was higher in mice inoculated with 18C pG-

pVAX1-ORF2 than that of the pVAX1-ORF2 group. Sylla*et al.* (2014) immunized mice thrice at 2 weeks interval with plasmid pEGFP-Cap having ORF2 gene of PCV2. In their experiment, capsid-specific IgG was detected on day 14 and it increased gradually. Silva Júnior *et al*. (2009) immunized two groups of mice with PCV2 DNA vaccine that expressed capsid protein @ 100 µg by intramuscular route in one group and 50 µg intradermally in another group, and boosted twice on day 15 and 30 after the initial dose. In both the vaccinated groups, there were significantly higher antibody titre compared to the control group on day 15 to 45. Moreover, the mice group immunized with 100 µg of DNA vaccine had higher level of anti-capsid antibody than the group injected with 50 µg of DNA vaccine. Lin *et al.* (2011b) experimented with a bicistronic DNA vaccine in mice model that expressed VP2 protein of porcine parvovirus along with ORF2 of PCV2. The mice were immunized with three recombinant plasmids, pIRES-ORF2, pIRES-VP2, pIRES-ORF2-VP2 and an empty vector pIRES as control. Three shots of injection were given at two weeks interval. The mice immunized with pIRES-ORF2 and pIRES-ORF2-VP2 started developing detectable level of antibodies against PCV2 at one week after the first immunization. The antibodies were at higher range from the second week; however, there was a slight decline in antibody titre on in the fourth week but it increased thereafter the second booster dose. Fu *et al.* (2011) constructed two experimental DNA vaccines namely, pc-Cap that encodes only capsid protein of PCV2 and pc-Ub-Cap that co-expresses ubiquitin along with capsid protein. Mice were injected with three doses at three weeks of interval. The antibodies against capsid protein of PCV-2 were detected from the second week but the titre of antibody was higher in pc-Ub-Cap group compared to that of the pc-Cap group.

A similar trend of humoral immune response was also noted when several experimental PCV2 DNA vaccines were tested in the primary host, pig. Chen *et al.* (2014) injected two recombinant plasmids: pBudCE4.1-ORF2/IL18 that encodes ORF2 of PCV-2 as well as IL18 of swine and pBudCE4.1-ORF2 that encodes only ORF2, and observed that both the DNA vaccine constructs elicited specific antibodies against PCV2 but the titre was non-significantly ($p>0.05$) higher in mice inoculated with the plasmid pBudCE4.1-ORF2/IL18 compared to that of pBudCE4.1-ORF2. In another investigation done in pigs by Li *et al.*

(2015) it was observed that a DNA vaccine construct with ORF2 of PCV2 and a CpG motif (CpG-pVAX1-ORF2) elicited high level of antibody production on day 14 post-immunization. Hou *et al.* (2019a) constructed a DNA vaccine with ORF2 from PCV2d genotype (pVAX1-ORF2) as well as a construct that contained ORF2 and the specific domain of the complement C3 cascade (C3d-P28) which was denoted as pVAX1-ORF2-C3d-O2. Both the vaccine constructs started inducing PCV2 specific humoral immune response in pigs from day 14 post-immunization to the day of challenge but the level of antibodies induced by pVAX1-ORF2-C3d-O2 vaccine was significantly higher than that of the pVAX1-ORF2 vaccine construct.

In the present study, the cell-mediated immune response was evaluated based on the lymphocyte transformation test (LTT) and the relative expression of three cytokine genes which included IL4, Interferon-γ (IFN-γ) and Tumour Necrosis Factor-α (TNF-α) in PBMCs of immunized mice 10 days post-booster dose. In this study, the stimulation index recorded in LTT was found to be significantly higher ($p<0.05$) in the groups of mice immunized with pCI-Cap and the commercial vaccine compared to the groups injected with pCI-neo empty vector and PBS, when stimulated with capsid antigen.

The cell-mediated immune response induced by a vaccine candidate can be accessed *in-vitro* in immune cells by LTT or Lymphocyte proliferation assays (Nikbakht *et al.*, 2019). The *in-vitro* proliferation of lymphocytes is observed if an individual is vaccinated with a particular antigen or have been infected by the pathogen carrying the same antigen. The high-rate of lymphocytes proliferation in mice vaccinated with the DNA vaccine (pCI-Cap) indicated that the DNA vaccine is capable of inducing cellular immune response. Moreover, the SI of DNA vaccine was slightly higher than the commercial vaccine. The LTT results obtained in this study is consistent with observations made by several other investigators. Shen *et al.* (2008) observed that the splenocytes of mice at 8 weeks post-immunization with experimental PCV2 DNA vaccine or capsid protein exhibited high level of lymphoproliferative response when stimulated with capsid protein. Similarly, Silva Júnior *et al.* (2009) recorded a significantly high proliferation rate of spleen cells in mice inoculated with DNA vaccine. In another PCV2 DNA vaccination trial conducted in mice, the splenocytes showed significant proliferation

when the cells were restimulated with PCV2 antigen *in-vitro* (Fu *et al*., 2011). The *in-vitro* T-lymphocyte proliferation in PBMCs of pigs vaccinated with DNA vaccines was found to be significantly higher ($p<0.05$) than that of the control groups (Chen *et al*., 2014). The *in-vitro* lymphocyte proliferation assay performed by Guo *et al*. (2015) also showed similar result in which the PBMCs collected from mice injected with the experimental DNA vaccine exhibited significantly higher SI value than the control group when stimulated with capsid protein.

Cytokines play a dynamic and vital role in inducing cell-mediated immune response in vaccinated individuals. In a previous study, it was observed that pigs immunized with PCV2 vaccine produced higher level of several cytokines (IL-1β, IL-8, IFN-γ and TNF-α) than pigs that were naturally infected with signs of PMWS (Borghetti *et al*., 2013). Likewise, in the current investigation, the level of relative expression of IL4, IFN-γ and TNF-α in PBMC of mice vaccinated with pCI-Cap and commercial vaccine was found to significantly upregulated ($p<0.05$) compared to mice injected with pCI-neo empty vector and PBS. The upregulation of IFN-γ in pCI-Cap and Circoflex groups of mice indicated the involvement of Th1-mediated immune response whereas the upregulation of IL-4 transcripts in the same groups indicated that the immune response was also mediated by Th2. Production of IFN-γ by the IFN-γ secreting cells is necessary to control PCV-2 infection because insufficient production of IFN-γ is found to be associated with severe viraemia and low neutralizing antibody titre (Meerts *et al*., 2005; Fort *et al*., 2009a; Fort *et al*., 2009b). However, the significant up-regulation of IFN-γ transcripts in pCI-Cap group of mice than the commercial vaccine or other control groups indicates that the DNA vaccine is actively involved in inducing higher level of cellular immune response which is an essential criteria of a potent vaccine candidate. Growth and development of B lymphocytes is induced by IL4, thereby it elicits humoral immune response by means of antibody production (Howard, 1983). Significantly higher level of IL4 transcript expression in commercial vaccine group compared to the pCI-Cap immunized group suggests the active role of commercial subunit vaccine in induction of humoral immune response by Th2 cells.The pro-inflammatory cytokine,TNF-α, plays an active role in eliciting adaptive immunity against viral infections (Kreikemeier *et al*., 2015). The expression of TNF-α gene was also found to be up-regulated in Circoflex and pCI-Cap group as compared to pCI-Vec and

control group. Thus up-regulated expression of IL4, IFN-γ and TNF-α in the vaccinated mice with pCI-Cap plasmid or commercial vaccine observed in the present study indicated the ability of the vaccines to induce cell-mediated immune response which may have potential to combat PCV2 infection. In consensus with the present study, Chen *et al.* (2014) estimated the concentration of IL4 and IFN-γ secreted by PBMCs of pigs vaccinated with experimental DNA vaccines and observed that the concentration of both the cytokines were significantly higher in the vaccinated group compared to the controls. As reported by Fu *et al.* (2011),the concentration of cytokine IFN-γ associated with Th1-mediated immune response was found to be in higher in the supernatants of cultured splenocytes collected from mice that were immunized with plasmids containing ORF2 of PCV2 in conjugation with ubiquitin or ORF2 alone. Sylla *et al*. (2014) estimated the serum IFN-γ concentration in mice vaccinated with a recombinant plasmid that encodes capsid protein and observed that the level of IFN-γ was quite higher post-vaccination compared to that of the control mice. Koinig et al. (2015) reported that immunization of pigs with commercial PCV2 vaccine increases the production of IFN-γ and TNF-α co-producing immune cells which might help in providing protection in challenged pigs. The IFN-γ secreting cells were found be in greater number in pigs that were immunized with commercial vaccine and an experimental PCV2 DNA vaccine encapsulated in liposome (Park *et al*., 2017). Similarly, Hou *et al.* (2019a) also noted a significant increase in IFN-γ secreting cells in PBMCs of pigs vaccinated with ORF2 encoding plasmids either fused with or without C3d domain of the complement protein. The relative expression of IFN-γ and TNF-α mRNA in PBMC of pigs vaccinated with VLPs formed by capsid protein of PCV2 were significantly higher in comparison to the control group when pigs were challenged with a live PCV2 virus (Liu *et al*., 2020).

Protective efficacy of the PCV-2 DNA vaccine

Vaccination of pigs with PCV2 vaccines significantly reduces viraemia, virus shedding as well as viral load in serum or different tissues after experimental infection (Sno *et al*., 2016; Wozniak *et al*., 2019; Cybulski *et al*., 2020). In order to determine the protective efficacy of the pCI-Cap DNA vaccine construct designed in this study, mice were challenged with the Assam-01 strain of PCV2 after 3 weeks post, booster vaccination and the viral load in different

organs were estimated by qPCR using the standard curve method. The PCV2 load in four organs which included spleen, lymph node, liver and lung were significantly lower ($p<0.05$) in mice groups vaccinated with the plasmid pCI-Cap and the commercial vaccines than the groups inoculated with pCI-Vec or PBS. However, the viral load was lower in the organs of mice immunized with the commercial vaccine than that of the pCI-Cap plasmid. The results were in consistent with the findings of many other workers. Li *et al.* (2016) observed that mice groups that were injected with recombinant plasmids 18CpG-pVAX 1-ORF2 and pVAX1-ORF2 had lower PCV-2 load compared to the control mice in different organs like lung, liver, kidney, heart, spleen, muscle and blood between the first and the third week after challenge. Similarly, Guo *et al.* (2015) determined the protection rate of different construct of PCV-2 DNA vaccines in mice model challenging all the mice with a live PCV2 strain. Protection rate was calculated based on the number of mice with undetected PCV2 genomic DNA/total number of mice in the group. The detectable PCV2 genomic DNA in different organs (heart, liver, lung, lymph node and spleen) of immunized mice was comparatively lower than that of the mice of control group. The protection rate was 60-86.7 % in mice vaccinated with different forms of DNA vaccine that encoded ORF2 and IL6. Between 2 and 6 weeks post-challenge, the viral load was significantly lower in the sera of mice that were vaccinated with experimental PCV2 DNA vaccine (pORF) alone or in combination with capsid protein than that of the control group (Shen *et al*., 2008). In a vaccination-challenge study conducted in mice by Fu *et al* (2011), the mice injected with DNA plasmids pc-Ub-Cap and pc-Cap were found to have lower viral load in sera from 2-8 weeks post-challenge as compared to the mice injected with the empty vector. Sylla *et al.* (2014) also assessed the sera of mice that were challenged with PCV2 virus which were vaccinated with plasmid DNA that encoded capsid protein of PCV2 and observed that PCV2 genomic load was very low in the vaccinated group as compared to that of the control. The piglets immunized with DNA vaccine construct with ORF2 of PCV2 encapsulated in liposome and a commercial vaccine had considerably lower PCV2 load in the serum after challenge. Hou *et al.* (2019a) also recorded very less viral load in sera of challenged mice that were injected with plasmids that contained ORF2 of PCV-2 or ORF2 plus C3d-P28. Thus, it was observed that the experimental DNA vaccine that expressed capsid protein of PCV2 had the ability to neutralize PCV2 infection and decrease the viral load.

In the present study, an experimental DNA vaccine construct was designed by incorporating the *cap* gene of PCV2d genotype with an extra lysine residue codon at the 3' end (pCI-Cap). In *in-vitro* expression analysis of pCI-Cap transfected in PK-15 cells, successful expression of the capsid protein was noticed. This vaccine construct when injected in experimental laboratory animals (mice) induced both the humoral as well as cell-mediated immune responses. The higher leve of lymphocyte proliferation and IFN-γ expression in PBMCs of DNA vaccine injected mice compared to other groups indicated that the designed DNA vaccine can induce promising cellular immunity which is essential to control PCV2 infection. Moreover, in challenge study, this DNA vaccine could decrease the viral load in different organs, thereby indicating that it might be able to protect the immunized animals from natural PCV2 infection. Thus, the results of this study and those reported in earlier investigations suggest the potential field application of the PCV2 DNA vaccine in future. Moreover, the overall efficacy of the DNA vaccine (pCI-Cap) was comparable to the commercial vaccine but its efficacy could probably be further enhanced by using appropriate adjuvant and delivery system because DNA vaccines formulated with adjuvant and targeted delivery systems were reported to show better results (Saade and Petrovsky, 2012; Li and Petrovsky, 2016;Park *et al*., 2017). Hence, the immunogenicity as well as protective efficacy imparted by the DNA vaccine designed in the present study needs to be further validated in the primary host (pig) after incorporating a superior quality adjuvant or after encapsulation with appropriate and safe particles that may ensure targeted delivery of the vaccine.

Summary and Conclusions

6 SUMMARY AND CONCLUSIONS

There is tremendous scope of development of the piggery sector in India, particularly in the north-eastern region. Pig is a prolific animal and its total population in India is 9.06 million (20th Livestock Census, 2019). Pork is a good source of animal protein for human, and pig husbandry provides a means of livelihood to many small and marginal farmers. However, the transboundary incursion of many exotic swine diseases in India through the porous international border has caused uncertainty for the future of the pig industry in India. Several exotic swine viruses have entered in the last decade which include Porcine reproductive and respiratory syndrome virus (PRRSV), African swine fever virus (ASFV) and Porcine Circovirus types 2 and 3.

Porcine Circovirus type 2 (PCV2) is an unenveloped single-standard DNA virus of the family *Circoviridae* belonging to the genus *Circovirus*. There are eight genotypes of PCV2 (PCV-2a-h), and the most pathogenic and common globally distributed genotypes include PCV2a, PCV2b and PCV2d. PCV2 is associated with several forms of diseases or syndromes, which include post-weaning multisystemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), enteric disease, respiratory disease and reproductive complications. It is an opportunistic and immunosuppressive virus that can have detrimental effects when there is a co-infection with other porcine viruses.

PCV2 is an emerging threat to the swine industry in India because many previous studies reported the circulation of PCV2 in the country including the north-eastern region where it entered almost a decade ago. Moreover, the dominant and pathogenic genotypes

(PCV2a, PCV2b and PCV2d) have already been reported from India. Although vaccination can prevent the spread of PCV2 infection, unfortunately there is no indigenous vaccine available in the country. Therefore, the Indian pig farmers have to depend solely on the exotic commercial vaccines which are very expensive and their supply is very limited. Moreover, the virus undergoes genotypic shift at periodic intervals which leads to vaccine failures in many cases and most of the commercial vaccines are developed from the older genotypes. Hence, it is necessary to develop a vaccine with the currently prevailing genotypes of PCV2, particularly with PCV2d.

Based on the socio-economic status of the Indian farmers and availability of cold chain infrastructure, DNA vaccine is expected to be a better option. DNA vaccine also has other benefits such as the production process is less complex and less expensive, can be produced in bulk in a short period of time, is more stable, and induces both humeral and cell-mediated immunity.

In this study, a PCV2 DNA vaccine was constructed by incorporating the *cap* gene of a PCV2 strain belonging to the genotype PCV2d in a mammalian expression vector. The inserted Cap gene codes for the capsid protein with 234 amino acid residues with an extra lysine codon (AAG) incorporated at the 3' end prior to the stop codon. This mutated *cap* gene was amplified by PCR using a primer set designed in-house (mCap-1 and mCap-2). The amplified gene was cloned into pJET1.2/Blunt cloning vector and the gene sequence was confirmed by sequencing. After confirming the mutated *cap* gene sequence which has an extra lysine codon at 3' end, the mutated gene was sub-cloned into the mammalian expression vector pCI-neo and the recombinant vector was named as pCI-Cap. The insertion of the gene of interest was confirmed by its release size of 717 bp from the vector following double digestion with restriction enzymes, *Xho*I and *Mlu*I. It was also confirmed further by sequencing.

The recombinant clone pCI-Cap was transfected in PK-15 cells by lipofection and *in-vitro* expression of the capsid protein was analysed by western blotting. The transfected PK-15 cells were lysed and subjected to SDS-PAGE. Finally, the proteins were transferred to PVDF membrane and the Cap protein was detected by using anti-capsid polyclonal antibodies and goat anti-rabbit HRPO conjugate as the primary and the secondary antibodies, respectively.

Immunogenicity and protective efficacy of the recombinant plasmid pCI-Cap was evaluated in experimental laboratory mice and the same was compared with that of an available commercial vaccine. For the immunological trial, 24 inbred albino mice were randomly divided in to four groups containing six mice in each. The first group received 100 µg of the DNA vaccine construct (pCI-Cap), the second group received 100 µl of a commercial vaccine (Circoflex), the third group received 100 µg empty pCI-neo vector (pCI-Vec) and the fourth group received only 100 µl of PBS which was kept as the negative control. A booster dose of the corresponding inoculum was given to the mice in each group two weeks after the primary vaccination, and serum samples were collected at weekly intervals starting from day 0 to day 35.

The humoral immune response was evaluated by estimating the antibody levels against the capsid protein in serum samples using an in-house designed indirect ELISA. The antibody level of pCI-Cap and Circoflex groups was found be higher from day 7 to day 35 compared to that of pCI-Vec and PBS groups of mice. However, the level of capsid antibodies was always higher in the commercial vaccine group than all the other groups, and on day 21, it was significantly higher in the circoflex group than that of pCI-Cap group.

The cell-mediated immune response of the vaccine was determined by LTT and relative expression analysis of selected cytokine genes. Both LTT and cytokine genes expression were analysed in PBMCs of all the mice groups collected 10 days after administration of the booster dose. The LTT exhibited higher rate of lymphocyte proliferation in the vaccinated groups when stimulated with the capsid antigen. Likewise, the relative expression of cytokine genes such as IL4, IFN-γ and TNF-α was up-regulated in the vaccinated groups than the control group. Although the expression of IL4 and TNF-α was highest in the Circoflex group, the fold change of IFN-γ expression was higher in the pCI-Cap group compared to all other groups. Thus, pCI-Cap was found to be capable of inducing better Th1-mediated immune response than Th2-mediated response.

All the experimental mice were challenged with a live virulent PCV-2 strain after three weeks of booster vaccination and the viral load was estimated in the organs like liver, lung,

spleen and lymph node by qPCR. The PCV2 genomic loads in all the organs were found to be in lower in the mice immunized with the commercial vaccine or the pCI-Cap vaccine when compared to the mice in pCI-Vec or PBS groups. There was no significant difference in the viral load between the pCI-Cap and the Circoflex group except for the lung tissue in which it was higher in case of the pCI-Cap group. However, the overall viral load was lower in the Ciroflex group among all the other groups.

In conclusion, it is appears to be the first report of experimental PCV2d DNA vaccine from India that has been evaluated in the mice model. The constructed DNA vaccine '**pCI-Cap'** was found to be capable of inducing both humoral and cell mediated immune responses in the experimental mice. The results of LTT and IFN-γ expression in DNA vaccine immunized mice group suggests that pCI-Cap is a potent future vaccine candidate to combat PCV2 infection. Moreover, it has shown the ability to decrease the viral load when the mice were experimentally infected following vaccination. Its overall performance was comparable to the commercial vaccine in respect of most of the estimated parameters. The immunogenicity of a DNA vaccine can be boosted further by using an appropriate adjuvant formulation and delivery system. Since, no indigenous PCV2 vaccine is so far available in India, the findings of the present study have certainly opened the scope for development of a new PCV2 DNA vaccine for pigs but it will need further improvement, refinement and validation before it reaches the commercialization stage.

Mini Abstract

7 MINI ABSTRACT

Porcine circovirus-associated disease caused by Porcine Circovirus 2 (PCV2) is an emerging threat to the global pig industry. PCV2 is the most prevalent type among the porcine circoviruses and it is one of the common globally distributed pig pathogens. PCV2 is almost endemic to India, particularly in the north-eastern states. Commercial vaccines available in the international market have the potential to minimize the spread of PCV2. However, majority of the commercial vaccines are formulated based on genotype PCV2a and due to occurrence of genotypic shift in PCV2 at certain intervals, sometimes these commercial vaccines fail to impart protection against subsequent natural infection. In India, so far there is no production of indigenous vaccines against PCV2 and the dependency on exotic vaccines causes a heavy financial burden for the farmers. Therefore, this study aimed to construct a DNA vaccine based on the *cap* gene of the virulent PCV2d genotype and to evaluate its immuno-potential in the mice model. Accordingly, a DNA vaccine (pCI-Cap) was constructed which encodes the capsid protein of PCV2d genotype with an extra lysine residue at the C-terminal and *in-vitro* expression of the capsid protein was confirmed by Western blot. For evaluation of immunogenicity of the DNA vaccine construct, four groups of mice were separately inoculated twice at two weeks interval with the plasmid pCI-Cap, a commercial vaccine, empty pCI-neo vector and phosphate buffer saline. The humoral immune response elicited by the pCI-Cap vaccine was specific to the capsid protein as determined by indirect ELISA and was significantly higher ($p<0.05$) compared to the control group. Based on lymphocyte transformation test and relative expression profile of cytokine mRNA transcripts (IL-4, IFN-γ and TNF-α), it was observed that pCI-Cap has the ability to induce cell-mediated immune response. In the challenge study, it was noticed that the viral load in different organs of pCI-Cap immunized mice were comparatively lower than that of the mice in the control groups. The designed DNA vaccine construct exhibited promising and broad immune response and its overall immunogenicity was comparable to the commercial vaccine. However, the immunogenicity of the pCI-Cap vaccine construct may be enhanced further by constructing a better vaccine formulation with the addition of an appropriate adjuvant. This appears to be the first experimental DNA vaccine for PCV2 reported from India as per our knowledge and it has certainly opened the new vista to design and formulate better vaccine candidates in future for combating PCV2 infection in pigs.

लघु सारांश

8 लघु सारांश

पोर्सिन सर्कोवायरस–2 (पीसीवी–2) के कारण होने वाली पोर्सिन सर्कोवायरस बीमारी वैश्विक शूकर उद्योग के लिए एक उभरता हुआ खतरा है। पीसीवी–2, पोर्सिन सर्कोवायरस में सबसे प्रचलित प्रकार का विषाणु है और यह विश्व स्तर पर व्यापक शूकर रोगजनकों में से एक है। पीसीवी–2, भारत के लिए खासकर उत्तर–पूर्वी राज्यों में लगभग स्थानिक (एंडेमीक) है। अंतराष्ट्रीय बाजार में उपलब्ध वाणिज्यिक टीकों में पीसीवी–2 के प्रसार को कम करने की क्षमता है। हालांकि, इनमें से अधिकांश टीके पीसीवी–2ए जीनोटाइप पर आधारित हैं और निश्चित अंतराल पर पीसीवी–2 में जीनोटाइपिक बदलाव की घटना के कारण, कभी–कभी ये टीके सुरक्षा प्रदान करने में विफल हो जाते हैं। भारत में, पीसीवी–2 के खिलाफ स्वदेशी टीकों का कोई उत्पादन नहीं होता और विदेशी टीकों पर निर्भरता, किसानों के लिए भारी वित्तीय बोझ का कारण बनती है। इसलिए, इस अध्ययन का उद्देश्य विषाणुजनित पीसीवी–2डी जीनोटाइप के कैपजीन के आधार पर एक डीएनए वैक्सीन का निर्माण करना और चूहों के मॉडल में इसकी प्रतिरक्षा क्षमता का मूल्यांकन करना था। तदनुसार, एक डीएनए वैक्सीन (पीसीआई–कैप) का निर्माण किया गया जो पीसीवी–2डी जीनोटाइप के कैप्सिड प्रोटीन को सी–टर्मिनल पर एक अतिरिक्त लाइसिन अवशेषों के साथ एन्कोड करता है और वेस्टर्न ब्लॉट द्वारा कैप्सिड प्रोटीन की इन–विट्रो अभिव्यक्ति की पुष्टि की गई। डीएनए वैक्सीन निर्माण की इम्युनोजेनेसिटी के मूल्यांकन के लिए, चूहों के चार समूहों को प्लास्मिड पीसीआई–कैप, एक वाणिज्यिक वैक्सीन, खाली पीसीआई–नियो वैक्टर और क्षारीय फॉस्फेट बफर के साथ दो सप्ताह के अंतराल पर दो बार अलग–अलग टीका लगाया गया। पीसीआई–कैप वैक्सीन कैप्सिड प्रोटीन के लिए विशिष्ट था जैसा कि अप्रत्यक्ष एलिसा द्वारा निर्धारित किया गया था और नियंत्रण समूह की तुलना में काफी अधिक (पी<0.05) था। लिम्फोसाइट परिवर्तन परीक्षण और साइटोकाइन एमआरएनए टेप (आईएल–4, आईएफएन–गामा और टीएनएफ–अल्फा) के सापेक्ष अभिव्यक्ति प्रोफाइल के आधार पर, यह देखा गया कि पीसीआई–कैप में सेल–मध्यस्थ प्रतिरक्षा प्रतिक्रिया को प्रेरित करने की क्षमता है। चुनौती अध्ययन में, यह देखा गया कि पीसीआई–कैप प्रतिरक्षित चूहों के विभिन्न अंगों में वायरल लोड नियंत्रण समूहों में चूहों की तुलना में तुलनात्मक रूप से कम था। डिजाइन किए गए डीएनए वैक्सीन निर्माण ने आशाजनक और व्यापक प्रतिरक्षा प्रतिक्रिया का प्रदर्शन किया और इसकी समग्र इम्युनोजेनेसिटी वाणिज्यिक वैक्सीन के लिए तुलनीय है। हालांकि, पीसीआई–कैप वैक्सीन निर्माण की प्रतिरक्षी क्षमता को उपयुक्त सहायक के साथ एक बेहतर वैक्सीन फॉमूलेशन का निर्माण करके और बढ़ाया जा सकता है। जानकारी के अनुसार यह वैक्सीन भारत से पीसीवी–2 के लिए पहला प्रायोगिक डीएनए वैक्सीन है और इसने शूकरों में पीसीवी–2 संक्रमण का मुकाबला करने के लिए बेहतर उम्मीदवार वैक्सीन को डिजाइन और तैयार करने के लिए आयाम दिया है।

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Appendix

APPENDIX

Ampicillin (100 mg/ml) stock solution

Ampicillin	500 mg
Distilled water	2.5 ml

Dissolve thoroughly and filter through 0.22 μM filter. Then make up the volume to 5ml with nuclease free water. Store at -20°C.

Antibody dilution buffer

Bovine Serum Albumin	3 g (w/v)
TBST	100 ml

Blocking buffer

Bovine Serum Albumin (BSA)	5 g
TBST	100 ml

Ethidium bromide (10 mg/ml)

Ethidium bromide	100 mg
Distilled water	10 ml

Gel loading dye (6X)

Bromophenol blue	1.0%
Sucrose in water (w/v)	40%

Phosphate Buffer Saline (PBS), pH 7.2-7.4

Sodium chloride	8.0g
Potassium chloride	0.2g
Disodium hydrogen phosphate	1.16g
Potassium dihydrogen phosphate	0.2g
Distilled water upto to	1000 ml

Phosphate Buffer Saline Tween-20 (PBST)

PBS	100 ml
Tween-20	0.5 ml

TAE (50X)

Tris base	242g
Glacial Acetic acid	57ml
0.5M EDTA (pH8)	100ml
Distilled water up to	1000 ml

Transfer buffer, pH 8.3

Tris Base	3.03 g
Glycine	14.4 g
Methanol	200 ml

10% SDS	10 ml
Distilled water up to	1000 ml

TBS (0.02 M Tris with 0.15 M Nacl, pH 7.4)

Tris	2.42 g
NaCl	8.77g
Distilled water up to	900 ml

The pH was adjusted to 7.4 with concentrated HCL and the volume was finally made up to 1000 ml with distilled water.

TBS-T (TBS with 0.05 % Tween 20)

TBS	1000 ml
Tween- 20	0.5 ml

30% Acrylamide:Bisacrylamide solution

Acrylamide	29g
N,N'-bis- methylene-acrylamide	1g
Distilled water upto	100ml

(Filter the solution through Whatman filter paper 1 and store in amber colour bottle at 4^{o}C)

10% APS

APS	0.2 g
Distilled water upto	2 ml

10% SDS

SDS	10 g
Distilled water upto	100 ml

70% Ethanol

Absolute ethanol	70 ml
Double distilled water	30 ml

100 mM Carbonate-bicarbonate buffer (pH 9.6)

Na_2CO_3	3.03 g
$NaHCO_3$	6 g
Distilled Water	1000 ml

1.5 M Tris-HCl (pH 8.8)

Tris base	36.342 g
Distilled water	120ml
(Adjust to pH 8.8)	
Add distilled water upto	200ml

0.5 M Tri HCl (pH 6.8)

Tris. HCl	78.8 g
Distilled water upto	1000 ml
(Adjust pH 6.8)	

0.5 M EDTA (pH 8.0)

EDTA. $2H_2O$	186.1 g
Distilled water upto	1000 ml
(Adjust pH 8.0)	

10X SDS-PAGE Running Buffer

Tris	30.3 g
Glycine	144 g
SDS	10 g
Distilled water up to	1000 ml

VITAE

Name	:	**Lukumoni Buragohain**
Date of birth	:	05th February, 1988
Gender	:	Male
Mother's name	:	Mrs. Bimala Buragohain
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Academic qualification

S No.	Institution	Degree Awarded	OGPA	Year
1.	College of Veterinary Science, AsaamAgricultural University, Guwahati- 781022	B.V.Sc & A.H	78.20	2012
2.	Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana-125004, India	M.V.Sc in Animal Biotechnology	8.34	2014

Honors/Awards

1. Qualified DBT-JRF in Biotechnology
2. Qualified ICMR-JRF in Life Science
3. Qualified ICAR-NET in Animal Biotechnology
4. Qualified CSIR-NET in Life Science
5. Qualified UGC-NET in Social Medicine & Community Health
6. Qualified ICAR-SRF in Animal Biotechnology with 2nd All India Ranking
7. Best Paper awarded on "Expression profile of MX2 gene and protein for early pregnancy diagnosis in murrah buffalo" in the Annual Convention and National Symposium of SVBBI held at OUAT, Bhubaneswar on 11th & 12th March, 2016
8. C certificate in NCC with A grade.