CHARACTERIZATION OF RECOMBINANT CAPSID PROTEIN OF PORCINE CIRCOVIRUS-2 (PCV-2) AS POTENTIAL DIAGNOSTIC ANTIGEN

Thesis

Submitted to the Guru Angad Dev Veterinary and Animal Sciences University in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE
in
ANIMAL BIOTECHNOLOGY
(Minor Subject: Veterinary Microbiology)

By

Rupinder Kaur
(L-2013-ABT-07-M)

School of Animal Biotechnology
Post Graduate Institute of Veterinary Education and Research

Ludhiana – 141 004

2015

CERTIFICATE – I

This is to certify that the thesis entitled, "**CHARACTERIZATION OF RECOMBINANT CAPSID PROTEIN OF PORCINE CIRCOVIRUS-2 (PCV-2) AS POTENTIAL DIAGNOSTIC ANTIGEN"** submitted for the degree of **M.Sc.,** in the subject of **Animal Biotechnology** (Minor subject: Veterinary Microbiology) of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, is a bonafide research work carried out by **Rupinder Kaur (L-2013-ABT-07-M)** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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(Dr. Dipak Deka)
Major Advisor
Assistant scientist
School of Animal Biotechnology
GADVASU, Ludhiana-141004

CERTIFICATE – II

This is to certify that the thesis entitled, "CHARACTERIZATION OF RECOMBINANT CAPSID PROTEIN OF PORCINE CIRCOVIRUS-2 (PCV-2) AS POTENTIAL DIAGNOSTIC ANTIGEN" **submitted by** Rupinder Kaur (L-2013-ABT-07-M) **to the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, in partial fulfillment of the requirements for the degree of** M.Sc., **in the subject of** Animal Biotechnology **(Minor subject: Veterinary Microbiology) has been approved by the Student's Advisory Committee along with Director of the School after an oral examination on the same, in collaboration with the external examiner.**

_____
(Dr. Dipak Deka)
Major Advisor
Assistant Scientist
School of Animal Biotechnology

_____
(Dr. Baldev Raj Gulati)
External Examiner
Principal Scientist,
National Research Centre on Equines, Hisar

_____
(Dr. Ramneek)
Director
School of Animal Biotechnology

_____
(Dr. Simrat Sagar Singh)
Dean, Postgraduate Studies

ACKNOWLEDGEMENT

On the accomplishment of the present study, I would like to take this opportunity to extend my deepest sense of gratitude and words of appreciation towards those, who dedicated their today for my tomorrow.

First and foremost, I express my deepest gratitude to ***GOD*** *for he has been with me from the beginning and for He shall be till the end.*

I take the privilege in expressing my deep sense of gratitude and indebtedness to my Major advisor ***Dr. Dipak Deka****, Assistant Scientist, School of Animal Biotechnology for his invaluable and critical suggestions, scientific acumen, perspicacious remarks, scholarly guidance, active persuasion and supervision, excellent unstinted help at every step, which served as a constant source of inspiration throughout the course of my study and research work.*

I feel indebted and convey my regards to ***Dr. Ramneek Verma,*** *Director, School of Animal Biotechnology (Dean PGS Nominee), for being a source of encouragement and inspiration at every step. I would also thank him for providing the best infrastructure and the facilities for my research work.*

I extend sincere thanks to the members of Advisory Committee ***Dr. Satprakash Singh, Assistant Professor,*** *School of Animal Biotechnology &* ***Dr. T.S.Rai,*** *Professor, Deptt. of Veterinary Microbiology, GADVASU, for their even willing help, edifying, criticism and conductive advice throughout the course of study. Other staff members of my department* ***Dr. G.S. Brah, Dr. B.V. Sunil Kumar, Dr. C.S. Mukhopadhyay, Dr. Jaspreet Arora, Dr. R.S. Sethi, Dr. Neeraj K. Singh*** *also deserve my sincerest thanks, their advices and assistance has meant more to me than I could ever express.*

The best outcome from these past two years is finding a friend ***Harpreet Kaur*** *who has been a true and great supporter and co-Partner in the research work and has unconditionally loved me during my good and bad times. My dearest comrades and my friends in the department****, Jaspal Kaur, Jaspreet Kaur, Harmanjot Kaur,Gagandeep Kaur, Navneet Kaur*** *were constant sources of laughter, joy, and support. You all have been like my family, bearing the brunt of the frustrations because of me, and sharing my happiness. I am very happy that, in many cases, my friendships with you have extended well beyond our shared time in Ludhiana.*

I would thank my best friends ***Maninder, Ramandeep, Preeni, Kawal, Gagan, Pawan, Amrinder and Aman*** *who shared my passions, thank you for rekindling my dreams and thank you for being a part of my life from the last six years and for lifetime.*

I extend my thanks to my seniors and juniors ***Drs. Swati, Sonal, Mamta, Jasdeep, Geetika, Sandeep, Sarabjot, Gagan, Rabia, Aman, Manmeet, Mohan, Amrit, Upkar, Rajinder*** *and* ***Chirag*** *and* ***Sadhika*** *who shared their knowledge with me during my research.*

I should thank the non-teaching staff ***Mrs Harleen, Anita and Sunil*** *for their time and availability. I also endorse the assistance of* ***Harman, Ashish, Rajat, Anuj, Deepak, Prince*** *and* ***Jugnu*** *in laboratory.*

I would not have contemplated this road without my parents, ***Nirmal Singh and Narinder Kaur,*** *my brother* ***Simarjeet Singh****, my sister in- law* ***Rupinder Kaur*** *who instilled within me a love of creative pursuits, science and language, all of which finds a place in this thesis. To my parents, thank you. I owe everything to them.*

Last but not the least I am glad to have one person in my life, my nephew, ***Harkirat Singh,*** *spending time with whom I forgot all my tension and worries.*

I would really thank you all for being a part of my life and for helping me in my tough times.

All may not be mentioned but none is forgotten.

Place:

Date: ***(Rupinder Kaur)***

Title of Thesis	:	**CHARACTERIZATION OF RECOMBINANT CAPSID PROTEIN OF PORCINE CIRCOVIRUS-2 (PCV-2) AS POTENTIAL DIAGNOSTIC ANTIGEN**
Name of Student	:	Rupinder Kaur
Admission No.	:	L-2013-ABT-07-M
Major Subject	:	Animal Biotechnology
Minor Subject	:	Veterinary Microbiology
Name and Designation of Major Advisor	:	Dr. Dipak Deka Assistant Scientist, SABT
Degree to be awarded	:	M.Sc
Year of Award of Degree	:	2015
Total Pages of thesis	:	85 + VITA
Name of University	:	Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana- 141004 (Punjab) India.

ABSTRACT

Porcine circovirus- 2 (PCV-2) is the primary causative agent of porcine circovirus associated disease (PCVAD), which has been a cause of considerable economic losses to the pig farmers. The capsid protein of PCV-2 contains high level of conserved epitopes and induces a strong immune response against sera from PCV-2 positive animals and thus it can be used as a potential diagnostic antigen for detection of PCV-2 infection. Therefore, this study was planned with the objective to characterize recombinant capsid protein of PCV-2 as potential diagnostic antigen. For this, a full length codon optimized PCV-2 *ORF-2* capsid gene (~702 bp) was successfully expressed in the bacterial expression system using pET302 NT His vector with a protein size of ~28 kDa which was purified using Ni-NTA affinity chromatography in denaturing conditions. The purified capsid protein showed good reactivity with the PCV-2 antisera as well as the anti-His tag antibody in western blot & dot blot. The purified protein in denaturing conditions was refolded using refolding buffer and concentrated following dialysis. The refolded capsid protein used as coating antigen in an indirect ELISA showed good reactivity against the PCV-2 specific antibodies. The codon optimized PCV-2 *ORF-2* gene (~699 bp) was also successfully expressed in baculovirus expression system with a protein size of ~38 kDa. The purified capsid protein showed good reactivity with anti-His tag protein antibody in dot blot. These results clearly showed the potential of expressed capsid protein as diagnostic antigen for serodiagnosis of PCV-2.

Key words: *Postweaning mutisystemic wasting syndrome , ORF-2, Western blotting, Dot blot, Indirect ELISA*

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Signature of Major Advisor **Signature of Student**

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ABBREVIATIONS

%	: per cent
µg	: microgram
µl	: microlitre
µm	: micro molar
aa	: amino acid
ATCC	: Association of swine veterinarians
AASV	: American type culture collection
bp	: base pair
BSA	: Bovine serum albumin
Conc.	: Concentration
CP	: Capsid protein
CsCl	: Cesium chloride
DAB	: Diaminobenzidenetetrahydrochloride
DDW	: Double distilled water
DNA	: Deoxyribonucleic acid
dNtp	: Deoxyribonucleotide triphosphate
DSN	: Diagnostic sensitivity
DSP	: Diagnostic specificity
E.coli	: *Escherichia coli*
EDTA	: Ethylene diamine tetra acetic acid
ELISA	: Enzyme linked immunosorbent assay
g	: gyrations
GAGs	: Glycosaminoglycans
gm	: gram
HRPO	: Horse radish peroxidase
hrs	: hours
IHC	: Immuno histochemistry
IIFA	: Indirect immunofluorescent assay
IIP	: Indirect Immunoperoxidase test
IPMA	: Immunoperoxidase monolayer assay
IPTG	: Isopropyl β- D -1-thiogalactopyranoside
ISH	: *In situ* hybridization
kbp	: kilo base pairs
kDa	: kilo Dalton

LB	: Luria Bertani
M	: molar
mA	: milli ampere
Mab	: Monoclonal antibody
MCT	: microcentrifuge tubes
mg	: milligram
ml	: millilitre
mm	: milli molar
M	: molar
NABARD	:National bank for agriculture and rural development
NCM	: Nitrocellulose membrane
NFW	: Nuclease free water
NLS	: Nuclear localization signal
Nt	: Nucleotides
Ni-NTA	: Nickel nitrilotriacetic acid
NTR	: Non translated region
ºC	: degree Celsius
OD	: Optical density
Orf	: Open reading frame
PAGE	: Polyacrylamide gel electrophoresis
PBS	: Phosphate buffered saline
PCR	: Polymerase chain reaction
PCV	: Porcine circovirus
PCVAD	:Porcine circovirus associated disease
PCVD	: Porcine circovirus disease
PDNS	: Porcine dermatitis and neuropathy syndrome
PEG	: Poly ethylene glycol
Pfx	: Platinum DNA polymerase
pH	: Hydrogen ion concentration
P.I.	: Post induction
PK	: Porcine Kidney
pmol	: pico mole
PMWS	:Postweaning multisystemic wasting syndrome
PPV	: Porcine parvo virus
PRRS	: Porcine reproductive and respiratory syndrome

PVDF	:Polyvinylidene fluoride
RCR	: Rolling circle replication
RE	:Restriction endonuclease
RF	:Replicative form
RNA	: Ribonucleic acid
rpm	: revolutions per minute
ROC	: Receiver operating characteristic
s	: second
S	: Sedimentation coefficient
SDS	: Sodium dodecylsulphate
TAE	: Tris acetate EDTA
TCA	:Trichloroacetic acid
Taq	: *ThermusAquaticus*
TE	: Tris EDTA
U	: Unit
UV	: Ultraviolet ray
V	: Volt
W	:Watt

CHAPTER I

INTRODUCTION

Amongst the different livestock species, pigs are considered as one of the best meat producing animal and efficient feed converters after the broiler chickens. The contribution of pork products in terms of value, works out to be 7% of the total animal protein (NABARD, 2012). As the production and consumption of pork is increasing significantly particularly in the North Eastern states, it has become important to control the economically important viral diseases which lead to reproductive failure in domestic pigs like the diseases associated with Porcine circovirus-2 infection, porcine reproductive and respiratory syndrome (PRRS) etc.

The disease syndrome associated with PCV-2 infection was first described as Postweaning multisystemic wasting syndrome (PMWS) based on the characterstic clinical signs of wasting, reduced weight gain, jaundice, respiratory complications, hepatitis, nephritis and lymphoid depletion in 8-15 week old piglets (Clark 1996; Harding and Clark 1997; Ellis *et al* 1998). PMWS is the disease of growing pigs, causing significant economic losses to the swine industry with high mortality and low morbidity. Due to the manifestation of other syndromes associated with PCV-2 infection such as reproductive disorders, enteric signs and porcine dermatitis and neuropathy syndrome (PDNS), PMWS was renamed as porcine circovirus-associated disease (PCVAD) in the U.S.A. and porcine circovirus disease (PCVD) in Europe (Opriessnig *et al* 2007).

PCV isolated from porcine kidney cell line as a persistant contaminant is non pathogenic and designated as PCV-1 and the pathogenic form of PCV associated with PMWS is designated as PCV-2. PCV-2 has been grouped into 2 distinct genotypes named as PCV-2 group-1 and PCV-2 group-2. Porcine circoviruses (PCV) are small non-enveloped DNA viruses containing a unique single-stranded circular genome of 1.7 kb size. Although the 2 genotypes differ in size with PCV-2 group 1 being 1,767 nucleotides and PCV-2 group 2 being 1,768 nucleotides, there is no difference in their pathogenesis. These viruses belong to the family *circoviridae* and the genus circovirus. PCV-2 has two potential open reading frames, ORF-1 and ORF-2, greater than 600 nucleotides in size. PCV-2 requires a capsid protein encoded by ORF-2 situated on the viral strand of the genome and two replicase proteins encoded by

ORF-1 in order to replicate and produce a functional virus. Rep and Rep' proteins join and form a complex that is involved in replication of the viral genome (Steinfeldt *et al* 2001). The ORF-2 of PCV-2 plays an important role in PCV-2 related diseases (Larochelle *et al* 2002). A third ORF has been described in PCV-2 which is involved in apoptosis.

The immune response to viral diseases is largely directed against the structural proteins of the virus which are present in the virion envelope and on the surface of the infected cells. The capsid protein (Cap) contains a high level of conserved epitopes and induces a strong immune reaction against sera from PCV-2 positive animals (Blanchard *et al* 2003). If any of these proteins can be produced by recombinant methods it can be used as a diagnostic antigen instead of whole viral antigen.

Diagnosis of PCV-2 infection is currently based upon immunohistochemistry and *in situ* hybridization (Choi *et al* 2000) in porcine lymphoid tissues. Immunoperoxidase monolayer assay (IPMA) (Allan *et al* 1999) and indirect immunofluorescent assay (IIFA) (Allan *et al* 1999) are the most widely used diagnostic methods for detecting PCV infection.

Enzyme linked immunosorbent assay (ELISA) based diagnostic assays are being used commonly for rapid seroprevalence studies against several diseases including PCV. The main disadvantage of these assays is the requirement of viral antigen in bulk. Large scale production of infectious virus (i.e. coating antigen) in tissue culture system is not only costly but also involves an additional risk of spreading the disease to susceptible animals. Earlier, whole viral antigen was used in ELISA as diagnostic antigen. Lately, recombinant proteins are being used as coating antigen as a replacement for whole virus. In recent years recombinant protein based diagnostic ELISAs are being developed as large scale production of viral protein is possible in a safe and cost effective manner.

Several types of ELISA procedures for diagnosis of PMWS have been developed thus gradually replacing IIFA (indirect immunofluorescent assay) or indirect immunoperoxidase (IIP) test for detection of antibody against PCV-2. Jittimanae *et al* 2012 developed in-house indirect enzyme linked immunosorbent assay using a recombinant nuclear localization signal truncated capsid (rntcap) protein of PCV-2, expressed in an *Escherichia coli* system and determined the diagnostic performance of the developed ELISA in comparison to the immunoperoxidase monolayer assays (IPMAs).

Several ELISA techniques for antibody detection have been described such as using antigenic domain of capsid protein (ORF-2) of PCV-2 (Sun *et al* 2010) and blocking ELISA for detection of serum neutralizing antibodies against PCV-2 (Huang *et al* 2011). Currently, whatever ELISA based diagnostic kits available in India are imported and costly thus hampering the sero-surveillance of the PMWS in India.

Keeping these points in view, the present study was planned with the following objectives:

1) Cloning and expression of complete *ORF-2* (capsid) gene of PCV-2 into bacterial/ baculovirus expression vector.
2) Characterization of the recombinant PCV-2 capsid protein as potential diagnostic antigen.

CHAPTER II

REVIEW OF LITERATURE

2.1 HISTORY

Porcine circovirus was first detected by Tischer in 1974 as a persistant cell culture contaminant of porcine kidney cell line PK-15 (ATCC-CCL33). In order to identify the contaminant, purified supernatant from cell culture was analyzed by electron microscopy which revealed viral particles with picornavirus like morphology. Biochemical and serological assays showed the virus had a circular ssDNA genome and identified pigs as the host of the virus. Subsequently, the novel virus was termed porcine circovirus (PCV) (Tischer *et al* 1982), which was later named as Porcine circovirus type-1 (PCV-1). Many of the serological surveys demonstrated the high prevelance of anti-PCV antibodies in the swine population worldwide (Tischer *et al* 1982; Tischer *et al* 1986; Dulac and Afshar 1989; Horner 1991; Allan *et al* 1994; Edwards and Sands 1994; Tischer *et al* 1995), but there was no disease association found with this virus in the PCV positive pig farms and experimental PCV-inoculated pigs (Tischer *et al* 1986; Allan *et al* 1995). So, at that time it was accepted that PCV-1 is the non-pathogenic form of this virus.

In 1991, Postweaning multisystemic wasting syndrome (PMWS), a newly emerging disease in pigs was first reported in Western Canada (Clark 1996; Harding 1996). Later on, a new DNA-virus, morphologically similar to the PK-15 origin PCV, was isolated from the tissues of PMWS-affected pigs in Europe and North America (LeCann *et al* 1997; Nayar *et al* 1997; Segales *et al* 1997; Allan *et al* 1998; Ellis *et al* 1998; Harding *et al* 1998; Kennedy *et al* 1998; Kiupel *et al* 1998), which was later named as Porcine circovirus type-2 (PCV-2).

PCV-2 is the important pathogen in dermatitis, and nephropathy syndrome (PDNS) (Rosell *et al* 2000), porcine respiratory disease complex (PRDC) (Kim *et al* 2003), and exudative epidermitis (Wattrang *et al* 2002). As more and more disease conditions such as reproductive disorders, enteric diseases, and respiratory signs were linked to PCV-2 infection, the disease was renamed porcine circovirus associated disease (PCVAD) in the United States and porcine circovirus disease (PCVD) in Europe.

Evidence of PCV-2 infection was traced back retrospectively to as early as 1973 in Northern Ireland (Walker *et al* 2000). Among the three distinct PCV-2 genotypes identified from pigs worldwide (Trible and Bowland 2012), PCV-2a and PCV-2b are associated with PCVAD with varying degrees of severity (Opreissnig *et al* 2007; Beach and Meng 2012), whereas PCV-2c was identified only from sporadic healthy swine herds in Denmark (Dupont *et al* 2008).

2.2 SEROPREVALENCE OF PORCINE CIRCOVIRUS

As PMWS cause the major economical as well as social damages in the pig industry, numerous studies were done to screen the disease prevalence in the swine population.

PCV-2 is widely distributed throughout the pig populations of Australia and New Zealand. Serological surveys based on the 2001/2002 Australian National Pig Serum Bank have demonstrated seroprevalence levels ranging from 75.8% to 86.9% (Finlason *et al* 2007) indicating the presence of PCV-2 in most, if not all pig herds.

In Europe, during epidemic stage from 1996 to 2004, on farm morbidity rates as high as 50 - 60% and post weaning mortality between 4-20% were reported (Madec *et al* 2000; Segales and Domingo 2002). In a recently completed study carried out in School of Animal Biotechnology, GADVASU, Ludhiana, the seroprevalence of porcine circovirus was found to be 26.18% in the North-Eastern states and in 25.96% in 9 districts of Punjab (Unpublished data).

The rates of seroprevalence of PCV-2 in affected countries like Canada (Magar *et al* 2000b), Spain (Rodriguez-Arrioja *et al* 2002), Taiwan (Wang *et al* 2004) and Australia (Finlaison *et al* 2007) have varied between 40 and 80%. However, the prevalence of PCV-2 as shown by the presence of PCV-2 antigen or viral DNA in tissue ranged from about 23% in Japan (Kawashima *et al* 2007), 50% in Taiwan (Wang *et al* 2004), 8% in Korea (Kim and Lyoo 2002).

Liu *et al* 2002 determined extent of exposure of normal pigs in Canada and Costa Rica to PCV-2. They used recombinant DNA techniques to produce an antigen from ORF-2 of PCV-2 that was suitable for the detection of antibody in swine sera. The presence of PCV-2 nucleotide sequences was detected using polymerase chain reaction (PCR) techniques. Using these tests, specific antibody and nucleotide sequences were demonstrated in sera from a cohort of pigs during a PMWS outbreak.

Antibody was detected in normal, healthy hogs slaughtered in Canada (82.4% of 386) and in Costa Rica (14.6% of 322).

Farnham *et al* 2003, examined the presence of PCV-2 in the stillborn fetuses of pigs. They collected sera from 171 still born fetuses and tested for the presence of PCV-2 antibodies using immunoperoxidase monolayer assay (IPMA) and out of 171 samples, 28 samples had antibody titres. Out of 28 samples, 13 samples had PCV-2 antibodies and viral DNA when tested by PCR assay. The amino acid sequences of the two isolates from stillborn pigs were shown to be nearly identical to each other, as well as to other PCV-2 isolates associated with reproductive failure.

Yang *et al* 2008, carried out a survey to understand the seroprevalence of Porcine circovirus type 2 in Hangzhou, Zheziang Province, China. The pig serum samples were analysed by enzyme linked immunosorbent assay (ELISA) for PCV-2 antibody and by PCR for *ORF-2* gene. Out of 1250 randomly collected serum samples, 500 (40%) were seropositive for PCV-2. The positive rate in 1- to 50-day-old pigs was 40%, 63% for those at 50- to 160-day-old, and 31.5% for boars and sows.

Wong *et al* 2008 studied the seroprevalence of PCV-2 in swine population of Tobago and Trinidad by employing multistage sampling design with non-proportional sampling between strata. A total of 274 samples were tested using PCV-2 indirect fluorescent antibody test kit by VMRD (USA). The overall seroprevalence of PCV-2 was 62.0%.

Mendoza *et al* 2009 carried a serological survey with 659 serum samples from pigs in Mexico between 1972 and 2000 and analyses was done by immunoperoxidase monolayer assay (IPMA). The overall prevalence of PCV-2 antibodies was 59%. This study showed evidence of enzootic PCV-2 infection in Mexico.

Csank *et al* 2011 determined prevalence of PCV-2 infection in the pig population in Slovakia. They tested sera from pigs suspected for post-weaning multisystemic wasting syndrome (PMWS) as well as clinically healthy pigs for viral DNA and specific IgM and IgG antibodies. Pigs were categorized to weaning, grower and fattening ones and sows. The results showed that PCV-2 antibodies were present in 53.4% of PMWS-suspects, in 50.0% of healthy pigs and in 69.0% of sows. In PMWS-suspect grower pigs, 40.7% were positive for IgM+IgG antibodies and 22.2%

for viral DNA. In PMWS-suspect fattening pigs, 50.0% were positive for IgM+IgG antibodies and 25.0% for viral DNA. In healthy fattening pigs, almost 90.0% were positive for IgG antibodies and 38.5% for viral DNA. The highest proportion of PMWS-suspects was in grower pigs and specific antibodies were increasing with the age of pigs.

In a study conducted by Monger and colleagues in 2014 in Bhutan, to determine the prevalence of antibodies to the viruses associated with pig diseases, the antibodies to the PCV-2 were found to be 73% in the government farms and 37% in the village backyard pigs. The serum samples were tested in acommercially available indirect ELISA Kit (Porcine Circovirus-2 Antibody Test Kit; BioChek BV, The Netherlands) (Monger *et al* 2014)

In a study conducted by Pearodwong *et al* in 2015 to determine the prevalence of PCV-2 in the uterine and ovarian tissues in gilts due to reproductive disturbance in Thailand, PCV-2 DNA was detected by PCR. The localization of the PCV-2 antigen was determined by immunohistochemistry (IHC) and the PCV-2 antibody was determined by ELISA. They concluded that the detection of PCV-2 in the reproductive organs reveals an important potential impact of this virus on the reproductive apparatus in gilts.

2.3 ECONOMIC LOSSES DUE TO PORCINE CIRCOVIRUS TYPE-2

Postweaning multisystemic wasting syndrome (PMWS) or PCVAD (Porcine circovirus associated disease) is a globally emergent epizootic disease of swine mainly caused by PCV-2 infection.

According to Darin Madson, DVM, Iowa State University Veterinary Diagnostic Laboratory, PCV-2 is one of the top three economically important swine diseases behind PRRSv and *Mycoplasma pneumonia*. If pigs are left unvaccinated, producers could see up to a $20 loss per pig, which equates to losses that could exceed $2 billion in the U.S. alone (Gillespie *et al* 2009).

The disease has a major economic, public health, and animal welfare impacts on the pig meat industry. The economic cost of PMWS across the European Union (EU) is estimated at between €562-900M, extrapolated from data for the Netherlands where the disease causes a reduction in the availability of pigs for slaughter by 4% p.a. (data supplied by the MLC). PMWS-associated immunosuppression results in

increased carcase contamination by food-borne pathogens (e.g. *Salmonella typhimurium, Campylobacter jejuni*, *Yersinia enterocolitica*), arising from increased prevalence of infection on-farm (Segales *et al* 2004) and increased shedding at slaughter. A second public health concern is increased use of antimicrobial agents in the attempted control of PMWS-associated disease, with consequently increased potential for selecting for antimicrobial resistant bacteria and drug residues in pig meat.

Factors underlying the economic losses include fewer pigs at slaughter, reduced feed conversion rates, increased costs for management and medication of sick pigs and costs of secondary diseases following PMWS-associated immunosuppression (Segales *et al* 2004). It was estimated in 2001 that costs on a typical UK farm were £10.39 per pig sold, or equivalent to an increase in the cost of production of £0.01 per kilogram of pig meat sold for each extra 1% mortality over previous losses (e.g. for a farm losing an extra 17% of pigs post-weaning the cost of production increases by £0.17 per kilogram) (Richardson P 2001).

2.4 TAXONOMY OF PORCINE CIRCO VIRUS

PCV-1 and PCV-2 belong to the family circoviridae (Allan and Ellis 2000; Ellis *et al* 2004; Segales *et al* 2005; Oppriessnig *et al* 2007) and the genus *circovirus* (Todd *et al* 2005). Other viruses belonging to this genus include canary circovirus, goose circovirus, pigeon circovirus and psittacine beak and feather disease virus. Viruses belonging to this family are small, non-enveloped, single stranded DNA viruses that effects birds, swine and other mammals.

Circoviruses are mainly host specific and have a narrow host range. PCV-1 is closely related to the beak and feather disease virus (Niagro *et al* 1998). Circoviridae family is most closely related to the family Nanoviridae, which includes the plant viruses. These viruses show similarities in the replication proteins and share a stem loop like structure at the origin of replication.

PCV-2 has been grouped into 2 distinct genotypes named as PCV-2 group-1 and PCV-2 group-2. Although the 2 genotypes differ in size with PCV-2- group 1 being 1,767 nucleotides and PCV-2-group 2 being 1,768 nucleotides, there is no difference in their pathogenesis. PCV-2 group-2 isolates were found in the United States, but in the late 2005 several outbreaks of higher than normal mortality (5-50%)

were reported in Kansas, North Carolina and Iowa which were found to be associated with PCV-2-group 1 isolates (Cheung *et al* 2007). At the same time, North American laboratories proposed grouping PCV-2 into North American isolates or PCV-2a and European like isolates or PCV-2b (Gagnon *et al* 2007). PCV-2b falls into PCV-2-group1 and PCV-2a falls into PCV-2-group 2 (Gagnon *et al* 2007).

2.5 GENOME ORGANISATION OF PORCINE CIRCOVIRUS

Porcine circoviruses are the non- enveloped and the smallest animal viruses with a diameter of 17 nm (Tischer *et al* 1982) and contain a covalently closed circular ssDNA genome. The virion has a icosahedral symmetry containing 60 capsid protein subunits arranged in 12 flat pentamerclustered units (Crowther *et al* 2003; Khayat *et al* 2011). The circular PCV genomeconsists of 1,758-1,760 nucleotides for PCV-1 and 1,766-1,769 nucleotides for PCV-2 (Fig. 1) (Allan *et al* 1998; Hamel *et al* 1998; Meehan *et al* 1998; Morozov *et al* 1998; Fenaux *et al* 2000; Huang *et al* 2011). PCV-1 and PCV-2 share 76% nucleotide sequence homology. Both PCV-1 and PCV-2 have a similar genomic organisation, which contains three major open reading frames (Orfs): ORF-1, ORF-2 and ORF-3.

ORF-1 is located on the positive strand of the viral DNA genome, and is oriented in clockwise direction (Fig 1). It encodes for the non-structural proteins Rep (35.7 kDa) and its alternatively spliced frame-shifted variant Rep' (20 kDa), which are involved in virus replication (Mankertz *et al* 1998; Mankertz and Hillenbrand 2001; Cheung 2003a; Mankertz *et al* 2003; Shang *et al* 2009). PCV-1 Rep and Rep' proteins consist of 312 and 168 amino acids (aa), respectively and PCV-2 Rep and Rep' proteins consist of 314 and 178 aa, respectively (Hamel *et al* 1998). ORF-1 is more conserved between PCV-1 and PCV-2 (Mankertz *et al* 2004; Steinfeldt *et al* 2007), with 83% nucleotide identity and 86% amino acid identity (Meehan *et al* 1998; Morozov *et al* 1998). Mutations in Rep or truncated Rep' proteins of PCV causes more than 99% reduction in viral protein synthesis and complete shut down of viral DNA replication (Cheung 2003b; Cheung 2004).

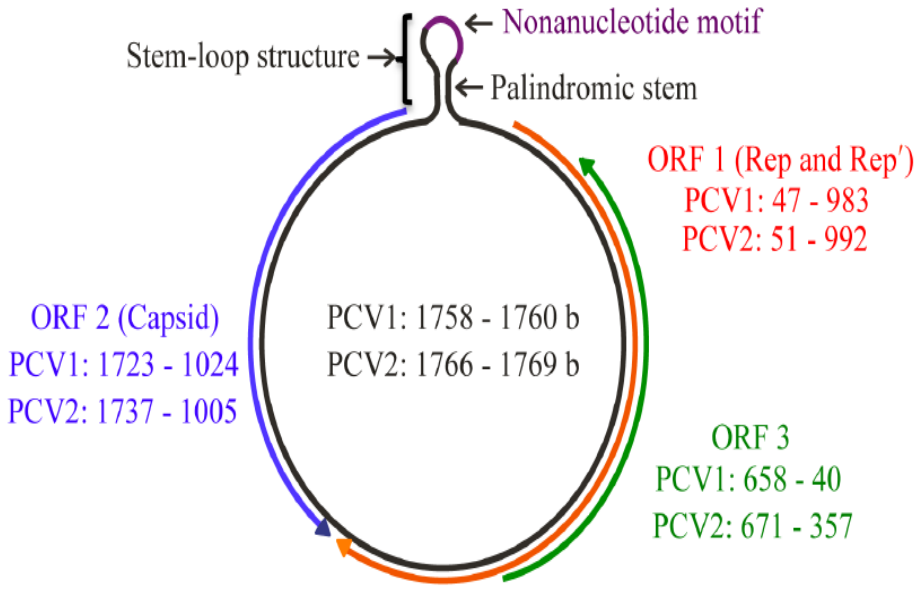


Fig. 1: Genome organization of PCVs. Red arrow-Open reading frame 1 (ORF-1), clockwise oriented and located on the positive strand, which encodes Rep and Rep' proteins. Blue arrow- Open reading frame -2 (ORF-2), counter clockwise oriented, located on the negative strand, which encodes the capsid protein. Green arrow- Open reading frame -3 (ORF-3), counter clockwise oriented, located on the negative strand, which overlaps the ORF-1. The figure was adapted from G. Misinzo,PhD thesis, Ghent University.

ORF-2 is located on the negative strand of the viral genome and is oriented in counter-clockwise direction (Fig 1), and encodes a structural capsid protein (Cap) (27.8 kDa) (Hamel *et al* 1998; Meehan *et al* 1998; Morozov *et al* 1998; Mahe *et al* 2000; Mankertz *et al* 2000; Nawagitgul *et al* 2000; Truong *et al* 2001). The capsid protein consists of 230-233 aa for PCV-1 and 233-236 aa for PCV-2 (Hamel *et al* 1998; Nawagitgul *et al* 2000; Lefebvre *et al* 2008a; Lefebvre *et al* 2009; Guo *et al* 2010; Huang *et al* 2011). ORF-2 is more variable between PCV-1 and PCV-2 with approximately 67% and 65% identity at nucleotide and amino acid levels, respectively (Hamel *et al* 1998; Meehan *et al*1998; Morozov *et al* 1998; Grierson *et al* 2004a; Knell *et al* 2005).

ORF-3 is completely overlapped with ORF-1 and located on the complementary strand with counter-clockwise orientation (Fig 1) (Hamel *et al* 1998; Meehan *et al* 1998; Morozov *et al* 1998). It encodes a non-structural protein of 206 aa

(23.2 kDa) for PCV-1 and 104 aa (11.9 kDa) for PCV-2. ORF-3 proteins have 62% identity between PCV-1 and PCV-2 (Hamel *et al* 1998 and Meehan *et al* 1998). PCV-2 ORF-3 has been shown to be involved with apoptosis *in vitro* and viral pathogenesis *in vivo* (Liu *et al* 2005; Liu *et al* 2006). PCV-1 ORF-3 has been shown to be more toxic to different cell types and induced more apoptosis than PCV-2 ORF-3 (Chaiyakul *et al* 2010).

2.6 REPLICATION CYCLE OF THE VIRUS

The mechanism involved in attachment and internalization of porcine circoviruses into their host's cells is unknown. The cellular receptors that bind porcine circoviruses initiate the replication cycle of the virus, remain to be discovered. PCV-2 uses a common cell receptor, because viral replication and PCV-2 antigen has been found in many different cell types (Darwich *et al* 2004). As a first step of attachment, PCV-2 binds to heparin sulfate and chondriotin sulfate, which are glycosaamino-glycans (GAG's). Another receptor is also used for viral entry as PCV-2 is also found in cells lacking GAG's. Replication of the circovirus genome once it is inside the cell is believed to occur through a mechanism known as the rolling cycle replication (Meehan *et al* 1997). Two types of Rolling cycle replication (RCR) mechanisms has been proposed, first being the 'cruciform' mechanism, which describes the replication of the viral genome from single 'leading strand', and the second is the 'melting pot' mechanism which describes the replication of the viral genome from both the leading and the lagging strand.

2.6.1 The Cruciform RCR mechanism

Once the PCV has infected a cell, the ssDNA genome of the virus is converted by host enzymes into a supercoiled dsDNA replicative form (RF). Upon formation of the RF, the PCV replication proteins, Rep and Rep', form a replication complex (RC) that binds to the origin of DNA replication. Binding of the RC destabilizes and unwinds the dsDNA at the origin which leads to the exposure of the nonamer sequence as ssDNA and the formation of a cruciform. The exposed nonamer sequence is then recognized and cleaved by the RC. Cleavage of the ssDNA nonamer is dependent upon the three conserved RCR motifs located within Rep and Rep'. Motif I is unclear, serves as a catalyst, Motif II is required for coordination of divalent metal cations, which are required for nicking the viral DNA for unwinding, Motif III

contains a tyrosine which performs the cleavage of the phosphodiester bond by nucleophilic attack. Cleavage by tyrosine causes the RC to be covalently attached to the 5' end of the viral genome and generates a 3'-hydroxyl that serves as a primer for DNA synthesis by the host DNA polymerase. Upon completion of a single genome, termination occurs when the newly formed leading strand displaces the positive sense coding strand and the RC covalently attaches the 5' and 3' ends of the genome, forming a circle. The positive circular ss parental DNA is then released leaving a ds-circular DNA molecule composed of the negative parental strand and the newly synthesized positive strand. At this point, the newly synthesized ssDNA molecule can either be encapsidated or be involved in a second round of replication (Faurez et al 2009).

2.6.2 The melting pot RCR mechanism

This mechanism is exactly same as the cruciform RCR mechanism until the binding of the RC and formation of the cruciform. Instead of forming a cruciform, all four strands of the inverted repeats are in a melted state with no hydrogen bonding between the plus and minus strands. However the strands remain in close proximity and are positioned in a four-stranded tertiary structure. Upon nicking of the nonamer by the RC, elongation proceeds into the palindromic region of the melting pot (through the right arm of the stem loop). Due to the positioning of the strands in the melting pot, both the complementary strand and the palindromic strand are available as templates. Upon completion of a single round of genomic replication, termination occurs when the leading strand ascends into the melting pot (along the left arm of the stem loop) and displaces the old strand. At this point in replication, both the newly synthesized strand and the complementary strand are available as templates. After this, events involving closure and release of the ssDNA viral genome are similar to the "cruciform" model.

2.7 INFECTION AND VIRUS LIFE CYCLE

The primary route of entry of PCV-2 into the host is the oro-nasal route (Bolin *et al* 2001 and Yu *et al* 2007). Upon entering the host, PCV-2 replicates within the tonsils and lymph nodes (Gillespie *et al* 2009). From there, the infection of B-cells or dendritic cells has been suggested as the mechanism of dissemination throughout the host (Darwich *et al* 2004 and Gillespie *et al* 2009). PCV-2 then establishes infection

in a wide range of cell types and antigen from the virus has been found in multinucleated giant cells, dendritic cells, histiocytes, as well as other cells of the monocyte macrophage lineage (Darwich *et al* 2004). Other cells harboring PCV-2 include kidney and respiratory epithelial cells, lymphocytes, vascular endothelial cells, enterocytes, hepatocytes, smooth muscle cells and pancreatic acinar and ductular cells (Misinzo *et al* 2006).

The first phase of a viral infection involves binding and entry of the virus into the host cell, which often dictates the cell and tissue tropism and can affect pathogenesis. Entry into the host cell can occur by direct penetration through the plasma membrane or through endocytic pathways preceding interaction with cell-surface receptors. PCV-2 binds to the heparin sulphate and chondroitin sulphate which are glycosaminoglycans (GAGs) as the first step of attachment (Misinzo *et al* 2006).

The next step in PCV-2 replication is transport of the DNA genome into the nucleus. These viruses depend on the host replication machinery for de novo DNA synthesis (Meerts *et al* 2005). Prior to replication, the viral DNA must pass through the nuclear envelope. But due to size limitations, the viral genome is unable to cross the nuclear envelope by passive diffusion (Heath *et al* 2006). The transport of proteins through the nuclear pore complex is signal mediated. Both Rep and Cap contain NLS sequences. PCV CP interacts with the viral genome and is transported through the nuclear pore complex into the nucleus. Upon entry into the nucleus, host cell factors convert the ssDNA into a dsDNA replicative form. Further steps are already mentioned in the section 2.6.

2.8 BIOLOGICAL AND PHYSICAL PROPERTIES OF PCV

PCV1 and PCV-2 are small (17 nm), icosahedral, nonenveloped viruses containing a single stranded circular DNA genome (Tischer *et al* 1982). The buoyant densityof PCV-1 in CsCl has been reported at 1.37 g/cm3 by Tischer (Tischer *et al* 1974) and 1.33–1.34 g/ml (Allan *et al* 1994) and a sedimentation coefficient of 57 S, when compared with the known sedimentation coefficient of a bovine enterovirus, has been reported (Allan *et al* 1994). PCV is resistant to inactivation after exposure to pH 3, chloroform, 56 C and 70 C (Allan *et al* 1994). Disinfectants which can dissolve lipids such as those based on alcohol, chlorhaxidine, iodine and phenol have no effect

on PCV-2. Inactivation of PCV-2 requires alkaline disinfectants (sodium hydroxide), oxidizing agents (sodium hypochlorite) or quaternary ammonium compounds (Grau-Roma *et al* 2011).

2.9 EPIDEMOLOGY OF THE VIRUS

2.9.1 Transmission

PCV-2 can be transmitted in several ways. The predominant route of transmission is oro-nasal, fecal and urinary routes (Bolin *et al* 2001). PCV-2 is shed in respiratory secretions, urinary secretions, oral secretions and faeces in the clinically infected, affected and apparently healthy pigs. Vertical transmission of the virus has also been reported in an experimental infection study. Park *et al* 2005 demonstrated that infection of a sow 6 weeks prior to farrowing caused reproductive failure. PCV-2 is also shed in colostrums (Shibata *et al* 2006) but whether it can result in an infection is still under investigation. The virus has been detected in both semen and colostrum, although there is no evidence that the virus is spread by insemination or by ingestion of colostrums (Pensaert *et al* 2004 and Park *et al* 2005). It has also been shown that PCV-2 infected pork products like lymphoid tissue, skeletal muscle and bone marrow, when fed to native piglets for 3 days, resulted in viremia and seroconversion to PCV-2 in all of the piglets (Opriessing *et al* 2009).

2.9.2 Factors modulating diseases caused by PCV-2

Infection caused by PCV-2 has been characterized by high prevalence of infection but low morbidity, and thus it has been shown that not all the animals infected by PCV-2 will develop the clinical signs of PCVAD (Calsamiglia *et* al 2002; Liu *et al* 2000). There are 4 main factors essential in the expression of PCV-2 related diseases: viral effects, host effects, effects of co-infection and immunomodulation (Opriessnig *et al* 2007).

2.9.2.1 Viral factors

PCV-2 is capable of causing several distinct disease syndromes and there is no difference in the virus genomes recovered from different syndromes. Sequence analysis of PCV-2 from PCVAD affected pigs and pigs with clinically unapparent infection, showed 95.6–100% sequence homology and no distinct patterns of sequence variations were evident between the 2 groups. Multiple PCV-2 strains of genotypes PCV-2a and PCV-2b isolated from a diseased or PMWS-affected pig,

which indicates that the co-infection with different PCV-2 strains might contribute to the development of PMWS in pigs (Zhai *et al* 2011). In a recent study, pigs infected with heterologous (PCV-2a/PCV-2b or PCV-2b/PCV-2a) or homologous (PCV-2b/PCV-2b) strains developed clinical disease and the severity of clinical disease was significantly higher in pigs inoculated with the heterologous strains than homologous strains (Harding *et al* 2010).

2.9.2.2 Host Factors

All breeds of pigs appear to be susceptible to infection, and clinical disease has been observed in many purebred and crossbred pigs (PG Halbur, unpublished data). Differences in the type of adaptive immune response against PCV-2 in different pigs may explain the host variation in the outcome of infection (Meerts *et al* 2005). There are significant differences in the replication patterns of PCV-2 in alveolar macrophage from different conventionally crossbred pigs (Meerts *et al* 2005). One the most important host factor which protects the host against the development of PMWS is the maternally derived immunity via colostrum which is considered to be antibody titre dependent (McKeown *et al* 2005).

2.9.2.3 Co-infection

Although PCV-2 is required to cause the characteristic lymphoid depletion of PCVAD, many strains of this virus require a cofactor to cause the full spectrum of clinical signs associated with PCVAD. Coinfection with several other viral and bacterial pathogens leads to an increase in incidence and a markedly more severe clinical course of disease. The agent implicated as creating the greatest risk is porcine reproductive and respiratory syndrome virus (PRRSV) (Pogranichniy *et al* 2002). Other agents include porcine parvovirus (PPV) (Balasch *et al* 1999) and *Mycoplasma hyopneumoniae* (Opriessnig *et al* 2004).

2.9.2.4 Immunomodulation

Co-infections with other infectious agents that activate the PCV-2 infection into the clinical expression of PMWS might be associated with immunostimulation (Gillespie *et al* 2009). One of the study showed that pigs that were immunostimulated with keyhole limpet hemocyanin developed clinical PCVAD when infected with PCV-2 (Krakowka *et al* 2001). In pigs vaccinated with the same antigen, but with oil-in water adjuvant was shown to cause a longer length of viremia, increased amounts of PCV-2 in serum and tissue and more severe lymphoid depletion when compared

with pigs vaccinated with aqueous and aluminum hydroxide products (Hoogland *et al* 2006).

2.10 PATHOGENESIS

The pathogenesis of PCV-2 infection and the major cell types that support PCV-2 replication are still not fully understood. Lymphoid depletion and lymphopenia in peripheral blood is a consistent feature in pigs that develop clinical PCVAD.

Pathogenesis is the mechanism by which a disease develops and includes where the pathogen goes and what it does within the body, and how the body reacts to that pathogen (Thacker B 2013).

The steps in the PCV-2 disease process include:

1) exposure,

2) initial infection

3) breakout infection

4) resolution

2.10.1 Exposure

Pigs are exposed to PCV-2 by contact with a contaminated environment, such as the floor, feeders, waterers, fences and possibly the air. The virus is transmissible from an infected pig to a healthy one by oronasal secretions or fecal matter.

2.10.2 Initial infection resulting in primary viremia

PCV-2 infects susceptible lymphocytes in surface-located lymphoid tissues, such as the tonsil and the Peyer's patches,which are found in the small intestine. At the cell level, susceptible lymphocytes must be permissive and allow the virus to enter the cell. In addition, the lymphocytes must be actively dividing in order for PCV-2 to replicate. This replication can be enhanced by immune stimulation from other co-infections like PRRS or *Mycoplasma hyopneumoniae*. After sufficient virus replication in the lymphocyte, the lymphocyte dies and releases infectious PCV-2 to the surrounding tissues. From there, the virus gains access to blood and lymph vessels, resulting in primary viremia or relatively low-level, non-detectable viremia. The primary viremia spreads the virus throughout the body causing infection of lymphocytes in multiple lymphoid tissues, including lymph nodes, spleen, liver and possibly bone marrow. The number of lymphocytes that become infected and die is

relatively small. Microscopically, no obvious lesions or aggregation of virus are detected.

2.10.3 Breakout infection resulting in secondary viremia, shedding and lymphoid depletion

This secondary viremia is readily detectable in a large number of lymphocytes and leads to an overwhelming level of infection in the pig. This secondary viremia is readily detectable in samples from the field or experimental studies. Shedding of PCV-2 via feaces and oronasal secretions starts at this point. Furthermore, large numbers of lymphocytes die, leading to depletion of lymphoid follicles or lymphocyte numbers in lymphoid tissues, which is visible by immunohistochemistry (IHC) under the microscope. Lymphoid depletion follows secondary viremia by a week or so. PCV-2 infection is now in multiple lymphoid tissues, and large amounts of virus is produced and circulates through the pig's system.

2.10.4 Lymphoid depletion and immunosupression

Lymphoid depletion and histiocytic replacement in lymphoid tissues are the hallmark lesions of PCV-2 infection and PCVAD (Opriessnig *et al* 2007). PCV-2 infection and its replication in lymphoid tissues can destroy the architecture of lymphoid follicles, thus leading to lymphoid depletions, which subsequently are replaced by histiocytic cells. Therefore, destruction of lymphoid follicles and leucopenia associated with PCV-2 infection can lead to immunosuppression in pig cells (Ramamoorthy and Meng 2008). The severity of lymphoid depletion is correlated positively with the amount of PCV-2 antigen detected in affected tissues.

2.10.5 Resolution resulting in chronic (granulomatous) inflammation

Following depletion of lymphocyte populations that were susceptible to infection, the body mounts an inflammatory response to clean up the dead lymphocytes and fill in the space previously occupied by those lymphocytes. This chronic inflammatory response is carried out by an infiltration of histiocytes, which are tissue-located macrophages that engulf and digest the dead cells. With PCV-2 infection, the lymphocyte death is a relatively quiet process. There is minimal acute inflammation, meaning that there is not the typical, early and acute inflammatory response characterized by infiltration of neutrophils (pus cells) and fluid (edema). Because PCV-2 is so durable, histiocytes can contain inactive PCV-2 virus particles, which are picked up during the cleaning process and unable to actively replicate. Chronic inflammation always lags behind lymphoid depletion by a week or so.

Because PCV-2 is a chronic disease, secondary viremia, shedding, lymphoid depletion and lymphoid inflammation can persist for months. This chronic disease occurs despite the emergence of PCV-2 antibodies, as measured by indirect immunofluorescence assay (IFA), at two to three weeks post-exposure.

2.11 PCV-2 ASSOCIATED SYNDROMES

Postweaning multisystemic wasting syndrome (PMWS) was recently replaced by the name porcine circovirus associated disease, which included all the syndromes associated with PCV-2 infection. According to the American Association of swine veterinarians (AASV), PCVAD can be subclinical or include one or more clinical manifestations including multisystemic disease with weight loss and high mortality, respiratory disease, porcine dermatologic and nephropathy syndrome, enteric signs including diarrhea, and reproductive disorders on an individual or herd basis.

2.11.1 Postweaning Multisystemic Wasting Syndrome

It is the most significant manifestation of PCVAD. Although it has been recognized in wild boars, but the source of infection is believed to be the domestic pigs (Schulze *et al* 2003). This disease affects pigs between 7 and 16 weeks. Morbidity is associated with the development of viremia and lymphopenia in piglets followed by the clinical manifestation of the disease. Mortality is usually 10% but can reach upto 50% (Allan and Ellis 2000 and Harding and Clark 1997).

Clinical signs of PCVAD include wasting with progressive weight loss, lethargy, dark-coloured diarrhea, lymphadenopathy, and paleness or jaundice. The main characteristic histopathologic lesions are lymphoid depletion with histiocyte replacement in lymphoid tissues, and intracytoplasmic inclusion bodies (Allan and Ellis 2000; Harding and Clark 1997). Early signs of reduced weight gain, ill-thrift, pale skin and rough hair coat often go unnoticed or are misdiagnosed. Later signs include dyspnea, tachypnea, anemia, diarrhea and jaundice (Harding and Clark 1997). Pigs can also have coughing and gastric ulceration, which most likely contributes to the anemia. On necropsy, the lungs fail to collapse and are mottled, tan colored, and in chronic cases some kidneys have white streaks or spots (Opriessnig *et al* 2007). Granulomatous lesions can also be found in the lungs, liver, kidney, heart, and intestines (Opriessnig *et al* 2007).

2.11.2 PCV-2 associated enteritis

This syndrome affects piglets from 8 to 16 weeks old and resembles chronic ileitis associated with Lawsonia intracellularis infection. Affected piglets have diarrhea, unthriftiness, retarded growth and increased mortality. Histopathologic lesions include a granulomatous enteritis and characteristic PCV-2 lesions in Peyer's patches but not in other lymphoid tissues. At necropsy, mesenteric lymph nodes are enlarged and the intestinal mucosa is grossly thickened. Histopathology is able to easily distinguish between Lawsonia versus PCV-2 infections (Jensen *et al* 2006).

2.11.3 PCV-2 Associated reproductive failure

It was first reported in Canada in 1999 (West *et al* 1999). The clinical signs include increased abortion, still births, fetal mummies and preweaning mortalities. The histopathologic lesions include a nonsuppurative to necrotizing or fibrosing myocarditis in still born and neonatal pigs (Mikami *et al* 2005). The time of infection determines the clinical course of the disease. Fetuses inoculated at 57 days of gestation had higher viral replication than those infected later in gestationand when killed at 21 days postinoculation had edema, enlarged livers and congestion. Late term infections at 86, 92, and 93 days of gestation caused an increase in reproductive abnormalities including still birth, fetal mummies and weak piglets (Jhonson *et al* 2002).

2.11.4 Porcine Dermatitis and Neuropathy Syndrome (PDNS)

This syndrome was first described in the United Kingdom in 1993 (Smith *et al* 1993) and was found associated with PCV-2 in the year 2000 (Rosell *et al* 2000). This disease is often fatal within 3 days of developmentand mostly affects grower pigs, but can affect pigs as young as 5 weeks old. Clinical signs include anacute onset of fever, lethargy, and raised purple skin lesions progressing to multifocal red-purple scabs with black centers being most prominent on the rear legs.

2.13 DIAGNOSIS

Diagnosis of PCV-2 is based on 3 criteria, and it is suggested to define the disease in single or group of animals: (1) the presence of clinical signs of wasting and weight loss, with or without dyspnea, jaundice, enlarged lymph nodes and icterus, (2) observation of the histopathological lesions including depletion of lymphocytes, granulomatous inflammation, and inclusion bodies in lymphoid tissues, lung and less

often liver and kidney and (3) detection of PCV-2 antigen within the tissue lesions (Grau-Roma *et al* 2007; Sorden 2000).

The PCV-2 antigen has been detected by several methods in a laboratory. *In situ* hybridization (ISH) and IHC were found to be highly sensitive and specific, and have been widely used to detect PCV-2 antigen or nucleic acid in all of the infected tissues (Rosell *et al* 1999). The level of lymphoid depletion or the amount of viral antigen has been suggested as a criterion for making a diagnosis of PMWS, but PCV-2 antigen has been detected in the lymphoid organs in pigs with or without clinical PMWS. The severe microscopic lesions of lymphocyte depletion were commonly observed in fatal cases of PMWS (Rosell *et al* 1999; Quintana *et al* 2001).

The PCR assay has been used as a specific and sensitive diagnostic method for the detection of PCV-2 in field samples (Calsamiglia *et al* 2002). Using the PCR assay, PCV-2 can readily be differentiated from PCV-2. One-step PCR is known to be sensitive enough to detect PCV-2 in tissue samples but it may not be sensitive for the serum or semen samples due to insufficient quantity of the viral DNA in the samples. Therefore, a nested PCR assay was developed and applied to detect PCV-2 in serum or semen samples (McIntosh *et al* 2006).

For the serological detection of PCV-2 infection, indirect immunofluorescence assay (IFA), immunoperoxidase monolayer assay (IPMA), and enzyme linked immunosorbent assay (ELISA) have been developed (Rosell *et al* 2000; Nawagitgul *et al* 2002).

Shang *et al* 2008 developed and validated a recombinant capsid protein-based ELISA for detection of antibody to PCV-2 by indirect ELISA using nuclear localization signal-truncated capsid protein of PCV-2 produced in *E. coli* (CAP ELISA). They validated assay by comparison with an indirect immunofluorescence assay (IIF) and PCV-2 based ELISA. The diagnostic sensitivity (DSN), specificity (DSP) and accuracy of the CAP ELISA were 95.3% and 93.9%, compared with IIF, and 93.3% and 84.2%, compared with the PCV-2-based ELISA, respectively.

Sun *et al* 2010 developed an ELISA using a protein encoded by ORF-2 antigenic domain of PCV-2 by indirect ELISA by using an antigenic domain (113-147AA) of ORF-2-encoded antigen, expressed in *E. coli*, for diagnosis of PCV infection. The specificity and sensitivity of I-ELISA were 87.7% and 93.57.

Yin *et al* 2010 established an ELISA based on a truncated soluble ORF-2 protein for the detection of PCV-2 antibodies in domestic pigs through expression in *E. coli*. The TcELISA was validated by comparison with an indirect immunofluorescence assay (IIFA). The diagnostic sensitivity (DSN), specificity (DSP), and accuracy of the TcELISA were 88.6%, 90.7% and 89.4%, respectively. A cross-reactivity assay showed that the method was PCV-2-specific by comparison with other sera of viral disease.

Huang *et al* 2011, developed a blocking ELISA for detection of serum neutralizing antibodies against PCV- 2 by monoclonal antibody (Mab) based blocking ELISA. The Mab with neutralizing activity, which was produced by immunizing a recombinant capsid protein of PCV-2 expressed in insect cells, was used as the detector antibody.

Jittimanee *et al* 2012, developed in-house indirect enzyme linked immunosorbent assay using a recombinant nuclear localization signal truncated capsid (rntcap) protein of PCV-2, expressed in an *E. coli* system and determined the diagnostic performance of the developed ELISA in comparison to the immunoperoxidase monolayer assays (IPMAs). Based on a receiver operating characteristic (ROC) curve analysis of the rntCap indirect ELISA, an optimum cutoff optical density (OD) of 0.330 was determined, which resulted in diagnostic sensitivity, diagnostic specificity, and accuracy of 98.33%, 93.33%, and 96.67%, respectively.

Because PCV-2 is an ubiquitous antigen, PCV-2 specific antibody could be detected in most pigs under natural conditions regardless of the presence of clinical PMWS. The antibody kinetics, therefore, have not been significantly accepted as a diagnostic method for clinical PMWS (Sorden 2000). Care must be taken in interpretation of the results of these tests because a slight degree of antigenic cross-reactivity between PCV-1 and PCV-2 isolates has been demonstrated (Allan *et al* 1998).

2.14 PREVENTION AND CONTROL OF PCV

Currently very little is known about control of PCV-2 associated diseases. Porcine circoviruses are highly resistant to inactivation by common detergents and disinfectants making the decontamination of infected premises difficult. Treatment of

individually affected pigs is supportive only and will vary greatly depending upon the clinical signs that the animal displays. Because many animals are coinfected, choosing appropriate treatment will also depend upon identificationof the other agents infecting the animal. Prior to the availability of PCV-2 vaccines, the treatment and control of PCVAD was primarily focused on ensuring good production practices that minimize stress, eliminating coinfections or minimizing their effect and eliminating potential triggering factors that induce immune stimulation and trigger progression of PCV-2 infection to PCVAD. A 20-point plan to control PCVAD on severely affected farms was proposed (Madec *et al* 2001). The main pointsof this plan have been summarized as the 4 golden rules (www.thepigsite.com) and include 1) limiting pig-to-pig contact, 2) reduction of stress, 3) good hygiene and 4) good nutrition.

Treatment of bacterial infections and prevention of cofactor-associated diseases is also a good practice in preventing PCV-2 diseases. Treating *M.hypopneumoniae* with chlortetracyclins was highly effective (Opreissnig *et al* 2006). Bleach (3–6% sodium hypochlorite) is an effective chemical in killing PCV-2, but has unknown field efficacy. Good housing management is critical in disease prevention. It has been shown that reducing stress, paying attention to proper hygiene, preventing mixing of ages, and utilizing all in/all out practices are effective in controlling disease. Whether PCV-2 can be found in insects or wild animals that could possibly transmit disease to pigs is not known. However, because circoviruses are highly species specific, it is unlikely that these animals, excluding feral boars, would pose a threat of PCV-2 transmission to domestic herds (Ramamorthy and Meng 2008).

2.15 VACCINATION

Vaccination induced antibodies against PCV-2 are known to be highly effective in controlling PCVAD. Since the late 2006, commercial PCV-2 vaccines including Circumvent TM PCV (Intervet Inc), Suvaxyn® PCV-2 (Fort Dodge), Ingelvac® CircoFLEX TM (Boehringer Ingelheim), and Circovac® (Merial Inc.) have been available in the swine industry, and the former three products have been licensed by the USDA.

The Circumvent TM PCV vaccine was developed based on the baculovirus vector system for ORF-2 gene transfection. The protective efficacy of the expressed

ORF protein was examined by the challenge trial in SPF pigs following inoculation of PCV-2 intratracheally and intramuscularly. The ORF-2 vaccine group was compared with a non-vaccine group, ORF-1 vaccine group, ORF-1 and ORF-2 combined vaccine group and DNA vaccine group. The results showed a significant improvement in the growth performance in all of the vaccinated groups as compared to the non-vaccine group (Madec *et al* 2000).

The Ingelvac® CircoFLEX was also produced using the baculovirus vector system and PCV-2 ORF-2 gene protein was expressed by the system in insect cells. The ORF2 protein was purified and substantially concentrated by the technical process of the Boehringer Ingelheim company. This vaccine was examined under commercial farm conditions and the results showed that clinical signs, mortality and lesions of the vaccinated group were significantly reduced as compared to the non-vaccinated group (Boehringer Ingelheim technical bulletin).

The Suvaxyn® PCV-2 was developed from the constructed chimeric virus of PCV-1 and PCV-2 in which *ORF-2* gene of PCV-2 was inserted into the backbone of PCV-1. The efficacy of the chimeric virus vaccine was evaluated with SPF pigs, and gross and microscopic lesions of the non-vaccinated group were significantly severer than those of the vaccinated group (Fenaux *et al* 2003).

The Circovac® was developed following inactivation of the whole virus and mixing with an oil adjuvant. The vaccine caused improvement in the performances by an experimental challenge in the piglets and sows, and has been suggested to use in pregnant sows (Charreyre *et al* 2005).

CHAPTER- III

MATERIALS AND METHODS

Bacterial expression and characterization of recombinant ORF-2 (capsid) protein of PCV-2

3.1. Codon optimization- The gene bank available PCV-2 *ORF-2* (capsid) gene was analysed, codon optimized for expression in *E. coli* and then synthesized commercially.

3.2.3.2. Cloning and sequencing of codon optimized PCV-2 *ORF-2* (capsid) gene

3.2.1. Preparation of *E. coli* (DH5α) competent cells

Solutions and buffers

1) RF I solution

Components (molecular weight)	**Final concentration**	**Amount**
Rubidium chloride (MW 120.9)	100 mM	6.00 gm
Manganese chloride (MW 197.91)	50 mM	4.95 gm
Potassium acetate (MW 98.15)	30 mM	1.47 gm
Calcium chloride (MW 147.02)	10 mM	0.75 gm
Glycerol	15% (w/v)	75 ml
DDW upto		500 ml

The pH of the solution was set at 5.8 and sterilized by filtration.

2) RF II Solution

Components (molecular weight)	**Final concentration**	**Amount**
MOPS (4-Morpholinopropanesulfonic acid (MW 209.3)	10 mM	1.55 gm
Rubidium chloride (MW 120.9)	10 mM	0.60 gm
Calcium chloride	75 mM	5.63 gm
DDW upto		500 ml

The solution was sterilized by filtration.

Procedure:

Two LB-agar plates, one with ampicillin and the other without ampicillin were prepared. DH5α strain of *E. coli* cells (obtained from frozen stock) were streaked on them and incubated for 16 hrs at 37°C. The bacterial colonies which appeared on LB-agar without ampicillin were selected.

1. Single colony was picked from the plate and transferred to 50 ml of LB medium in 500 ml flask and incubated at 37ºC overnight in a shaker incubator at 150 rpm.
2. 2 ml of DH5α cells were inoculated for sub-culturing into 100 ml of LB (1:100 ratio) and kept in shaker incubator for 3-4 hours at 37°C and 200 rpm to reach an OD of 0.6.
3. The culture was then transferred to sterile 50 ml centrifuge tubes and chilled on ice for 10 minutes.
4. The tube was centrifuged at 6500 rpm at 4°C for 10 minutes.
5. The supernatant was discarded and the cells were resuspended in 30 ml of cold RF I solution and chilled on ice for 30 minutes.
6. The tubes were centrifuged at 6500 rpm for 10 minutes at 4°C.
7. The supernatant was discarded and the cells were resuspended in 1 ml of RF II solution and chilled on ice for 15 minutes.
8. 100 µl aliquots were prepared in pre chilled sterile 1.7 ml MCTs. Stored at -80ºC for further use.

3.2.2. Transformation of DH5α competent cells

Codon optimized PCV-2 *ORF-2* coding sequences in the pUC57 plasmids was transformed into DH5α strain of *E. coli* as per the protocol given by Sambrook *et al* 2001.

Luria Bertani (LB) Broth (HiMedia)

Casein enzymic hydrolysate	10.0 g
Yeast Extract	5.0 g
Sodium Chloride	10.0 g
DDW	1000 ml

Autoclaved for 20 min at 15 lb pressure.

Luria Bertani (LB) Agar

Casein enzymic hydrolysate	10.0 g
Yeast Extract	5.0 g
Sodium chloride	10.0 g

Agar	15.0 g
DDW	1000 ml

Autoclaved for 20 min at 15 lb pressure

Ampicillin Stock

Ampicillin	100.0 mg
DDW	1 ml

SOB Medium

Tryptone	20 g
Yeast Extract	5.0 g
Sodium chloride	0.5 g
250 Mm KCl	10.0 ml

pH was adjusted to 7.0. Total volume was made upto 1000 ml by DDW then autoclaved at 15lbs/sq inch pressure for 15 min.

Just before use 5 ml of sterile solution of 2M $MgCl_2$ was added.

SOC Medium

2.0 ml of filter sterilized 1M glucose was added, to 100 ml of SOB.

Procedure:

DH5α competent cells were transformed by the following method.

1. 1 µl PUC57 plasmid (~1 ng) having codon optimized PCV-2 *ORF-2* gene sequence was added to one vial of DH5α competent cells (100 µl) and the tube was kept on ice for 30 minutes.
2. The cells were subjected to heat shock for 45 seconds at 42ºC in a preequilibrated water bath.
3. Then the tube was immediately transferred to ice and kept for 2-3 minutes.
4. Then 300 µl of SOC was added to the tube and incubated at 37ºC for 1 hr at 200 rpm in a shaker incubator.
5. The transformed cell mixture (~100 µl) was then spreaded over LB agar plates containing ampicillin (100µg/ml) and incubated at 37°C overnight in an incubator.

3.2.3 Selection and screening of clones

The agar plates were observed for the development of colonies. White Colonies (5-6 nos) were picked by sterile pipette tips and inoculated separately into 10 ml Luria-Bertani (LB) broth containing ampicillin (100µg/ml) and incubated at

37^{o}C overnight in a shaker incubator at 150 rpm. After overnight incubation the broth cultures were used for plasmid extraction.

3.2.4 Plasmid Isolation by alkaline lysis method (Sambrook *et al* 2001)

Solutions for plasmid extraction

Alkaline lysis solution I

Components	Final concentration	Salt amount (grams)
Glucose	50 mM	0.9008
Tris-Cl (pH-8.0)	25 mM	0.37224
EDTA (pH-8.0)	10 mM	0.394
DDW		100ml

Solution I was sterilized by autoclaving for 15 minutes at 15lbs/sq inch pressure and stored at 4°C.

Alkaline lysis solution II

Components	Final concentration	Salt amount
NaOH (0.2 N stock)	0.2 N	0.78 g
SDS	1.0 %	1.0 g
DDW		100 ml

Solution II was prepared freshly.

Alkaline lysis solution III

Components	Final concentration	Amount
5M potassium acetate	5 M	60 ml
Glacial acetic acid	11.5 %	11.5 ml
DDW		100 ml

Solution III was stored at 4°C.

Procedure:

The Bacterial plasmids were extracted from the broth cultures as per the method of Sambrook *et al* (2001) with slight modifications.

1. Broth cultures (8 ml) in 15 ml centrifuge tubes were centrifuged at 8,000 rpm for 10 minutes.
2. Supernatant was discarded and the pellet was resuspended in 200 µl ice cold alkaline lysis solution I. Mixed by pipetting and kept at room temperature for about 10 minutes.
3. Then, 200 µl of freshly prepared alkaline lysis solution II was added and mixed gently by inverting the tubes 6-8 times to obtain clear lysate. The tubes were kept on ice for 5 minute.
4. 300 µl of alkaline lysis solution III was added, mixed by inverting the tubes several times and then kept on ice for 10 minutes.
5. The tubes were centrifuged at 12,000 rpm for 10 minutes at 4°C.
6. The supernatant was transferred to a new tube, equal volume of phenol: choloform: isoamyl alcohol (25:24:1) was added and mixed by inverting the tube several times.
7. The suspension was centrifuged at 12,000 rpm for 10 minutes at 4°C.
8. The aqueous phase was collected in a new tube and RNaseA treatment (1U/µl) was given for about 1hour at 37ºC.
9. After that $1/10^{th}$ volume of 3M sodium acetate (pH-5.2) and equal volume of isopropanol was added to it.
10. Gently mixed and kept the tubes at -80°C for 1-2 hrs.
11. The tubes were then centrifuged at 12,000 rpm for 10 minutes at 4°C.
12. Supernatant was discarded and the pellet was washed with 70% ethanol by centrifuging at 12,000 rpm for 10 minutes at 4°C. Washing step was repeated once more.
13. The pellet was air dried and then 50 µl of TE buffer or NFW was added to dissolve the pellet. The plasmids were stored at -20°C for further use.

3.3.5 Cloning of PCV-2 *ORF-2* gene into PET 302 NT His expression vector

The PET 302 NT His expression vector, available in the laboratory, was used for expression of PCV-2 ORF-2 capsid protein. The expression vector carries a T7 *lac* promoter for high-level expression of the gene of interest in *E. coli.* It has multiple cloning site for restriction enzyme digestion and ligation of gene of interest and the N terminal 6x His tag for detection and purification of protein. It also carries a ampicillin resistance gene for selection in *E. coli* and a pBR322 origin for low-copy replication and maintenance of the plasmid in *E. coli.* It also contains *lac*I gene

encoding the lac repressor to reduce basal transcription from the T7*lac* promoter (Fig. 2).

3.2.5.1 RE digestion to release the codon optimized PCV-2 *ORF-2* coding sequences from the PUC57 plasmids and to linearise PET 302 NT His vector for cloning

The recombinant pUC57 vector having PCV-2 *ORF-2* insert and pET302 NT His expression vector were subjected to *Eco* RI and *Bam* HI RE digestion, separately. The double restriction enzyme digestion (*Eco* RI and *Bam* HI) reactions were set up in 50 µl volume for the release of PCV-2 *ORF-2* insert from PUC57 plasmid as well as to linearise the pET302 NT His vector for cloning purpose. The reaction mixture was incubated at 37ºC in a water bath for 3-4 hrs.

Components	**Volume/reaction**
Plasmid (~ 500 ng/ µl)	25.00 µl
Reaction buffer (2X Tango Buffer)	10.0 µl
Enzyme (*Eco* RI) (10U/ µl)	3.00 µl
Enzyme (*Bam* HI) (10U/ µl)	5.00 µl
NFW	7.00µl
	50.0µl

3.2.5.2 Purification of RE digested products by agarose gel electrophoresis

Solutions and buffers

Working solution of TAE buffer (1 X)

Tris-acetate	40.0 mM
EDTA	1mM

Agarose gel

Agarose	0.3 g
1X TAE	30.0 ml
Ethidium bromide (10mg/ml)	1.5µl

6X Loading Dye

Bromophenol blue	0.25%	25 mg
Xylene cyanol	0.25%	25 mg
Glycerol	30%	3.3 ml
DDW		6.7 ml (total 10 ml)

3.2.5.2.1 Agarose gel electrophoresis of the PCR amplified PCV-2 *ORF-2* gene

1. One percent agarose was prepared in 1X TAE buffer in a conical flask and heated to dissolve the agarose completely using a microwave.
2. The dissolved agarose was cooled down to a temperature of around 55-60^{o}C and ethidium bromide solution (stock conc. 10mg/ml) was added to make the final concentration 0.5 µg/ml.
3. Dissolved agarose was poured into a gel casting tray assembly (Bio-Rad) having comb of appropriate number and sizes hung into that and then allowed to solidify at room temperature.
4. Once solidified, the gel was placed in electrophoresis tank containing 1X TAE and then the comb were removed from the gel.
5. PCR products and 6X loading dye were mixed and were loaded in the wells with the help of micropipette. 6 µl of DNA ladder (1 kilo base pair plus, Fermentas) was also loaded in one of the wells to access the size of PCR products.
6. The electrophoresis was carried out for 30-45 minutes at 80 volts and the gel was viewed under Geldoc (Bio-Rad) system & photographed.

The RE digested pET302 NT His vector and PCV-2 *ORF-2* fragments were gel purified separately by using commercial gel extraction kit (Wizard SV Gel and PCR Clean up system, Promega).

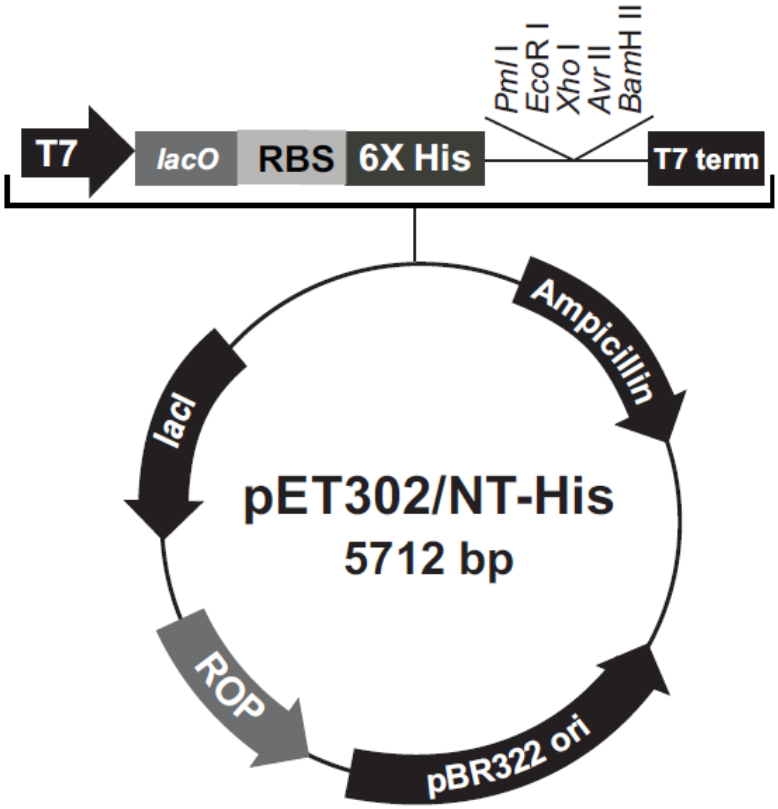
Procedure:

A. Dissolving the gel slice

1. The RE digested products were run on 1% agarose gel prepared in TAE (Tris Acetate EDTA) buffer.
2. After electrophoresis, the appropriate DNA bands were excised from the gel with help of a BP blade and taken in a microcentifuge tubes.
3. 10 µl of membrane binding solution was added per 10 mg of gel slice. The mixture was then vortexed and incubated at 60ºC until the gel slice was completely dissolved.

B. Binding of DNA

1. The SV Minicolumn was inserted into the collection tube.
2. The dissolved gel mixture was transferred to the minicolumn assembly and incubated atroom temperature (RT) for 1 minute.



Comments for pET302 NT-His
5712 nucleotides

T7 promoter: bases 20-36
T7 promoter priming site: bases 20-39
lac operator (*lac*O): bases 39-63
Ribosome binding site (RBS): bases 93-101
6X His Tag: bases 112-129
T7 transcription termination region: bases 182-310
T7 reverse priming site: bases 221-241
bla promoter: bases 615-719
Ampicillin (*bla*) resistance gene: bases 714-1574
pBR322 origin: bases 1785-2516 (c)
ROP ORF: bases 2760-2951 (c)
lacI ORF: bases 4263-5375 (c)

(c) = complementary strand

Fig. 2: Map and features of pET 302 NT His expression vector

3. The mixture was centrifuged at 16,000 x *g* for 1 minute. The flow through was discarded and the minicolumn was reinserted into collection tube.

C. Washing

1. 700 µl of Membrane wash solution (ethanol added) was added and centrifuged at 16,000 x *g* for 1 minute. The flow through was discarded and the minicolumn was reinserted into collection tube.
2. 500 µl of Membrane wash solution (ethanol added) was added and centrifuged at 16,000 x *g* for 5 minute. The flow through was discarded and the minicolumn was reinserted into collection tube.
3. Empty spin was given to the column assembly for 1 minute at 16,000 x *g*.

D. Elution

1. The minicolumn was transferred to a clean 1.5 ml microcentriguge tube.
2. 35 µl of nuclease free water was added to the minicolumn. Incubated at room temperature for 1 minute and centrifuged at 16,000 x *g* for 1minute.
3. Minicolumn was discarded and the DNA was stored at -20 ºC.

3.2.5.3 Ligation of the gel purified, codon optimized PCV-2 *ORF-2* gene fragment into pET302 NT His Vector

The gel purified, codon optimized PCV-2 *ORF-2* gene fragment was ligated into pET 302 NT His vector in a vector insert molar ratio of 1:2 as follows:

$$\text{ng insert required} = \frac{\text{ng vector x kbp insert}}{\text{Vector size (kbp)}}$$

Components	Volume/reaction
pET 302 NT His vector(36ng/µl)	2.0 µl
Insert PCV-2 *ORF-2* fragment (9ng/µl)	1.30 µl
10X ligation buffer	2.0 µl
T4 DNA Ligase (Fermentas, 3 weiss unit)	1.0 µl
NFW	13.7 µl
	20.0µl

The ligation mixture was incubated at 22ºC for 3-4 hours in an eppendOrf thermocycler.

3.2.5.4 Transformation of DH5α competent cells

After ligation, 10 µl of the ligation mixture was added into DH5α competent cells for transformation as per the protocol described in section 3.2.2.

The clones (10-15 Nos) obtained after transformation, were picked and streaked on the LB agar plates containing ampicillin (100 µg/ml) and incubated at 37ºC overnight in an incubator.

3.2.5.5 Screening of recombinant clones by colony PCR

The recombinant clones were screened for the presence of desired insert by PCR using sequencing primers for the presence of insert. The following reaction mixture was prepared for amplification of *ORF-2* gene by *Taq* DNA polymerase (Invitrogen) using universal T7 forward and T7 reverse sequencing primers.

T-7F: 5′-TAATACGACTCACTATAGGG-3′

T-7R: 5′-TAGTTATTGCTCAGCGGTGG-3′

Composition of PCR reaction mix	**Amount**
Nuclease free water (NFW)	40 µl
$MgCl_2$ (50mM)	1.5 µl
10X Buffer	5.0 µl
dNTPs (10 mM)	1.0 µl
T7 Forward primer (20pmol/µl)	1.0 µl
T7 Reverse primer (20pmol/µl)	1.0 µl
Template (bacterial colony)	Clone (picked up by sterile pipette tips)
Taq DNA polymerase(5U/µl, invitrogen)	0.5 µl
Total volume	50 µl

The following conditions were used for the amplification of PCV-2 *ORF-2* gene in a thermocycler (Eppendorf).

Steps	**Temperature**	**Time**	**Cycles**
Initial denaturation	94 ^oC	3 min	1
Denaturation	94 ^oC	30 sec	35
Annealing	50 ^oC	45 sec	
Extension	72 ^oC	1 min	
Final extension	72 ^oC	8 min	1

3.2.2.6 Selection of the clones

After PCR, the amplified product was run in 1.5% TAE agarose gel as mentioned in the section 3.2.5.2.1. The recombinant clones which showed the presence of desired insert by PCR amplification were selected for further studies.

Positive white Clones (5-6 nos) showing desired gene insert in PCR were picked by sterile pipette tips and inoculated separately in to 50 ml Luria-Bertani (LB) broth containing ampicillin (100µg/ml) and incubated at 37^{o}C overnight in a shaker incubator. After overnight incubation plasmids were isolated from the broth cultures by alkaline lysis method as per the protocol described earlier (section 3.2.4).

3.3 Expression of codon optimized PCV-2 *ORF-2* gene in bacterial expression (BL21(DE3) star Chem. Competent Cells, Life technologies) system

3.3.1 Transformation of BL21 competent cells

The recombinant Pet 302 NT His-*ORF-2* plasmid DNA extracted from the positive DH5α clone was transformed into BL21 (DE3) *E. coli* competent cells for protein expression.

3.3.2 Optimization of protein expression by IPTG induction

pET 302 NT His *ORF-2* plasmid carrying BL21 clones were subjected to induction by IPTG for optimization of recombinant PCV-2 ORF-2 protein expression.

1. A single recombinant colony was picked from the LB ampicillin plate and inoculated into 5 ml of LB carbenicillin (100µg/ml) broth.
2. The broth was incubated overnight at 37^{o}C with the rotary shaking speed of 200 rpm.
3. Overnight culture 0.2 ml was inoculated into 20 ml of LB carbenicillin broth (5 tubes) and grown at 37°C with agitation. An OD_{600} of 0.5-1.0 was achieved within 3hrs.
4. Two ml of uninduced culture was removed and centrifuged for 1min in a microcentrifuge tube.
5. The supernatant was discarded and the pelleted cells were stored in -20°C.
6. IPTG at 1.0 mM concentration was added to the culture tubes and continued to incubate the culture as described above.
7. Two ml aliquots of cells were removed at 1, 2, 3, 4, 6 hours and overnight post-induction from each tube for protein expression study.
8. The cells were centrifuged as mentioned above and the pellets were stored in -20°C till further use.

3.4 Solutions/buffers for SDS-PAGE

Solution A (30% acrylamide stock solution):

Acrylamide	29.2 g
Bis (N, N'-Methylenebisacrylamide)	0.80
DDW upto	
100 ml	
Stored in dark at 4°C	

Solution B (1.5% M Tris-HCl buffer, pH 8.8):

Tris base (Tris hydroxymethyl aminoethane)	18.20 g
SDS (Sodium dodecyl sulfate)	0.40 g
DDW up to	100 ml
pH adjusted to 8.8 with 1N HCl. Stored at 4°C	

Solution C (0.5 M Tris-HCl buffer, pH 6.8):

Tris	6.10 g
SDS	0.40 g
DDW upto	100 ml
pH adjusted to 6.8 with 1N HCl. Stored at 4°C	

Solution D (10% ammonium persulfate):

Ammonium persulfate	100 mg
Dissolved in 1 ml DDW. (Prepared fresh before use)	

15 % SDS-PAGE resolving gel cocktail (100 ml):

Water	25.5 ml
30% Acrylamide (Solution A)	49.5 ml
1.5 M Tris-HCl, pH 8.8 with 0.4% SDS (Solution B)	25 ml

5% SDS-PAGE stacking gel cocktail (100 ml):

Water	58 ml
30% Acrylamide (Solution A)	17 ml
0.5 M Tris-HCl, pH 6.8 with 0.4% SDS (Solution C)	25 ml

15% Resolving gel (5ml)

15% resolving gel cocktail	4.95 ml
Ammonium persulphate (APS)	0.05 ml
TEMED	0.002 ml

5% Resolving gel (1ml)

5% resolving gel cocktail	0.99 ml
Ammonium persulphate (APS)	0.01 ml
TEMED	0.001 ml

Tris- Glycine buffer (Running buffer 5X)

Glycine	144 g
Tris	30 g
10% SDS	50 ml
Volume made up to 1000 ml with DDW	

1X Running Buffer:

Glycine	9.40 g
Tris base	1.50 g
10% SDS	5 ml
DDW up to	500 ml

2X Loading buffer

10% SDS	4.0 ml
β- Merceptoethanol	0.8 ml
Sucrose	2.0 g
1 M Tris (pH 6.8)	1.25 ml
Bromophenol	1.00 ml
DDW upto	10.0 ml

Staining solution

Acetic acid	10%
Methanol	45%
DDW	45%
Coomasie brilliant blue	0.15%

Destaining solution

Acetic acid	10%
Methanol	45%
DDW	45%

3.5 Expression study of recombinant PCV-2 ORF-2 protein by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis

SDS-PAGE analysis was carried out in a vertical minigel electrophoresis apparatus (Bio-Rad) as follows:

1. Glass plates were cleaned and set in a gel casting assembly of the Bio-Rad's electrophoresis system and 15% resolving gel solution was prepared and poured between the two SDS-PAGE glass plates.
2. Immediately, 1 ml of distilled water was poured over the gel for making an even surface for the stacking gel.
3. After polymerization, water was removed by tilting the plates.
4. Then 5% stacking gel was prepared, poured over the resolving gel and a suitable comb was inserted.
5. The polymerized gel was mounted into the electrophoresis chamber after removing the combs and buffer reservoir was filled with 1 X Tris glycine running buffer.
6. Before loading, sample buffer (2X) was added to equal volume of each sample (bacterial cell pellet) and then boiled for 10 minutes in a water bath at 95ºC.
7. The samples were then centrifuged at 10,000 rpm for 15 mins and the supernatants (25µl from each sample) were loaded into the wells of SDS-PAGE gels.
8. A prestained protein molecular weight marker (Thermo scientific) was included in a well along with the samples.
9. Electrophoresis was carried out at a constant current of 70V, till the tracking dye reached the bottom of the gel.
10. The gel was taken out from the plates and stained with coomassie brilliant blue for 2 hours and then destained with several changes of the destaining solution.
11. The desired size of the expressed recombinant protein was compared with the protein ladder.

3.6 Solutions for protein purification at native conditions

3.6.1 Binding/Lysis buffer (100ml, pH 7.4)

20 mM NaH_2PO_4	0.2396 g
500 mM NaCl	2.92 g
15 mM Imidazole	0.10 g
DDW	100 ml

Firstly 50 ml of distilled water was added into the salts and mixed properly to dissolve the salts. pH was adjusted to 7.4 using 1N NaOH/ HCL and then the final volume was made upto 100ml by DDW.

3.6.2 Washing buffer (100ml, pH 7.4)

20 mM NaH_2PO_4	0.2396 g
500 mM NaCl	2.92 g
Imidazole	20-40 mM
DDW	100 ml

pH was adjusted to 7.4 using 1N NaOH/ HCL

3.6.3 Elution buffer (100ml, pH 7.4)

20 mM NaH_2PO_4	0.2396 g
500 mM NaCl	2.92 g
350 mM Imidazole	2.38 g
DDW	100 ml

pH was adjusted to 7.4 using 1N NaOH/ HCL

3.7 Purification of recombinant PCV-2 ORF-2 protein by Ni-NTA column chromatography under native conditions

3.7.1 Preparation of lysate

1. 50 ml culture was inoculated in 1000 ml LB broth and incubated for 3 hours at 37°C with shaking at 250 rpm until OD600 reached 0.6.
2. After 3 hrs of incubation, uninduced culture (10 ml) was collected into a 15 ml centrifuge tube and IPTG was added into the rest of the culture (990 ml) at a final concentration of 1mM. The cells were harvested at 6hr post induction.
3. The harvested culture was pelleted by centrifugation at 8000 rpm for 5 min, washed with PBS and the pellet was resuspended in 2ml per gram wet weight of lysis/binding buffer (pH 7.4).
4. Added 8 mg lysozyme and incubated on ice for 30 minutes.
5. Using a sonicator equipped with a microtip, sonicated the solution on ice using six 10-second bursts at high intensity with a 10-second cooling period between each burst.
6. Added RNase A (10 μg/ml) and DNase I (5 μg/ml) and incubated on ice for 10–15 minutes.
7. The lysate was then centrifuged at 3,000 × *g* for 15 minutes to pellet the cellular debris and the supernatant was transferred to a fresh tube for purification.

3.7.2 Purification of recombinant capsid protein

Preparation of the column:

1. The Ni-NTA Agarose (invitrogen) was resuspended in its bottle by inverting and gently tapping the bottle repeatedly.
2. Two ml of 50% Ni-NTA slurry (invitrogen, Cat.No.R901-15) was poured into a purification column. The slurry was allowed to settle completely by gravity until the ethanol was removed.
3. Then 6 ml of distilled water was poured over it and the resin was resuspended by alternately inverting and tapping the column. Allowed the resin again to settle under gravity.
4. The column was equilibrated using the denaturing lysis buffer as described in the step 2-3.

Purification under native conditions:

1. After preparing the column, 10ml of lysate was mixed with Ni-NTA slurry by shaking gently for 1-2 hrs at room temperature or overnight at 4°C on a rotary mixer (Tarsons), for binding of the His-tagged recombinant protein.
2. Then the mixture was loaded into an empty column and the resin was allowed to settle by gravity and the flow through was collected.
3. After removal of the flow through, the column was washed with 10 ml of washing buffer (pH7.4) three times containing different concentrations of imidazole i.e., 20mM, 40mM, 60mM, respectively.
4. The 1 ml fractions of flow through were collected at each step of washing for SDS-PAGE analysis
5. The recombinant protein was eluted with 8 ml of elution buffer (pH 7.4) and stored at -20ºC after adding protease inhibitor, PMSF (phenylmethylsulfonyl fluoride) at a final concentration of 1mm.
6. After purification the resin was washed using 0.5 M NaOH for 30 min and poured 30 ml of distilled water into the column. For equilibration, binding buffer was poured into the column and kept at 4°C till next use (within a week).

3.7.3 SDS-PAGE analysis of purified PCV-2 ORF-2 recombinant protein

SDS-PAGE analysis was carried out in a vertical minigel electrophoresis apparatus (Bio-Rad) as mentioned earlier in the section 3.5. Here the samples were bacterial cleared lysates, purified PCV-2 ORF-2 protein samples, wash flow throughs

at different stages of washing. The yield of the protein was very less under native conditions indicating that the protein was present mostly in insoluble form in the cells not in the soluble form. So, to check whether the protein is present in the insoluble form, the bacterial cell pellet left after the native cell lysate preparation was resuspended in the denaturing lysis buffer and checked by SDS-PAGE analysis. A high level protein expression was seen in denaturing conditions, so the purification was carried out under denaturing conditions using 6M Urea in large scale.

3.8 Solutions for protein purification at denaturing conditions

3.8.1 Binding/Lysis buffer (100ml, pH 7.4)

20 mM NaH_2PO_4	0.2396 g
500 mM NaCl	2.92 g
6M Urea	57.19g
15 mM Imidazole	0.10 g
DDW	100 ml

Firstly 50 ml of distilled water was added into the salts and mixed properly to dissolve the salts. pH was adjusted to 7.4 using 1N NaOH/ HCL and then the final volume was made upto 100ml by DDW.

3.8.2 Washing buffer (100ml, pH 7.4)

20 mM NaH_2PO_4	0.2396 g
500 mM NaCl	2.92 g
6M Urea	57.19g
Imidazole	20-40 mM
DDW	100 ml

pH was adjusted to 7.4 using 1N NaOH/ HCL

3.8.3 Elution buffer (100ml, pH 7.4)

20 mM NaH_2PO_4	0.2396 g
500 mM NaCl	2.92 g
6 M Urea	57.19 g
350 mM Imidazole	2.38 g
DDW	100 ml

Firstly 70 ml of distilled water was added into the salts mixed properly to dissolve the salts. pH was adjusted to 7.4 using 1N NaOH/ HCL and then the final volume was made upto 100ml by DDW.

3.9 Large scale Purification of recombinant PCV-2 ORF-2 protein by Ni-NTA column chromatography under denaturing conditions

3.9.1 Preparation of lysate

1. 50 ml culture was inoculated in 1000ml LB broth and incubated for 3 hours at 37°C with shaking at 250 rpm until OD600 reached 0.6.
2. After 3 hrs of incubation, uninduced culture (10 ml) was collected into a 15 ml centrifuge tube and IPTG was added into the rest of the culture (990 ml) at a final concentration of 1mM. The cells were harvested at 6hr post induction.
3. The harvested culture was pelleted by centrifugation at 8000 rpm for 5 min, washed with PBS and the pellet was resuspended in 5ml per gram wet weight of lysis/binding buffer (pH 7.4).
4. The tube containing bacterial cell suspension was stirred for 15-60 minutes in a rotary mixer (Tarsons) at room temperature with by gentle vortexing intermittently, taking care to avoid foaming. Cell lysis was complete when the solution became translucent.
5. The lysate was centrifuged at 10,000 rpm for 30 min at 22°C to pellet the cellular debris.
6. After centrifugation, the supernatant was transferred to a new tube for purification.

3.9.2 Purification of recombinant capsid protein

The recombinant protein was purified under denaturing conditions using the buffers mentioned in the section 3.8. The protocol for purification of the protein was similar to the protocol mentioned in the section 3.7.2.

3.9.3 SDS-PAGE analysis of purified PCV-2 ORF-2 recombinant protein

SDS-PAGE analysis was carried out in a vertical minigel electrophoresis apparatus (Bio-Rad) as mentioned earlier in the section 3.5. Here the samples were bacterial cleared lysates, purified PCV-2 ORF-2 protein samples, wash flow throughs at different stages of washing.

3.10 Solutions for protein refolding

3.10.1 Protein refolding buffer (50 ml)

2M Urea	6.006g
20mM Tris (pH-7.4)	121.14 mg
10% Sucrose	5.0 g
100Mm KCL	372.75 mg
2Mm $MgCl_2$	9.521 mg

The salts were mixed properly in DDW, pH was adjusted to 8.0 using 1N NaOH/ HCL and then the final volume was made up to 50ml by adding DDW.

Procedure

1. The Ni-NTA column purified capsid protein in the denaturing elution buffer (~10ml) was slowly added drop by drop into the protein refolding buffer (~ 40ml) kept in a beaker at room temperature to get a final urea concentration of 2M and the solution was stirred continuously for around one hour by using teflon coated magnetic bar over the magnetic stirrer.
2. Then the refolded protein was dialyzed by using Snake Skin Pleated Dialysis tubing (3.5 k Da, Fisher Scientific) against several changes of Phosphate buffered saline (PBS pH 7.4).
3. The protein was concentrated by keeping the dialysis bags in PEG-8000 (polyethylene glycol 8000) which absorbed the PBS and reduced the volume.
4. Then the concentrated protein was recovered from the dialysis bag and protease inhibitor cocktail was added and stored at -80°C deep freezer till further use.

3.11 Solutions for Western Blotting

Transfer buffer

Tris Base	25 mM
Glycine	192 mM
Methanol	20%
SDS (Sodium dodecdyl sulphate)	0.1%

Blocking buffer

Bovine Serum Albumin (BSA)	0.5%
Skimmed milk powder	5%
Lactalbumin Hydrolysate (LAH)	1%
Dissolved in PBS	

Antibody dilution buffer

50% of Blocking buffer in PBS.

Primary antibodies

1. Porcine circovirus Type-2 (PCV-2) antisera (porcine origin) (VMRD) (Cat .No. PAB –PCV-2)

2. Anti- His Tag Protein mouse antibody (Calbiochem) (Cat.No. D00159204)

Secondary antibodies

1. Anti-pig IgG-HRP (Sigma) rabbit raised (Cat.No. A5670-1ML) was used as secondary antibody in western blot, dot blot and in Indirect ELISA.
2. Goat Anti –Mouse IgG-HRP (Santa cruz), (Cat .No. sc-2005)

Stable DAB

Readymade solution of diamino benzidine (DAB Stable, Invitrogen) was used.

3.12 Western Blot analysis of the recombinant capsid protein

Expressed recombinant PCV-2 ORF-2 protein was characterized by western blot analysis in order to confirm the specificity/reactivity with PCV antisera as well as Anti-His antibody. The following protocol was used to perform the western blotting:

3.12.1 Transfer of proteins into PVDF membrane

1. The protein samples were subjected to SDS-PAGE along with prestained protein marker (Puregene) as mentioned earlier in the section 3.5.
2. After electrophoresis the gel was taken out from the plates, washed twice with double distilled water and then kept on transfer buffer.
3. On the other side, cassette was placed with the gray side down on a clean surface and pre-wetted fibre pad was then kept on the gray side of the cassette of Mini Trans blot cell module, Biorad (Cat.No. 1703810)
4. A sheet of filter paper was placed on the fibre pad and the equilibrated gel was placed on the filter paper.
5. After that, the pre-wetted PVDF membrane (Novex) was placed on the gel and the sandwich was completed by placing a piece of filter paper on the PVDF membrane. The PVDF membrane was dipped in 20% methanol for 1 min before use, dried and then equilibrated in transfer buffer for 20 minutes. At last, the last fiber pad was placed. Air bubble, if any was removed using roller.
6. The cassette was closed firmly, being carefully not to move the gel and filter paper sandwich. The cassette was locked using white latch.
7. The cassette module was placed in an assembly in the proper direction and frozen blue cooling unit was placed in the buffer tank, to keep the buffer cool during electrophoresis.
8. Then the buffer tank was filled with transfer buffer upto the blotting mark.

9. Teflon coated magnetic bar was placed at the bottom of the assembly. The assembly was placed onto the magnetic stirrer to maintain even buffer temperature and ion distribution in the tank. The speed was set to keep ion distribution even.
10. The electrophoresis was done at 100 V, constant 350 mA for 2 hrs.
11. After transfer, the gel was stained with staining solution to check the efficiency of transfer and the membrane was subjected to immunological detection.

3.12.2 Development of the Western Blot

1. The membrane, after transfer, was blocked by incubating over night at 4°C in presence blocking buffer prepared in PBS.
2. After blocking, the membrane was washed thrice with PBS for 5 minute each, and incubated with 1:200 diluted PCV-2 antisera, porcine origin (primary antibody) or anti –His Tag protein mouse antibody(1µg/ml) for 1hr at 37°C on a dancing shaker.
3. Washed the membrane thrice with PBS as mentioned above and secondary antibody (anti pig IgG HRP (1:10,000 dilutions) or anti- mouse –IgG-HRP (1µg/ml) was added, and again incubated it for 1 hr on a dancing shaker.
4. After three washings with PBS, the antigen antibody reaction was detected by incubating the membrane with 10-15 ml of stable DAB (Invitrogen).
5. The colour reaction was terminated by washing the membrane with distilled water to prevent background colouration.

3.13 Analysis of recombinant PCV-2 ORF-2 protein by Dot blot

Procedure

1. A strip of nitrocellulose membrane (NCM) or NCM comb was used.
2. The recombinant protein (112 ng/µl) was blotted into the NCM comb along with the uninduced cell lysate.
3. Samples without primary antibody and secondary antibody were kept as primary antibody and secondary antibody control, respectively.
4. Then the membrane was allowed to air dry for 15-20 minutes.
5. Then the membrane was blocked with blocking buffer for 1 hour at room temperature on a dancing shaker. Then the blocking buffer was removed and the membrane was washed once with PBS (pH7.4).

6. Primary antibody, PCV antisera (porcine origin) at 1:200 dilution or anti His Tag protein mouse antibody at 1µg/ml concentration prepared in antibody dilution buffer was used and the membrane was incubated for 1 hr at room temperature.
7. The membrane was then washed 3 times (5 minutes each) with PBS (pH7.4) on a dancing shaker.
8. Then the membrane was incubated with secondary antibody anti pig IgG HRP at 1:10,000 dilution or anti- mouse –IgG-HRP (1µg/ml) prepared in antibody dilution buffer for 1 hour at room temperature.
9. Again the membrane was washed 3 times (5 minutes each) with PBS (pH7.4) on a dancing shaker.
10. The blot was developed by using stable DAB (invitrogen).
11. After development of the blot the membrane or the NCM comb was washed with distilled water and photograph was taken.

3.14 Development of recombinant PCV-2 ORF-2 protein based Indirect ELISA

3.14.1 Reagents for Indirect ELISA

100 mM Carbonate-bicarbonate buffer (pH 9.6) for antigen coating

Na_2CO_3	3.03 g
$NaHCO_3$	6 g
DDW	1000 ml

PBS (pH 7.2)

NaCl	8.0 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g
DDW	1000 ml

Autoclaved at 15 lbs/sq inch for 15 min.

Blocking buffer

Bovine Serum Albumin (BSA)	0.5%
Skimmed milk powder	0.5%
Lactalbumin Hydrolysate (LAH)	1%

Dissolved in PBS

Substrate solution

One tablet of Sigmafast buffer with urea H_2O_2 (golden foil) and one tablet of Sigmafast OPD (silver foil) of the Sigmafast OPD tablet set (P9187-50SET) were properly dissolved in 20 ml of distilled water in a amber coloured 50 ml centrifuge tube and used immediately.

3 M H_2SO_4 stop solution

Volume of H_2SO_4 (mol wt 98)	29.4 ml
DDW upto	100 ml

3.14.2 Procedure:

1. Flat-bottomed (Nunc, Maxisorp) plate was coated with 50µl of antigen (purified, diluted ORF-2 protein) by mixing it with coating buffer and the plate was kept at 4ºC overnight.
2. Next morning the antigen was discarded and 150 -200 µl blocking buffer (5% Skimmed milk in PBS) was added into each well and incubated at 37ºC in a ELISA plate shaker for 1 hr at ~40rpm.
3. Washing was done thrice with PBS (pH7.4) buffer and plate was tapped dry over blotting paper pads.
4. 50 µl of the primary antibody (porcine raised PCV-2 antisera) diluted in antibody dilution buffer (50% of blocking buffer in PBS) was added into the wells keeping appropriate controls.
5. The plate was covered with the plastic cover and incubated in a ELISA plate shaker for 1 hr at 37°C with constant mixing at 40 rpm.
6. After incubation the plate was washed with PBS (pH7.4) buffer thrice and then the plate tap dried.
7. Secondary antibody (Anti-pig IgG-HRP) diluted 1:20,000 in antibody dilution buffer was added @ 50 µl per well. The plate was incubated for one hour as mentioned above.
8. Again the plate was washed with PBS (pH7.4) buffer thrice and then tap dried.
9. 200µl of OPD substrate was added into the wells and kept at room temperature till the color appears (10 – 30 mins).
10. The reaction was stopped following colour development by adding 50µl of 3M H_2SO_4 into each well and the plate was read spectrophotometrically at 492nm on Synergy H1 Hybrid Reader (BioTek).

3.14.3 Optimization of antigen and antibody concentration

a) Coating Antigen

The optimum antigen concentration was selected by checkerboard titration using two fold serial dilution of antigen (recombinant capsid protein). Ni-NTA column purified recombinant PCV-2 ORF-2 protein (conc. 560 ng/µl) was serially diluted 2 fold (1:2 to 1:4096) in bicarbonate-carbonate coating buffer (pH 9.6) and coated the ELISA plate by putting 50 µl diluted antigen per well.

b) Antibody Optimisation

Primary antibody (porcine raised PCV-2 anti sera) at dilution 1:50, 1:100, 1:200 and 1:400 were tested for reactivity. Anti- pig IgG HRP-conjugate at 1:20,000 dilutions was used every time as the secondary antibody.

c) Standard Curve of Indirect ELISA

The O.D. values obtained against different dilutions of primary antibody and different antigen concentrations were plotted to draw a standard curve and the coefficient of determination (R^2) values were derived.

d) Indirect ELISA for field sample testing

The procedure for Indirect ELISA was followed as described earlier in Section 3.12.2. The known positive and the negative pig sera samples tested earlier by commercial ELISA kit (Pig Circovirus Ab ELISA, Glory Sciences Co.,ltd) were used at 1:50 dilutions. The secondary antibody (Anti-pig IgG HRPO) was used at 1:20,000 dilutions.

BACULOVIRUS MEDIATED INSECT CELL EXPRESSION OF PCV-2 CAPSID PROTEIN (ORF-2)

3.15. Generation of recombinant pFast HBM-TOPO entry clones containing gene of interest

3.15.1 PCR Amplification of PCV-2 *ORF-2* (capsid) gene codon optimized gene

The following reaction mixture was prepared for amplification of *ORF-2* gene using new set of primers. The following primers were used for the amplification the *ORF-2* gene.

PCV-HBM-F - 5’ACTTACCCCGCCGTTAC 3’

PCV-HBM-R- 5’TTTCGGGTTGAGGGGTGGGTCTT 3’

Composition	Amount
Nuclease free water (NFW)	32.75µl
10X buffer (Platinum pfx buffer)	5.0 µl
Enhancer	5.0 µl
$MgSO_4$ (50 mM)	1.5 µl
dNTPs (10 mM)	2.0 µl
Forward primer (PCV-HBM-F) 20 pmol/µl	1.0 µl
Reverse primer (PCV-HBM-R) 20 pmol/µl	1.0 µl
Template (plasmid)	1.0 µl
Platinum Pfx DNA polymerase(2.5 U/µl)	1.0µl
Total	**50.0 µl**

The following conditions were used for the amplification of PCV-2 *ORF-2* gene in a thermocycler (Eppendorf).

Steps	Temperature	Time	Cycles
Initial denaturation	94 ^oC	8 min	1
Denaturation	94 ^oC	45 sec	35
Annealing	56 ^oC	45 sec	
Extension	68 ^oC	3 min	
Final extension	68 ^oC	10 min	1

Amplified PCR product was analyzed by 1 % agarose gel electrophoresis.

3.16 Purification of PCR amplified products by agarose gel electrophoresis

The PCR amplified products were analysed by agarose gel electrophoresis as per the procedure mentioned in the section 3.2.5.2. The desired band (PCR product) was cut out of the gel using a scalpel under the UV transilluminator and kept in a MCT. The PCR products were gel purified using commercial kit (Wizard SV Gel and PCR Clean up system from Promega) as mentioned in the section 3.2.6.

3.17 Blunt end cloning of PCV-2 *ORF-2* (capsid) gene into pFastBacHBM-TOPO cloning vector (Life technologies)

The pFastBacHBM-TOPO vector allows the rapid generation of an expression construct containing the gene of interest under the control of a baculovirus-specific

strong polyhedrin (PH) promoter and in frame with the Honey Bee Mellitin (HBM) secretion signal coding sequence. The expression of the gene of interest is controlled by the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) polyhedrin (PH) promoter for high-level expression in insect cells. This expression cassette is flanked by the left and right arms of Tn7. The cassette also contains a gentamicin resistance gene andan SV40 polyadenylation signal to form a mini Tn7. The presence of the N-terminal Honey Bee Mellitin (HBM) secretion signal coding sequence on the plasmid facilitates the secretion of the cloned gene product into the extracellular medium and the C-terminal polyhistidine tag allows easy purification of the secreted protein.

3.17.1 Ligation of purified PCR products with pFastBacHBM-TOPO cloning vector (Fig. 3)

The gel purified, codon optimized *ORF-2* gene fragment was ligated into pFastBacHBM-TOPO vector in a vector insert molar ratio of 1:2 as follows:

$$\text{ng insert required} = \frac{\text{ng vector x kbp insert}}{\text{Vector size (kbp)}}$$

pFastBac/HBM-TOPO vector (10ng/μl)	1.0 μl
Insert PCV-2 *ORF-2* gene fragment (5ng/μl)	0.9 μl
Salt solution	1.0 μl
NFW	3.1 μl
	6.0μl

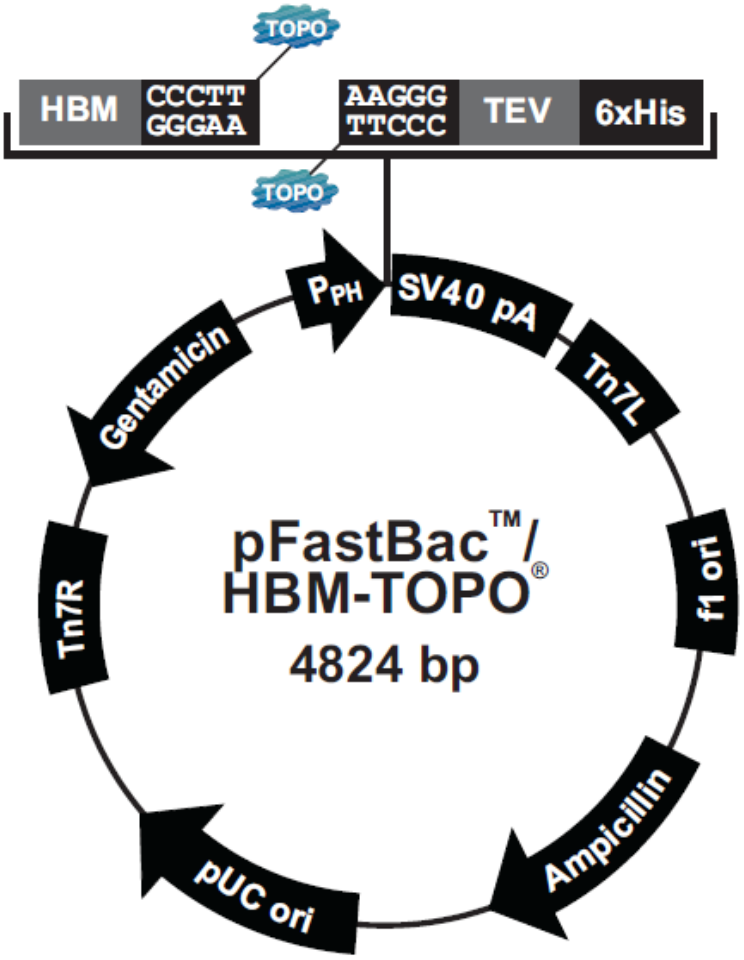
The ligation mixture was incubated at 22ºC for 3-4 hours in an eppendorf thermocycler.

3.17.2 Transformation of DH5α competent cells

The ligated product was transformed into DH5**α** strain of *E. coli* as per the protocol given by Sambrook *et al* (2001) described in the section 3.2.2.

3.17.3 Screening of recombinant clones by colony PCR

The clones were screened by PCR using combination of gene specific and sequencing primers for the presence of insert. The following reaction mixture was prepared for amplification of PCV-2 *ORF-2* gene by *Taq* DNA polymerase (Invitrogen) using a combination of two sets of primers. The first combination was gene specific PCV-HBM forward primer and the universal SV40 Poly A reverse



Comments for pFastBac™/HBM-TOPO® vector
4824 nucleotides

Polyhedrin promoter (P_{PH}): bases 1-129
Honey Bee Mellitin (HBM) secretion signal: 141-210
TOPO cloning site: bases 215-216
TEV recognition site: bases 222-242
6xHis tag: bases 243-260
SV40 polyadenylation signal: bases 305-545
Tn7L: bases 574-739
f1 origin: bases 923-1377
Ampicillin resistance gene: bases 1508-2368
pUC origin: bases 2513-3186
Tn7R: bases 3432-3656
Gentamicin resistance gene: bases 3723-4256 (complementary strand)

Fig. 3: Map and features of pFastBacHBM-TOPO Vector

primer and the second was polyhedrin forward sequencing primer and gene specific PCV-HBM reverse primer.

PCV-HBM-F : 5′-ACTTACCCCGCCGTTAC 3′

SV40 Poly A-R : 5′-GGTATGGCTGATTATGATC-3′

PCV-HBM-R : 5′-TTTCGGGTTGAGGGGTGGGTCTT-3′

Polyhedrin-F : 5′-AAATGATAACCATCTCGC-3′

Composition	**Amount used**
Nuclease free water (NFW)	40 µl
$MgCl_2$ (50mM)	1.5 µl
10X Buffer	5.0 µl
dNTPs (10 mM)	1.0 µl
PCV-HBM-F or Polyhedrin -F (20pmol/µl)	1.0 µl
PCV-HBM-R or SV40 Poly A-R (20pmol/µl)	1.0 µl
Template (clone)	Bacterial colonies picked up by pipette tips
Taq DNA polymerase(5U/µl, invitrogen)	0.5 µl
Total volume	50 µl

The following conditions were used for the amplification of PCV-2 *ORF-2* gene in a thermocycler (Eppendorf).

Steps	**Temperature**	**Time**	**Cycles**
Initial denaturation	94 ^{o}C	6 min	1
Denaturation	94 ^{o}C	30 sec	35
Annealing	50 ^{o}C	45 sec	
Extension	72 ^{o}C	1.30 min	
Final extension	72 ^{o}C	8 min	1

The PCR amplified products were subjected to 1.5% agarose gel electrophoresis.

3.17.4 Selection of the clones

The positive white clones showing the presence of desired gene insert detected by PCR were picked by sterile pipette tips and inoculated separately in to 10 ml Luria-Bertani (LB) broth containing ampicillin (100µg/ml) and incubated at 37^{o}C overnight in a shaker incubator. After overnight incubation plasmids were isolated from the broth cultures.

3.17.5 Plasmid Isolation by alkaline lysis method (Sambrook *et al* 2001)

Procedure

The Bacterial plasmids were extracted from the broth cultures as per the method of Sambrook *et al* (2001) with slight modification already described in the section 3.2.4.

3.17.6 Transformation of DH10Bac *E. coli* competent cells

Requirements for transformation and cloning

LB Agar plates with tetracycline, Kanamycin, Gentamycin, X-Gal (5-bromo-4chloro-3-indolyl-β-D-galactopyranoside) and IPTG (Isopropyl β-D-1-thiogalactopyranoside):

LB Agar(Himedia)	40.0 g
DDW	1000 ml

After autoclave, at 55ºC temperature, tetracycline, Kanamycin, Gentamycin, X-gal and IPTG were added.

Tetracycline Stock

Tetracycline	10.0 mg
Ethanol (100%)	1.0 ml

Filtered by filter (0.2 µm) under aseptic conditions.

Gentamycin Stock

Gentamycin	7.0 mg
DDW	1.0 ml

Filtered by filter (0.2 µm) under aseptic conditions.

Kanamycin Stock

Kanamycin	10.0 mg
DDW	1. 0 ml

Filtered by filter (0.2 µm) under aseptic conditions.

X-gal

X-gal	40.0 mg
Dimethyl formamide	1.0 ml

IPTG (100 mM)

IPTG	0.238 g
DDW	10.0 ml

Filtered by filter (0.2 µm) under aseptic conditions.

Once pFastBac HBM construct containing gene of interest was constructed, transformed purified plasmid DNA into DH10Bac *E. coli* competent cells for transposition into the bacmid.

Procedure:

DH10Bac competent cells were transformed by the following method.

1. Thawed on ice one vial of MAX Efficiency DH10Bac competent *E. coli* cells for each transformation.
2. Added appropriate amount of plasmid DNA (1ng) to 100 µL of DH10Bac cells and mix gently.
3. Incubated the cells on ice for 30 minutes.
4. The cells were subjected to heat shock for 45 seconds at 42ºC in a preequilibrated water bath.
5. Then the tube was immediately transferred to ice and kept for 2-3 minutes.
6. Then 500 µl of SOC medium was added to the tube and shaked the tubes at 200 rpm for 4-5 hrs at 37ºC in an incubator.
7. Plated 100 µL of transformed culture onLB agar plate containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 100 µg/ml X-gal, and 40 µg/ml IPTG.
8. Incubated the plates for 48 hours at 37ºC in an incubator.

3.17.7 Selection of clones

Colonies containing the recombinant bacmid were white in a background of blue colonies that harbored the unaltered bacmid. Selected white colonies for analysis**.** True white colonies tend to be large. White colonies were picked and restreaked on fresh LB agar plates containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10µg/ml tetracycline, 100 µg/ml X-gal, and 40 µg/ml IPTG. Incubated the plates overnight at 37ºC in an incubator.

3.17.8 Screening of the clones by colony PCR

The clones were screened by PCR using sequencing primers for the presence of desired insert in the bacmid. The following reaction mixture was prepared by using *Taq* DNA polymerase (Invitrogen) and pUCM13 forward and reverse sequencing primers (Fig 4).

pUCM13 Forward 5′-CCCAGTCACGACGTTGTAAAACG-3′

pUCM13 Reverse 5′-AGCGGATAACAATTTCACACAGG-3′

Composition	Amount
Nuclease free water (NFW)	39.75 µl
$MgCl_2$ (50mM)	1.5 µl
10X Buffer	5.0 µl
dNTPs (10 mM)	1.0 µl
pUC/M13 Forward(20pmol/µl)	1.0 µl
pUC/M13 Reverse(20pmol/µl)	1.0 µl
Template (clone)	Bacterial colonies picked up by pipette tips
Taq DNA polymerase(5U/µl, invitrogen)	0.75 µl
Total volume	50 µl

The following conditions were used for PCR amplification of the recombinant bacmid in a thermocycler (Eppendorf).

Steps	Temperature	Time	Cycles
Initial denaturation	94 ^{o}C	3 min	1
Denaturation	94 ^{o}C	45 sec	35
Annealing	55^{o}C	45 sec	
Extension	72 ^{o}C	5 min	
Final extension	72 ^{o}C	7 min	1

3.17.9 Selection of the clones

After PCR, the amplified product was run in 0.8% agarose gel. The selection of the positive clones was done on the bases of required size. Positive white clones showing desired gene insert in PCR were picked by sterile pipette tips and inoculated separately in to 100 ml Luria-Bertani (LB) broth containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 100 µg/ml X-gal, and 40 µg/ml IPTG and incubated at 37^{o}C overnight in a shaker incubator. After overnight incubation bacmid were isolated from the broth cultures by alkaline lysis method as per the protocol described earlier in the section 3.2.4. After the isolation of the bacmid, it was kept in the water bath at 70ºC for 15-20 mins, to make it endotoxin free. After that the bacmid was stored at 4ºC till further use.

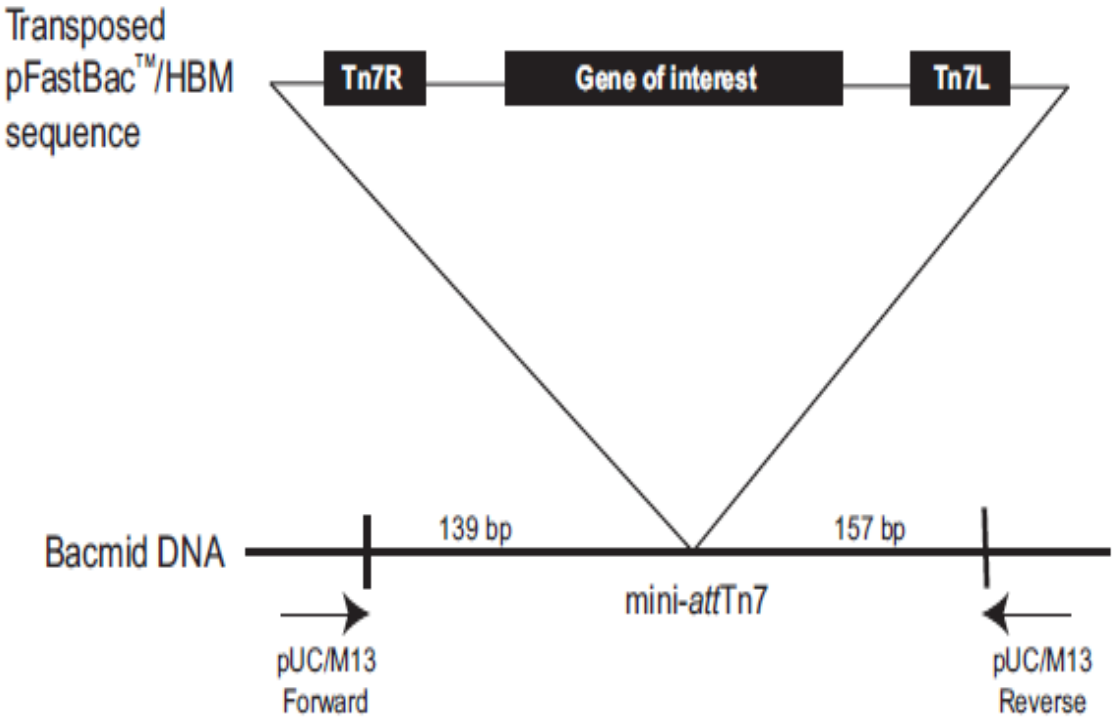


Fig. 4: Analyzing the recombinant bacmid DNA by PCR

3.18 Culturing of the Sf-9 (*Spodoptera frugiperda)* insect cells

3.18.1 Culture media compostion

SF-900 II SFM (Gibco, U.S.A)	100ml
Fetal bovine serum	10 ml
L-glutamine (50mg/ml)	500 mg
Gentamycin (7µg/ml)	0.07 ml

Filtered by filter (0.2 µm) under aseptic conditions.

3.18.2 Initiation of Sf -9 Cell Culture from Frozen Stock

1. One vial of Sf-9 cells were taken out of the liquid nitrogen cryocan and immediately thawed by placing it in perwarmed double distilled water at 37ºC in a beaker.
2. Thawed rapidly with gentle agitation until cells were almostthawed.
3. The vial of cells was removed from the beaker and placed in a biosafety cabinet.
4. The outside of the vial was decontaminated by treating with 70% ethanol.
5. 1 ml cell suspension was directly transferred into the 9 ml of SF-900 II SFM medium in a 15 ml centrifuge tube.
6. The cell suspension was then centrifuged at 600 rpm for about 10 minutes at 26 ºC.
7. The supernatant containing the freezing mixture was discarded and the the pellet was resuspended in 5 ml of fresh medium and transferred to 25 cm^2 flask.
8. The cells were then observed under inverted microscope and the flask was incubated at 27 ºC in a incubator.
9. The medium was changed after 24-48 hrs and if needed subculturing was done.

3.18.3 Subculturing Monolayer Cultures

1. The medium and the floating cells were aspirated and discarded cells from a 80% to 90% confluent monolayer.
2. To each 25 cm^2 flask, added 4 to 6 ml of SF-900 II SFM (Gibco, U.S.A.) containing 10% serum, L-Glutamine and sterile antibiotic (gentamicin @7µg/ml) equilibrated to room temperature.
3. Cells were resuspended by pipetting the medium across the monolayer with a disposable 5 ml Pasteur pipette.
4. The cell culture flask was observed using an inverted microscope to ensure adequate cell detachment from the surface of the flask.

5. The viable cell count of harvested cells (e.g., using a hemocytometer and trypan blue dye exclusion) was determined.
6. Cells were then inoculated at 2×10^4 to 5×10^4 viable cells/cm^2 into 25 or 75-cm^2 flasks.
7. Cultures were then incubated at 27°C ± 0.5°C in the incubator.
8. The flasks were again subcultured when the monolayer reached 80% to 100% confluency, approximately 2 to 4 days, after first subculturing.

3.19. Culturing of the High Five insect cells

High Five cell line was used for high level expression of recombinant proteins. They were grown in Express Five serum-free medium and were adapted to suspension culture for producing high levels of recombinant protein.

3.19.1 Initiation of Hi-five Cell Culture from Frozen Stock

The Hi-Five cell culture was initiated from the frozen culture as per the the protocol described earlier in the section 3.15.1. The Hi-Five cells were cultured in Express Five SFM (Gibco, U.S.A).

3.19.2 Adapting Monolayer Cells to Suspension Culture

Insect cells are not generally anchorage dependent, they adapt easily to suspension culture conditions. It is important to proceed slowly when adapting stationary cultures to suspension culture. A drop in viability and increased clumping was observed through the first three to five passages. 6-10 confluent 75-cm2 monolayer flasks were sufficient to initiate a 100-ml suspension culture.

Protocol

1. The cells were dislodged from the bottom of the flasks by pipetting repeatedly.
2. The cell suspension was pooled and the viable cell count was determined using a haemocytometer by trypan blue dye exclusion method.
3. The cell suspension was then diluted to approximately 5×10^5 viable cells/ml in complete serum-supplemented or serum-free growth medium equilibrated to room temperature.
4. Incubated at 27.0°C ± 0.5°C with a stirring rate of 80 rpm in shaker flasks.
5. The cells were subcultured when the viable cell count reached 1×10^6 to 2×10^6 cells/ml (3 to 7 days post-subculturing).

3.20 Transfection of the insect cells by recombinant baculovirus

The recombinant bacmids carrying gene of interest were transfected into the insect cells to produce recombinant baculovirus.

3.20.1 Transfection conditions

Condition	Amount
Tissue culture plate size	6-well (35 mm) plate (Nunc) (one well/bacmid)
Sf -9 cells to transfect	8 $x10^5$ cells/well
Bacmid DNA	2-3 µg
Cellfectin reagent	10 µL

Procedure:

1. The cell density was determined (1.5 $x10^6$–2.5 x 10^6 cells/ml) by using a hemocytometer and 8 x 10^5 Sf-9 cells were seeded in each well of 6- well cell culture plate.
2. The SF-900 II SFM (Gibco, U.S.A) was removed from the cells and the cells were then washed with Grace's Insect Medium, Unsupplemented (without antibiotics and serum).
3. Two ml of Grace's Insect Medium, Unsupplemented (without antibiotics and serum) was added in each well.
4. The cells were allowed to attach for 15 minutes at room temperature in the hood.
5. The cellfectin II Reagent was mixed and 10 µl was diluted in 100 µl of Grace insect medium unsupplemented (without antibiotics and serum). Vortexed briefly to mix.
6. Three µg baculovirus DNA was diluted in 100 µL Grace's Insect Medium, unsupplemented (without antibiotics and serum) and mixed gently.
7. The diluted DNA was mixed with diluted cellfectin II (total volume ~210 µL). The mixture was vortexed briefly and incubated at room temperature for 15-30 minutes.
8. ~210 µL DNA-lipid mixture or transfection mixture was added dropwise onto the cells (prepared in step 4) and the cells were incubated at 27°C for 3–5 hours.
9. The transfection mixture was removed and replaced it with 2 ml of complete growth medium (Grace's Insect Medium, Supplemented and 10% FBS and gentamicin antibiotic @ 7µg/ml).

10. The cells were incubated at 27°C for 72 hours or until the signs of viral infection were seen.

3.20.2 Isolation of the P1 viral stock

Budded virus was released into the medium 72 hours after transfection.

Procedure

1. The transfected cells showed the signs of late stage infection i.e., cells stopped growing, appearance of virus budding and vesicular appearance of cells, after 72 hrs of transfection.
2. The medium containing the virus was collected from each well in 15 ml of centrifuge tubes.
3. Centrifuged at 500 x *g* for 5 minutes to remove the cells and the debris.
4. The clarified supernatant was transferred to 15 ml of centrifuge tubes. This was the P1 viral stock. Stored at 4ºC, protected from light.

3.20.3 Amplification of the P1 baculovirus stock

The P1 viral stock is a small-scale, low-titer stock. So, this stock was used to infect Sf-9 cells to generate a high-titer P2 stock.

Procedure:

1. 250 µl P1 viral stock was added to the cells in a 25 cm^2 flask (vented cap, Nunc).
2. The cells were then incubated at 27ºC in a humidified incubator.
3. 5 ml of medium containing virus from the flask was collected after 48 hrs of infection and transferred the virus to sterile 15 ml centrifuge tubes. The tubes were centrifuged at 500 x *g* for 5 minutes to remove cells and large debris and clarified baculoviral stock was obtained i.e., P-2 viral stock. Stored at 4ºC, protected from light.

3.20.4 Amplification of the P-2 baculovirus stock

P-2 baculovirus stock was used to produce high titer P-3 baculovirus stock as per the procedure described earlier in the section 3.20.3.

3.21 Production of PCV-2 ORF-2 recombinat protein in the High-Five cells

The P-3 baculovirus stock was used to infect the High-five insect cells for the expression of recombinant protein. The High-Five cells adapted to suspension culture in the Express Five serum free medium were used for expression of the secreted protein. The Hi-Five cell line provided 5-10 fold higher expression than the Sf-9 cells.

Protocol:

1. Added the pFastBac HBM-TOPO P-3 baculovirus stock to the shaker flask at the desired MOI. The positive control, pFastBac Gus control baculovirus previously characterized recombinant baculovirus was also included.
2. The shaker flasks were incubated at 27°C with a spin rate of 80 to 90 rpm.
3. 5 ml aliquots of cells were removed at designated time points i.e., at 72 hrs and 96 hrs and transferred to 15 ml of centrifuge tubes.
4. The cells were centrifuged at 800 x g for 10 minutes at 4°C. The samples were kept at 4°C or on ice to prevent proteolysis.
5. The supernatant was transferred to a new tube.
6. The tubes containing the supernatant and cell pellet were labeled properly and kept at 4ºC.

3.21.1 Analysis of the recombinant capsid protein by SDS –PAGE

3.21.1.1 Preparation of cell lysates by detergent lysis method

Detergent lysis is a quick and efficient way to lyse cells and extract intracellular protein.

Composition of the lysis Buffer:

NaCl	8.0 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g
Triton X-100	0.1%
DDW	1000 ml

Procedure:

1. The cell pellets from the different time courses were placed on ice along with the control sample (Serum free media control).
2. The cell pellets were then resuspended in 1ml of lysis buffer.
3. Added protease inhibitor phenylmethylsulfonylfluoride (PMSF) at the working concentration of 1Mm to the samples having the stock concentration of 100 Mm.
4. Vortexed each cell sample to break up the cell pellet to begin lysis.
5. The samples were then incubated on ice for 30–45 minutes, then vortexed at 10 minute intervals to assist lysis.

6. Then, pelleted the cellular debris at 1,000 x*g* for 10 minutes at 4°C.
7. The supernatant was collected for SDS-PAGE analysis. 2X SDS loading buffer was added and samples were boiled for 10 minutes at 95ºC.

3.21.1.2 Preparation of supernatant protein sample for SDS-PAGE analysis

The protein sample from the supernatant was prepared using Trichloroacetic acid (TCA) precipitation method.

TCA stock solution: 100% (w/v) Trichloroacetic acid (TCA)

Recipe: dissolved 10 g TCA into 10 ml of DDW. Stored at room temperature.

Procedure:

1. Added 1 volume of TCA stock to 4 volumes of protein sample.
2. Incubated for 1 hour at 4°C.
3. Centrifuged the tubes at 14,000 rpm, 15 minutes.
4. Supernatant was removed leaving protein pellet intact.
5. Pellet was washed with 200µl cold acetone.
6. Then centrifuged at 14,000 rpm for 5minutes.
7. Repeated steps 4-6 for a total of 2 acetone washes.
8. Pellet was dried by placing tubes in 95°C heat block for 5-10 min to drive off acetone.
9. For SDS-PAGE analysis, 2X SDS loading buffer was added and samples were boiled for 10 minutes at 95ºC.

3.21.2 Solutions and buffers for SDS-PAGE analysis

The solutions for SDS-PAGE were prepared as per the protocol described earlier in the section 3.4.

3.21.3Expression study of recombinant PCV-2 ORF-2 protein by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis

SDS-PAGE analysis was carried out in a vertical minigel electrophoresis apparatus (Bio-Rad) as per the the protocol described earlier in the section 3.5.

3.22 Protein purification under denaturing conditions from the cell lysate

3.22.1 Solutions for protein purification

The buffers used for the protein purification from the cell lysate were prepared as mentioned in the section 3.8.

3.22.2 Preparation of the lysate

1. The cells after 96 hrs of infection were pelleted at 800 x *g* for 10 minutes at 26ºC.
2. The cell pellets were then resuspended in 2ml of the denaturing lysis buffer.

3. The tube containing cell suspension was stirred for 15-60 minutes in a rotary mixer (Tarsons) at room temperature with gentle vortexing intermittently, taking care to avoid foaming. Cell lysis was complete when the solution became translucent.
4. The lysate was centrifuged at 10,000 rpm for 30 min at 4°C to pellet the cellular debris.
5. After centrifugation, the supernatant was transferred to a new tube for protein purification by Ni-NTA column chromatography.

3.22.3 Preparation of the column

The Ni-NTA purification column was prepared as per the protocol mentioned in the section 3.7.2.

3.22.4 Purification of the protein

The protein purification under the denaturing conditions was carried out as per the protocol mentioned in the section 3.7.2.

3.22.5 SDS-PAGE analysis of purified PCV-2 ORF-2 recombinant protein

SDS-PAGE analysis was carried out in a vertical minigel electrophoresis apparatus (Bio-Rad) as mentioned earlier in the section 3.5. Here the samples were non-infected cell lyaste, purified PCV-2 ORF-2 protein samples, wash flow throughs at different stages of washing, positive Gus control cell lysate.

3.23 Protein refolding

The protein refolding was carried out as per the protocol mention in the section 3.10.

3.2.4 Analysis of recombinant PCV-2 ORF-2 protein by Dot blot

Procedure

1. A strip of nitrocellulose membrane (NCM) or NCM comb was used.
2. Different concentrations of recombinant protein (1 µl) was blotted into the NCM comb along with the non- infected cell lysate, positive GUS control and the PBS control.
3. Then the membrane was allowed to air dry for 15-20 minutes.
4. Then the membrane was blocked with blocking buffer for 1 hour at room temperature on a dancing shaker. Then the blocking buffer was removed and the membrane was washed once with PBS.
5. Primary antibody Anti His Tag protein mouse antibody at 1µg/ml concentration prepared in antibody dilution buffer was used and the membrane was incubated for 1 hr at room temperature.

6. The membrane was then washed 3 times (5 minutes each) with PBS on a dancing shaker.
7. Then the membrane was incubated with secondary antibody Anti-mouse-IgG-HRP (1µg/ml) prepared in antibody dilution buffer, for 1 hour at room temperature.
8. Again the membrane was washed 3 times (5 minutes each) with PBS on a dancing shaker.
9. The blot was developed by using stable DAB (invitrogen).
10. After development of the blot the membrane or the NCM comb was washed with distilled water and photograph was taken.

CHAPTER IV

RESULTS AND DISCUSSION

Postweaning multisystemic wasting syndrome (PMWS) is nowdays considered as a global endemic disease causing considerable economic losses to the swine industry. Porcine circovirus type-2 is the major causative agent of PMWS and also of the dermatitis and the neuropathy syndrome, respiratory disease complex and reproductive abnormalities in pigs.

In case of many viral diseases, the immune response is largely directed against the structural proteins of the virus which are present on the virion envelope and on the surface of the infected cells. These proteins can be produced by recombinant methods to use as a diagnostic antigen instead of whole viral antigen.

Secondly, a highly sensitive and specific large scale screening test like ELISA is a prerequisite to any disease control programme. Currently available PCV-2 diagnostic kits in India are all imported and costly thus hampering the sero-surveillance of the PCV-2 in India, so there is a need to develop cheap indigenous ELISA kits for routine diagnosis. Considering the severity of the PCV-2 infection, it is important to diagnose the disease as early as possible using highly sensitive and specific assays.

Keeping these points in view, an attempt has been made to obtain a high level expression of the recombinant ORF-2 (capsid) protein in the bacterial as well as baculovirus expression system by cloning the codon optimized *ORF-2* gene into the expression vectors and further to characterize its potential as a diagnostic antigen by dot blot, western blot and indirect ELISA.

4.1 Bacterial expression of PCV-2 *ORF-2* codon optimized synthetic gene

4.1.1 Release of the codon optimized PCV-2 *ORF-2* coding sequences from the PUC57 plasmids by RE digestion and linearization of PET 302 NT His expression vector

The *Eco* RI and *Bam* HI double RE digestion could successfully release the codon optimized *ORF-2* gene insert from the recombinant pUC 57 plasmid with a product size of ~720 bp (Fig. 5a) and linearized the pET 302 NT His expression vector with a product size of ~5712 bp (Fig.5b). Both products were then gel purified successfully using the commercial gel extraction kit (Promega), as evident by the

specific band of the eluted product in the agarose gel electrophoresis. Ligation of the eluted products with a vector insert molar ratio of 1:2 and then transformation into DH5α competent *E. coli* cells resulted in development of numerous white colonies on the selective medium (LB agar plates with ampicillin) indicating optimum transformation efficiency. The recombinant white clones thus obtained following overnight incubation were screened for the presence of desired insert.

4.1.2 Screening of recombinant pET 302NT His clones by colony PCR

The white colonies that appeared on the LB ampicillin (100µg/ml) plates after overnight incubation were then screened by PCR using, T7 promoter forward and T7 reverse sequencing primers. PCR amplification resulted in a band of ~952 bp size on 1% agarose gel (Fig. 6). The correct orientation of the gene insert in frame with the N-terminal 6X His tag of the pET 302 NT His vector was further confirmed by commercial sequencing of the plasmids extracted from the PCR positive clones.

4.1.3 Expression of PCV-2 *ORF-2* gene in BL21 DE3 Star *E. coli* cells

The positive clones screened were picked up with the help of sterile pipette tips and were grown in LB broth containing ampicillin and plasmids were isolated from the cultured clones. The purified plasmid carrying the PCV-2 *ORF-2* gene (lacking the nuclear localization signal) in pET302 NT His expression vector was then used to transform the competent BL21DE3 star competent *E. coli* cells for protein expression purpose. The transformed mixture was then plated over the LB ampicillin (100µg/ml) plates. 5-6 white colonies were selected randomly and were grown overnight in LB broth containing carbenecillin. Next day the fresh cultures were subjected to IPTG induction (1mM) for *ORF-2* gene expression at 37 ºC. The protein expression was seen after 1hour of IPTG induction and the expression level was found to be highest at 6 hrs post induction following SDS-PAGE analysis and a ~28 kDa size band of the overexpressed protein was detected after staining with commassie brilliant blue R-250 staining (Fig. 7).

The PCV-2 *ORF-2* gene encodes the capsid protein and is difficult to express in *E. coli* because of the existence of arginine and rare codons at the N-terminal part. The N-terminal part of ORF2 is mainly composed of the NLS sequence, which contains 41 aa (Liu *et al* 2001). It has been reported that the deletion of NLS could lead to high-level expression of *ORF-2* gene in *E. coli* (Zhou *et al* 2005). The whole

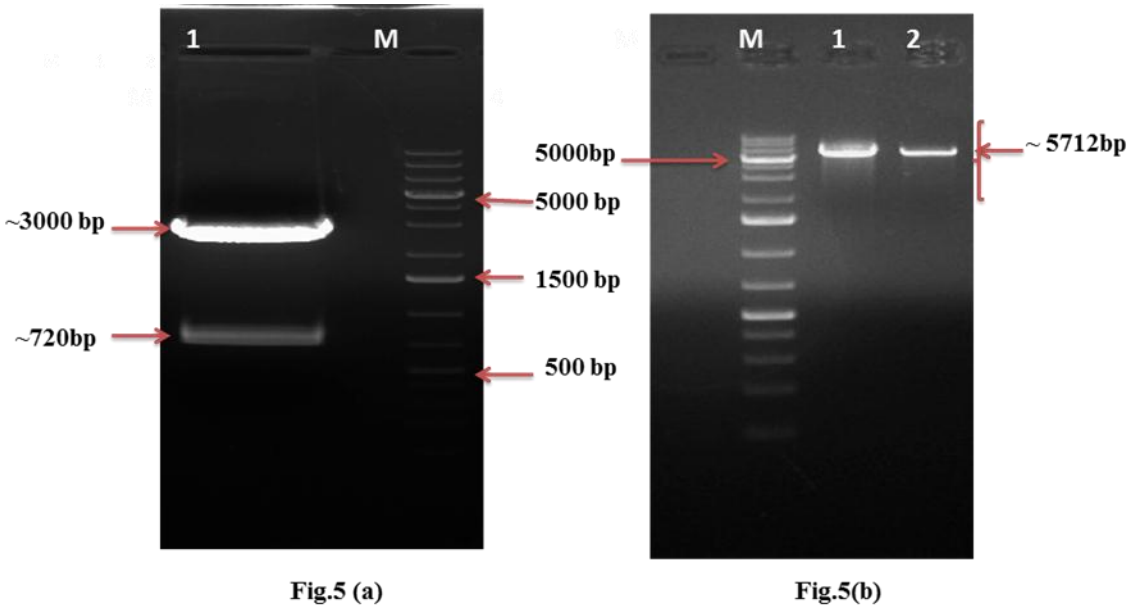


Fig.5 (a)

Fig.5(b)

Fig. 5(a): Release of codon optimized PCV-2 *ORF-2* insert from the recombinant PUC57 plasmid *Eco* RI and *Bam* HI RE's

M - 1kb plus ladder (Thermo scientific)

Lane 1 - codon optimized *ORF-2 i*nsert (720 bp) released from pUC 57 plasmid

Fig. 5(b): Linearised pET 302 NT His vector using *Eco* RI and *Bam* HI RE's

M - 1kb plus ladder (Thermo scientific

Lane 1 & 2 - Linearized pET 302 NT His vector

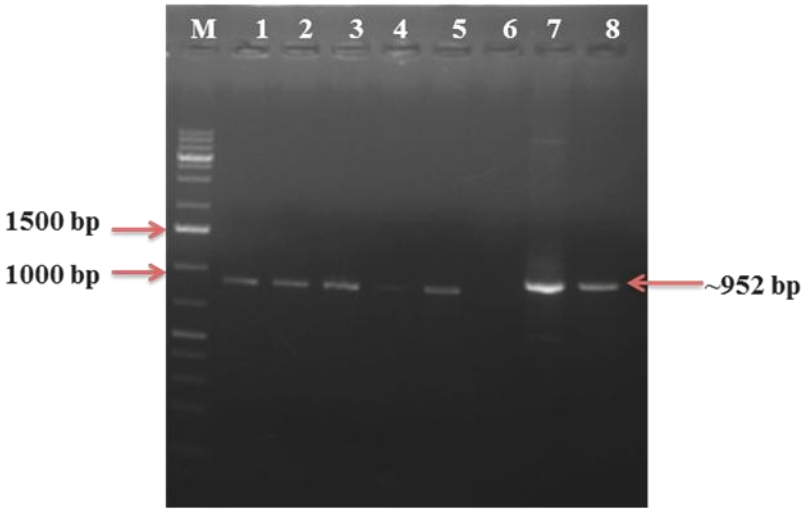


Fig. 6: Screening of recombinant pET 302 NT His clones for the presence of *ORF-2* insert by colony PCR

M : 1kb bp plus DNA ladder (Thermo scientific)

Lane 1 -3,5,7,8 : *ORF-2* insert (~952 bp)

Lane 4 & 6 : No insert

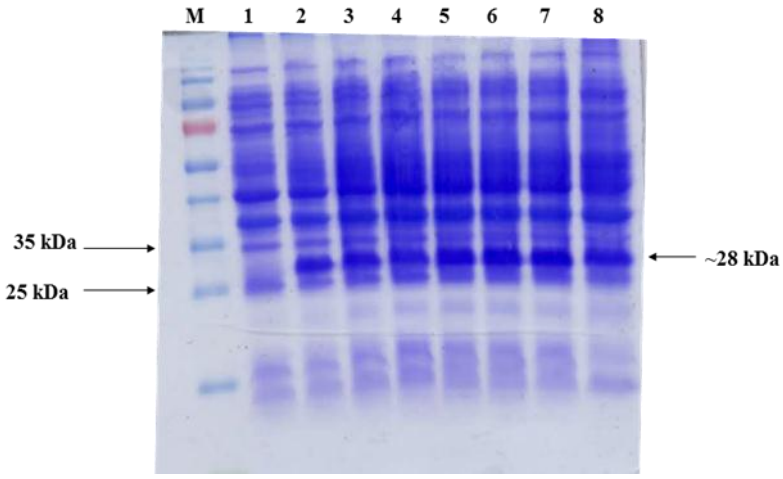


Fig. 7: SDS-PAGE analysis of the recombinant ORF-2 protein expressed at different time intervals following induction

M : Prestained protein ladder (Thermo scientific)
Lane 1 : Uninduced cell lysate
Lane 2 : Cell lysate of 1 hr P.I
Lane 3 : Cell lysate of 2 hrs P.I
Lane 4 : Cell lysate of 3 hrs P.I
Lane 5 : Cell lysate of 4 hrs P.I
Lane 6 : Cell lysate of 5 hrs P.I
Lane 7 : Cell lysate of 6 hrs P.I
Lane 8 : Cell lysate of 20 hrs P.I

ORF-2 gene has also been expressed in codon optimized *E. coli* cells [BL-21-Codon-Plus (DE3)-RIPL cells], containing extra copies of the argU, ileY, leuW, and proL tRNA genes, but the level of expression was low (Liu *et al* 2001 and Trundova *et al* 2007). So, considering the previous reports, to obtain the high level expression of recombinant capsid protein, the *ORF-2* gene lacking the NLS was synthesized commercially in the present study.

4.2 Purification of the bacterial expressed recombinant PCV-2 ORF-2 protein by Ni-NTA chromatography and confirmation by SDS-PAGE

The initial attempts to purify the recombinant capsid protein under native conditions using the Ni-NTA affinity chromatography was not satisfactory as the expressed protein was mostly present in insoluble form rather than the soluble form. Protein expression was found to be drastically reduced when attempts were carried out to express the protein by growing the culture at lower temperature (25-30ºC) for harvesting the protein under native conditions. Therefore to enable the purification of the capsid protein, denaturing conditions in the presence of 6M urea were utilized. The recombinant PCV-2 capsid protein was expressed in large scale by inducing 10 litres of the bacterial culture grown at 37ºC for 6 hrs with 1mM IPTG. The bacterial cell pellet was lysed in the denaturing binding buffer and the cleared lysate was then subjected to Ni –NTA column chromatography. The purified protein was then analysed by 15% SDS-PAGE and a protein band of ~28 kDa could be detected after staining with commasie brilliant blue R-250 staining, which was absent in the lysate prepared from the uninduced culture (Fig. 8). The purified denatured protein was refolded using the refolding buffer to bring the protein to its natural native conformation. The yield of the purified protein was approximately 70 µg/gm of the bacterial cell pellet.

4.3 Characterization of the bacterial expressed recombinant PCV-2 ORF-2 protein by western blot and dot blot analysis

After refolding the small sized contaminant proteins and salt (urea) was removed from the refolded protein by dialysis using Snake Skin Pleated dialysis bags against 1X PBS (pH 7.4) and concentrated using PEG8000. Dot blot and western blot were performed for the characterization of the refolded, dialyzed recombinant capsid proteins using the PCV-2 antisera (porcine origin) at 1:200 dilution and anti-His tag protein mouse antibody at 1µg/ml concentration as primary antibodies and anti-pig

IgG-HRP (rabbit raised) at 1:20,000 dilution and anti-mouse IgG-HRP at 1µg/ml concentration as the secondary antibodies. In dot blot, a brown coloured spot appeared on the site where protein was spotted on the NCM comb while no colour appeared on the control sites on the NCM comb (Fig. 9a and 9b). Western blot was performed to confirm the size of expressed recombinant protein using the same antibodies used in the dot blot resulted in intense colour reaction at ~28 kDa indicating the correct size of the expressed protein [(Fig. 10(a), 10(b), 11(a), 11(b)].

Recombinant PCV-2 ORF-2 protein has been produced in variety of expression systems including bacteria, baculovirus/insect cell system, yeast, adenovirus (Sun *et al* 2010, Lou *et al* 2011, Tu *et al* 2013, Jittimanae *et al* 2012) with each system having its own advantages and disadvantages. Marcekova *et al* 2009 expressed the PCV-2 *ORF-2* gene lacking the nuclear localization signal using pET28b-cap-His expression vector in the BL21 DE3 star competent cells with a protein size of 26 kDa and further purified using Ni-NTA affinity chromatography and evaluated in indirect ELISA to monitor the levels of PCV-2 specific antibodies in piglets experiencing PCV-2 infection. Sun *et al* 2010 expressed the ORF-2 capsid protein (~29kDa) as a GST-tagged fusion protein (GST-ORF-2-E) in bacterial cells, which showed a good reactivity with the anti- GST monoclonal antibody and the porcine serum in western blot. They also reported an enzyme linked immunosorbent assay using the the purified ORF-2 protein as an antigen for the diagnosis of PCV-2. Lou *et al* 2011, used the pET-32a vector for the expression of truncated capsid proteins in *E. coli*. The proteins were purified by Ni-NTA affinity chromatography, which showed a good reactivity with anti-PCV-2 antisera in ELISA. Kong *et al* 2011 expressed the capsid protein using the codon optimized *ORF-2* gene sequence without the NLS sequence in the *E. coli* cells. The immunoreactivity of the expressed protein was confirmed by western blotting using the anti-Cap monoclonal antibody. Jittimanae *et al* 2012 used pGEX-5x-3 vector for the expression of the capsid protein and the protein was purified by affinity chromatography and the reactivity of protein was tested with monoclonal mouse anti PCV-2 antibody.

4.4 Development of ELISA

4.4.1 Optimization of antigen and antibody concentrations

The concentration of the purified protein used as the coating antigen was 560 ng/µl. The selection of optimum concentration of the coating antigen was determined

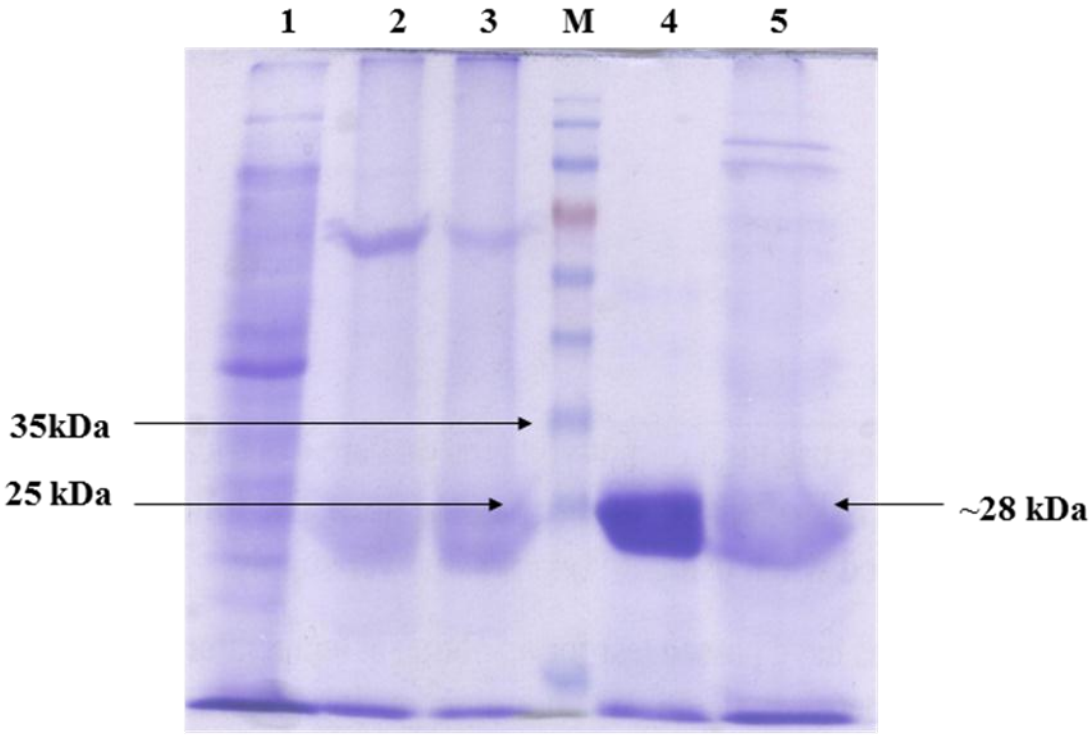


Fig. 8: SDS-PAGE analysis of the Ni-NTA purified recombinant proteins

Lane 1	**: Cell lysate of uninduced culture**
Lane 2&3	**: Ni-NTA Purified proteins (Native condition)**
M	**: Prestained protein ladder (Thermo scientific)**
Lane 4&5	**: Ni-NTA Purified proteins (Denaturing condition)**

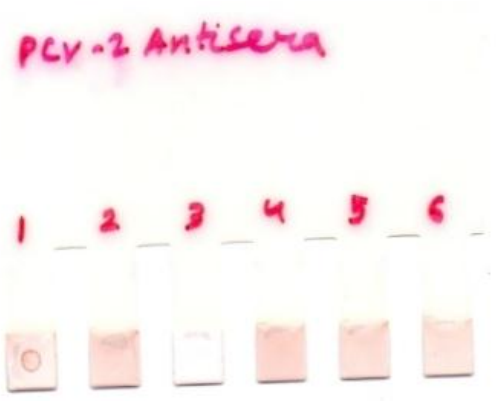


Fig. 9(a): Dot blot analysis of the purified recombinant ORF-2 protein with PCV-2 antisera as primary antibody and anti-Pig IgG as secondary antibody

Lane 1	**: Purified protein showing reactivity with PCV-2 Antisera (brown spots)**
Lane 2	**: Primary Antibody control**
Lane 3	**: Secondary Antibody control**
Lane 4 & 5	**: Negative serum control**
Lane 6	**: PBS control**

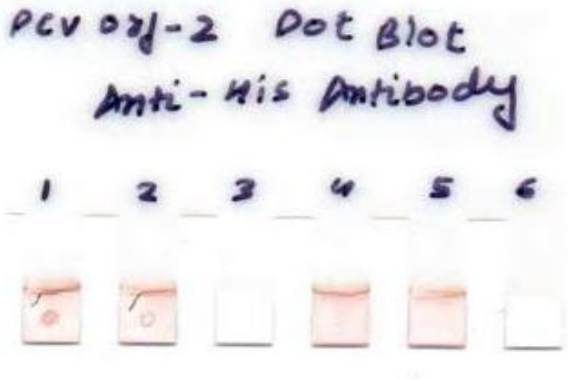


Fig. 9(b): Dot blot analysis of the purified recombinant ORF-2 protein with anti-His antibody as primary antibody and anti-mouse IgG-HRP as secondary antibody

Lane 1&2	**: Purified protein showing reactivity with anti-His antibody (brown spots)**
Lane 3	**: Primary Antibody control**
Lane 4	**: Secondary Antibody control**
Lane 5	**: Negative serum control**
Lane 6	**: PBS control**

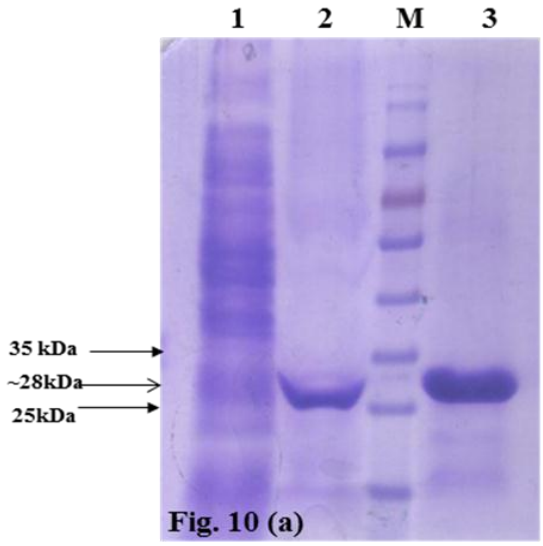


Fig. 10(a): SDS PAGE analysis of purified ORF-2 protein

Lane 1 : Uninduced cell lysate
Lane 2 : Purifiedprotein (Deanturing)
M : Prestained Protein ladder
Lane 3 : Purified protein (Denaturing condition)

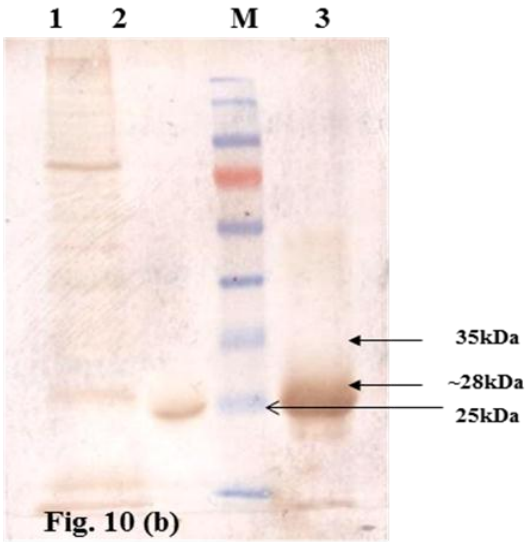


Fig. 10(b): Reactivity of purified ORF-2 protein with PCV-2 anti sera in Western blot

Lane 1 : Uninduced cell lysate
Lane 2 : Purified protein showing reactivity with PCV-2 antisera
M : Prestained Protein ladder/ marker
Lane 3 : Purified protein reacted with PCV-2 antisera

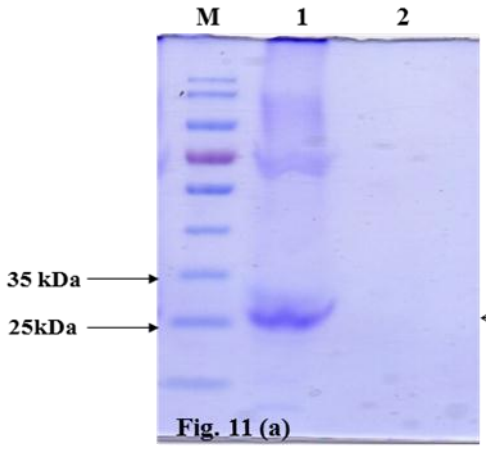


Fig. 11(a): SDS-PAGE analysis of purified ORF-2 protein

M : Prestained Protein ladder (Thermo scientific)
Lane 1 : Purified protein
Lane 2 : Cell lysate of uninduced culture

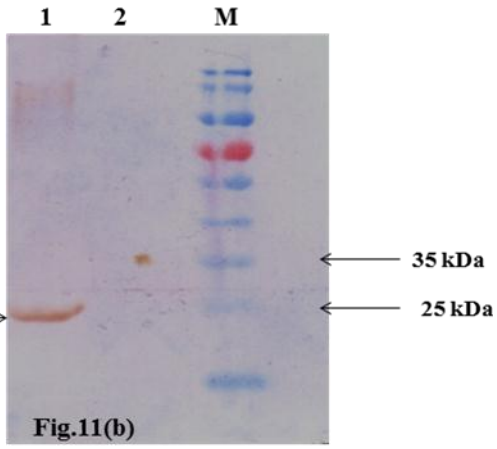


Fig. 11(b): Reactivity of purified ORF-2 protein with anti-His antibody in Western blot

Lane 1: Purified protein showing reactivity with Anti-His antibody
Lane 2: Cell lysate of uninduced culture
M : Prestained Protein ladder(Thermo scientific)

by checkerboard dilution method in bicarbonate-carbonate coating buffer (pH 9.6) against a fixed dilution of PCV-2 antisera (1:200), as the primary antibody and Anti-pig IgG-HRP (1:20,000) as the secondary antibody. The maximum reactivity was observed with the antigen at a concentration of 112 ng/µl and the same concentration of the antigen was used for optimization of other parameters (Fig. 12).

Primary antibody (porcine raised PCV-2 antisera) at different dilutions (1:50, 1:100, 1:200, 1:400) were tested in the indirect ELISA where the maximum reactivity was found at 1:200 dilution. The secondary antibody (Anti- rabbit IgG HRP) showed the optimum reactivity at 1:20,000 dilution out of three dilutions (1:5000, 1:10,000 and 1:20,000) tested.

4.4.2 Standard Curve Antigen concentration versus O.D. values

The antigen (recombinant capsid protein) having concentration of 112 ng/ µl was serially diluted in bicarbonate-carbonate coating buffer. The standard curve was plotted considering the different O.D. values at 492 nm obtained against different antigen (recombinant capsid protein) concentrations yielded a coefficient of determination value of 0.901 which indicated ~90% accuracy and significance of this assay (Table 1, Fig. 13).

Table 1: Average O.D values against different antigen concentration

Antigen conc (ng/µl)	Average OD value
112	0.71
56	0.63
28	0.49
14	0.42
7	0.352
3.5	0.33
1.75	0.30
0.875	0.29

4.4.3 Standard Curve Antibody dilution versus O.D. values

The standard curve was plotted considering the different O.D. values at 492 nm obtained against different antibody (PCV-2 antisera) dilutions yielded a coefficient of determination value of 0.960 which indicated ~96% accuracy and significance of this assay (Table 2 & Fig.14).

Table 2: O.D. values against different primary antibody dilutions (PCV-2 antisera)

Antibody dilution	Average OD Value
0.02 (1:50)	0.541
0.01 (1:100)	0.428
0.005 (1:200)	0.338
0.0025 (1:400)	0.261

4.4.4 Field sample testing

The purified, refolded capsid protein was tested as a coating antigen against the field pig sera samples (anti PCV-2 antibody positive and negative sera samples) in indirect ELISA. The recombinant antigen showed a good reactivity with the PCV-2 known positive pig sera samples and no reactivity was seen with the known negative pig sera samples tested earlier by commercial ELISA kit (Pig Circovirus Ab ELISA, Glory Sciences Co.,ltd). These results clearly indicated the potential use of the recombinant protein as diagnostic antigen for sero diagnosis of PCV-2 (Fig.15, Table 3, Fig. 16)

Table 3: Average O.D. values obtained following reaction of recombinant antigen against different PCV-2 positive and negative sera samples

Antibodies	Average O.D values
PCV- 2 antisera	0.24
PCV-2 positive field pig serum sample	0.87
PCV-2 positive field pig serum sample	0.73
Normal rabbit serum sample	0.076
PCV-2 negative field pig serum sample	0.086
Primary antibody control	0.083
Secondary antibody control	0.081
Chromogen control	0.082

PCV-2 antibodies have been detected by indirect immunofluorescence (Ellis *et al* 1998), IPMA (Allan *et al* 1998), and by indirect ELISA based on either PCV-2

Fig. 12: Optimization of coating antigen concentration by checkerboard dilution method for development of an indirect ELISA

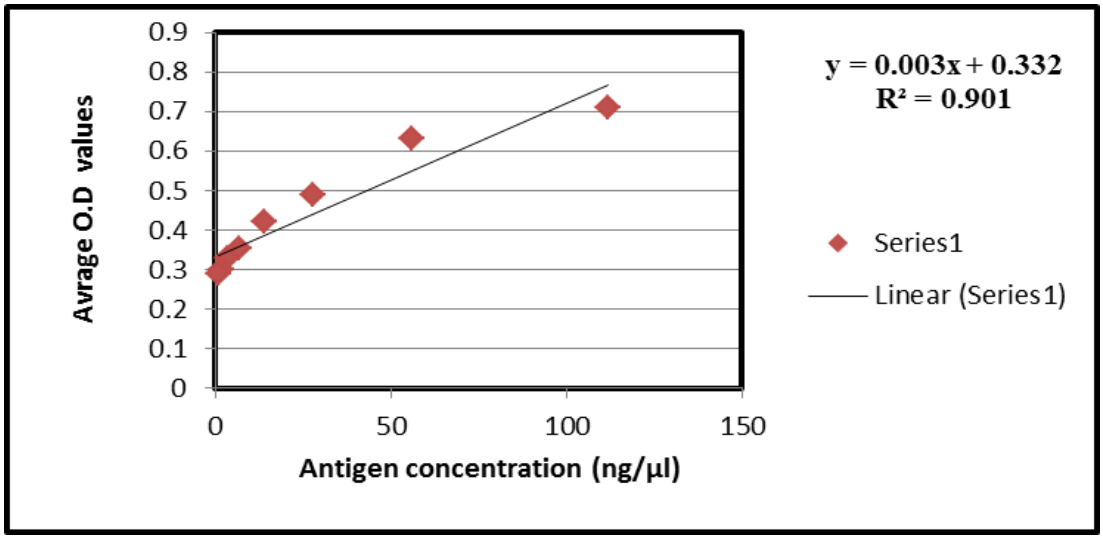


Fig. 13: Standard curve of antigen concentration versus O.D. value at a fixed primary antibody (PCV-2 antisera) dilution(1:200)

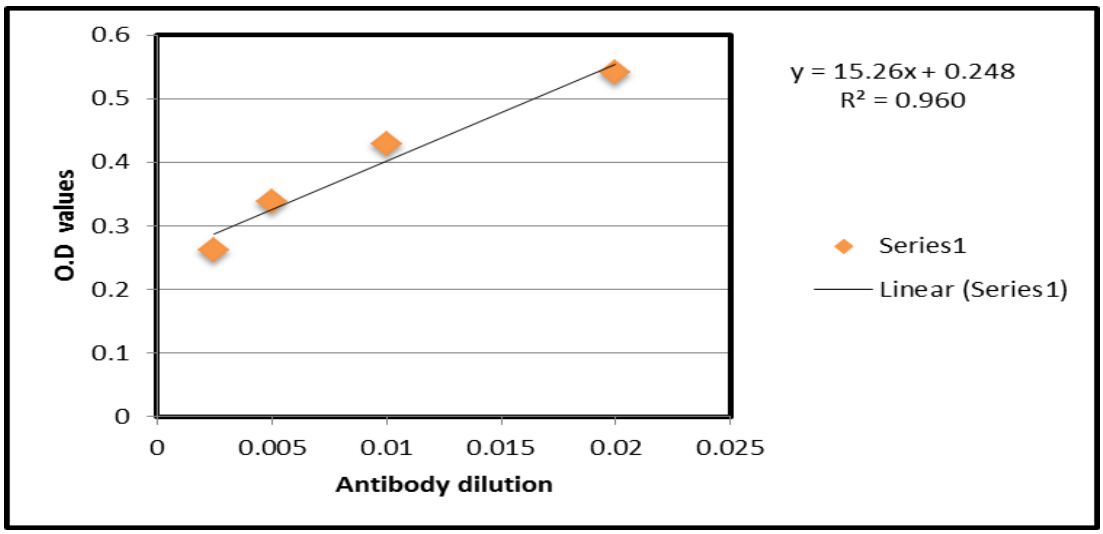


Fig.14 Standard curve of antibody dilutions versus OD value

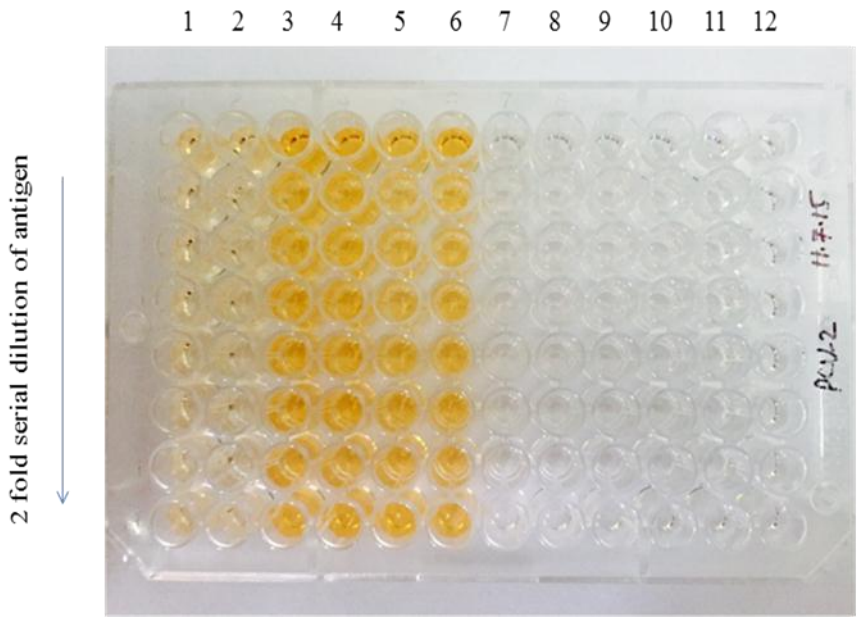


Fig.15: Indirect ELISA showing the reactivity of bacterial expressed PCV-2 ORF-2 recombinant antigen against field sera samples

Column 1 & 2	**:**	**PCV-2 antisera(1:200)**
Column 3& 4	**:**	**Field positive sample 1 (1:50)**
Column 5 & 6	**:**	**Field positive sample 2(1:50)**
Column 7	**:**	**Normal rabbit serum sample**
Column 8 & 9	**:**	**Field negative sample (1:50)**
Column 10	**:**	**Primary antibody control**
Column 11	**:**	**Secondary Ab control**
Column 12	**:**	**Chromogen control**

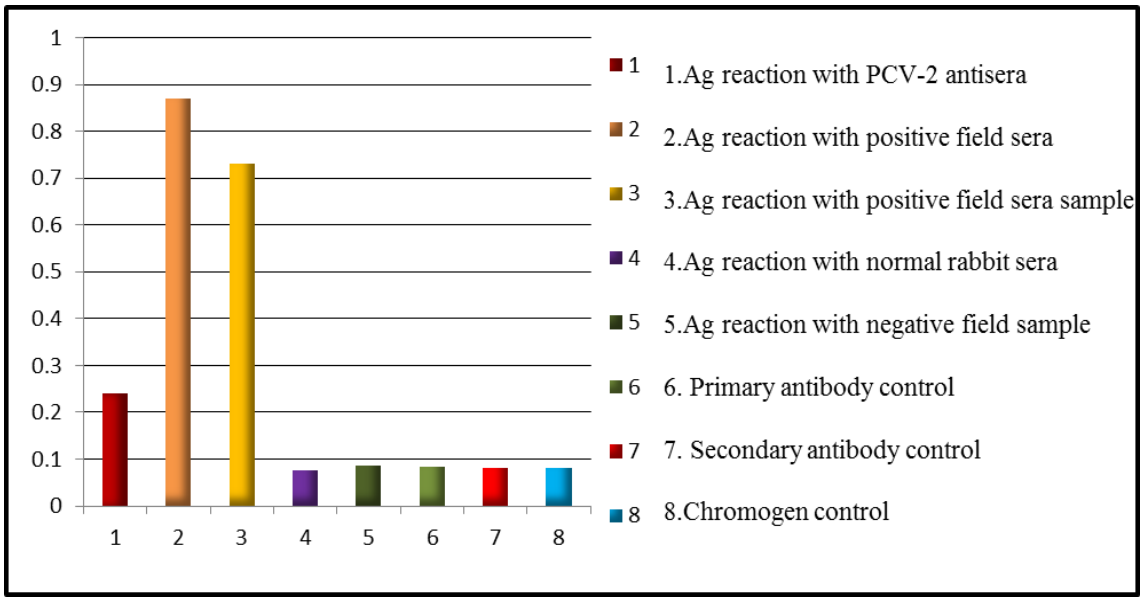


Fig. 16: Graphical comparison of average O.D. values obtained following reaction of recombinant antigen (capsid protein) with different PCV-2 positive and negative sera samples

viral particles (Nawagitgul *et al* 2002) or recombinant PCV-2 capsid protein expressed in baculovirus (Nawagitgul *et al* 2002; Blanchard *et al* 2003; Liu *et al* 2004). However, both the IIF and IPMA are highly demanding and not suitable for large scale survey of the PCV-2 infection whereas ELISA is a very suitable assay for the examination of large number of sera samples. So, a diagnostic test developed with the recombinant antigen (capsid protein) not only provide a high sensitivity and specificity but also takes into account the safety aspects associated with handling of the whole live virus in the laboratory. An indirect ELISA based on recombinant NLS-truncated PCV-2 capsid protein expressed in *E. coli* has been established by Shang *et al* 2008, Sun *et al* 2010 and Jittimanae *et al* 2012).

4.5 Baculovirus mediated insect cell expression of the codon optimised PCV-2 *ORF-2* gene

4.5.1 PCR Amplification and blunt end cloning of codon optimised PCV-2 *ORF-2* gene in pFastBacHBM TOPO vector

PCR amplification of the codon optimised PCV-2 *ORF-2* gene using proofreading Platinum *Pfx* DNA polymerase and newly designed primers resulted in a blunt end PCR product of 699 base pair size as seen in 1.5% agarose gel (Fig. 17).

The amplified PCV-2 *ORF-2* gene product was gel purified and ligated into pFastBacHBM-TOPO vector. The appropriate quantity of the cloning reaction mixture was used for the transformation of the chemically competent *E. coli* (DH5α) cells, which resulted in the development of white colonies on the selection plates (containing ampicillin 100 μg/ml), thus indicating optimum transformation efficiency. Randomly six white colonies were picked up and grown overnight in LB broth (containing ampicillin 100 μg/ml).

The overnight grown broth culture was used for plasmid isolation by standard protocol. PCR when carried out to confirm the presence of PCV-2 *ORF-2* gene insert in the recombinant pFastBacHBM-TOPO plasmids by using the combination of gene specific PCV-HBM forward primer and SV 40 Poly A reverse sequencing primers, an amplicon of ~800 bp could be amplified indicating correct orientation of the cloned gene (Fig. 18).

Similarly, PCR carried out using the combination of polyhedrin forward sequencing primer and gene specific PCV-HBM reverse primers could successfully

amplify ~880 bp *ORF-2* insert from some of the recombinant clones which further confirmed the correct orientation of the cloned gene (Fig. 18).

4.5.2 Generation and screening of the recombinant bacmid in DH10Bac *E. coli* cells

The purified pFastBacHBM-TOPO plasmid carrying the PCV-2 *ORF-2* gene in correct orientation was then transformed into the DH10Bac *E. coli* competent cells for transposition into the bacmid. The transformation resulted in development of mostly white colonies on the selective medium with few blue colonies indicating the optimum transformation efficiency. White colonies indicated that the transposition hasd occured between the mini-Tn7element on the pFastBacHBM vector and the mini-*att*Tn7 target site on the bacmid resulting in the generation of a recombinant bacmid. Few blue colonies appeared due to unalteration of the bacmid. Distinct and large white colonies (5-10) suspected to carry the recombinant bacmid were selected for further screening (Fig. 19). The selected white colonies that appeared on the LB plates, when screened using colony PCR using the pUC M13 forward and reverse sequencing primers, a product size of ~3300 bp (699 bp of *ORF-2* gene + 2500 bp of vector) could be successfully amplified indicating the presence of desired gene insert in the recombinant bacmid (Fig. 20).

4.5.3 Production of recombinant baculovirus

The purified recombinant bacmids were used to transfect the Sf-9 cells to produce recombinant baculovirus using the cationic lipid cellfectin reagent to obtain highest transfection efficiencies. After 72 hrs post-infection the cells stopped growing as compared to the cell only control and the signs of viral budding and vesicular appearance of cells were visible. This was the P1 viral stock which was collected and kept at 4ºC and then the P-1 viral stock was used to re-infect the fresh Sf-9 cells and high titre P-2 viral stock was generated (Fig. 21a and 21b). Similarly, the P-2 viral stock was used to further amplify the baculovirus stock to generate a high titre P-3 viral stock.

4.5.4 Expression of the recombinant PCV-2 ORF-2 protein in the baculovirus mediated insect cell expression system

The high titre P-3 baculovirus stock was then used to infect the suspension culture of High-Five cells grown in Express Five serum free medium for the expression of the recombinant protein. The culture supernatant as well as the cell

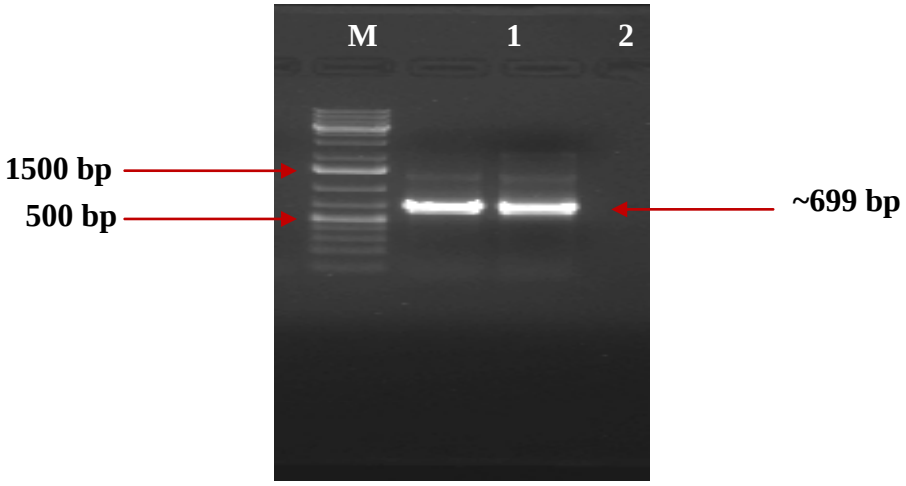


Fig. 17: Amplification of codon optimized PCV-2 ORF-2 coding sequences by PCR

Lane M : 100 bp plus DNA ladder (Thermo scientific)

Lane 1& 2 : Amplified *ORF-2* gene (~699bp)

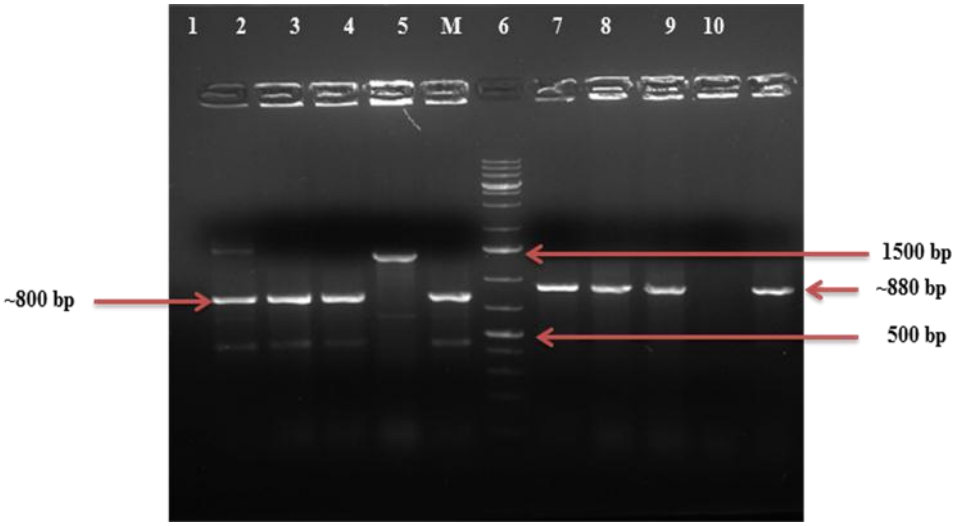


Fig.18: Colony PCR for screening of recombinant pFastBac HBM-TOPO clones for the presence of desired *ORF-2* insert with different primer combinations

M : 1kb plus ladder (Thermo scientific)

Lane 1,2,3,5 : Recombinant *ORF-2* clone (~800 bp)

Lane 6,7,8,10 : Recombinant *ORF-2* clone (~880 bp)

Lane 6 to 10 : Clone 1-5 (~880 bp)

Lane 4 : Non-specific amplification

Lane 9 : No amplification

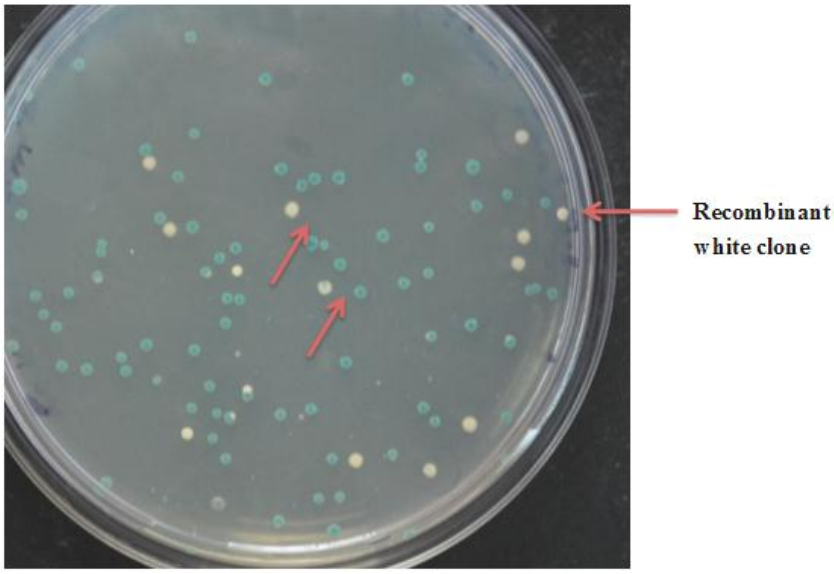


Fig. 19: Blue white screening of recombinant DH10Bac clones

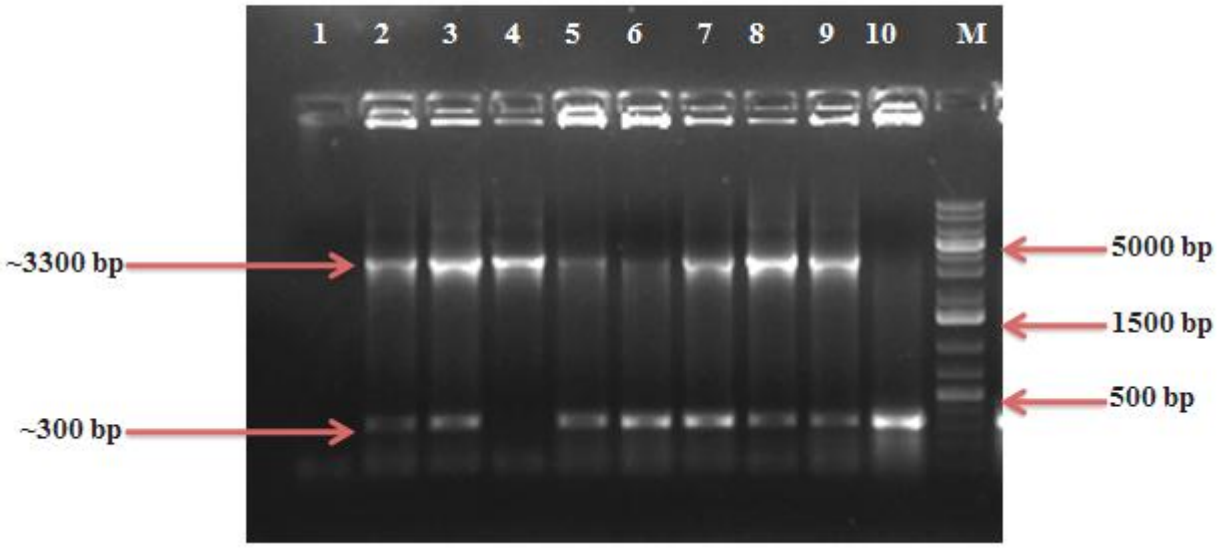


Fig. 20: Confirmation of recombinant bacmid by colony PCR

M : 1kb plus ladder (Thermo scientific)

Lane 1 : Non-template control

Lane 2,3, 5-9 : Empty and recombinant bacmid (mixed colonies)

Lane 4 : Recombinant bacmid containing ORF-2 insert

Lane 10 : Empty bacmid

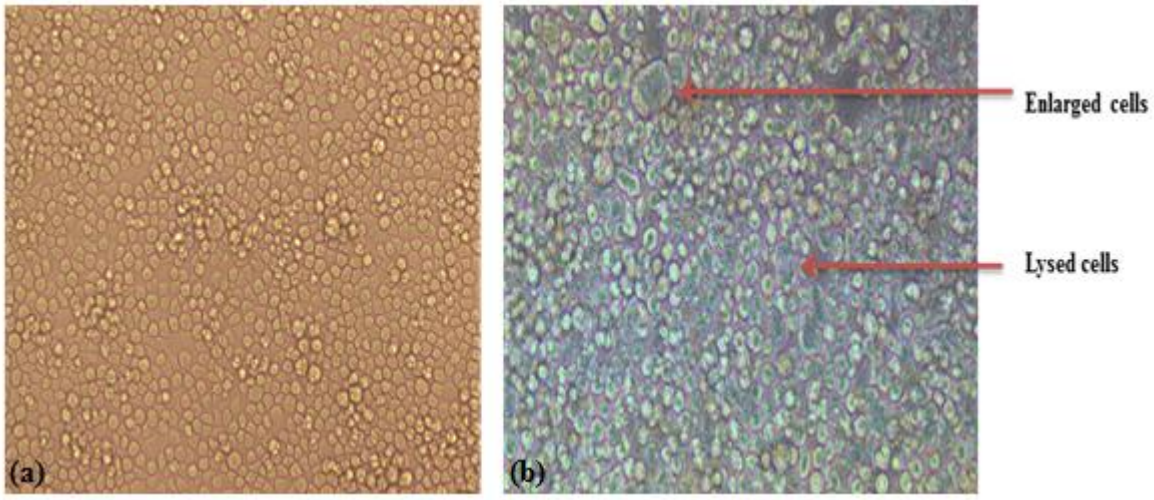


Fig. 21(a): Confluent monolayer of Sf-9 cells (Resolution 10X)

(b) Sf-9 cells showing cytopathic effects following 96 hrs post infection with P-3 viral stock (Resolution 10X)

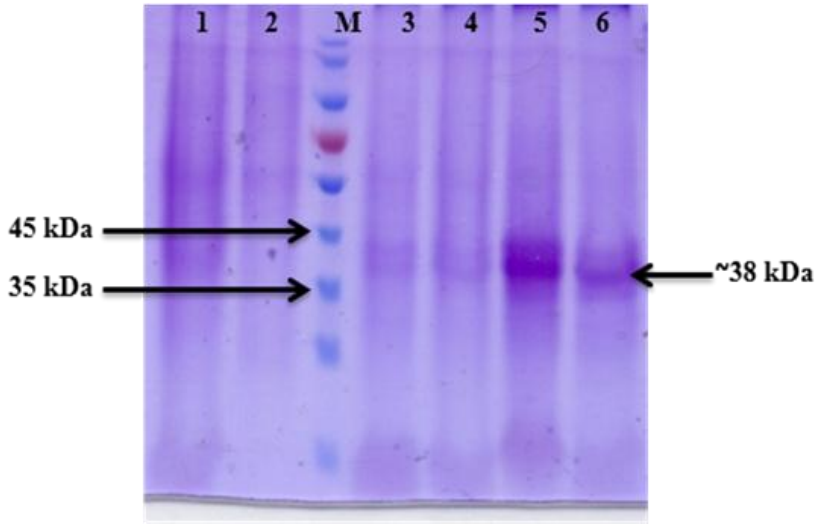


Fig. 22: SDS-PAGE analysis of the insect cell (High-Five) expressed recombinant ORF-2 protein

Lane 1	**: Serum free media Express control**
Lane 2	**: Non- infected cell lysate**
M	**: Prestained protein ladder**
Lane 3	**: PCV-ORF-2 native cell lysate (96 hrs)**
Lane 4	**: PCV-2 ORF-2 native cell lysate (72 hrs)**
Lane 5	**: PCV-2 ORF-2 denatured cell lysate (96 hrs)**
Lane 6	**: PCV-2 ORF-2denauredcell lysate (72hrs)**

pellet were collected at different time intervals i.e., 72 hrs and 96 hrs post infection (P.I) to check the protein expression by SDS-PAGE analysis. The culture supernatant collected was processed by TCA precipitation method and the cell lysate was prepared by using NP-40 lysis buffer for checking the expression of the recombinant ORF-2 protein. A band of ~38 kDa protein could be observed in the SDS-PAGE analysis after staining with commasie brilliant blue R-250 staining in the lysate prepared from the cell pellet collected at 96 hrs post-infection (Fig. 22). There was no protein observed in supernatant indicating the intracellular expression of recombinant protein rather than the secretory expression as expected.

4.5.5 Purification of the baculovirus expressed recombinant PCV-2 ORF-2 protein by Ni-NTA column chromatography and confirmation by SDS-PAGE

Suspension culture of Hi-Five cells (~2.7x 10^6 cells/ml) were infected with the P-3 recombinant baculovirus stock and harvested after 96 hrs of post infection. The initial attempts to purify the recombinant capsid protein from the cell pellet using the Ni-NTA affinity chromatography under native conditions was not satisfactory, as the yield of the purified protein was very less, indicating that the the protein was present mostly in the insoluble form rather than in the soluble form. Therefore, purification of the recombinant capsid protein from the cell lysate was carried out in denaturing conditions in the presence of 6M urea using Ni-NTA column chromatography. The purified protein was then analysed by 15% SDS-PAGE where a protein band of ~38 kDa could be detected after staining with commasie brilliant blue R-250 staining, which was absent in the lysate prepared from uninfected cells (Fig. 23). The size of the insect cell expressed ORF-2 protein was found to be slightly larger than the calculated size of ~28 kDa, which might be due to the post translational modifications like glycosylation. The purified denatured protein was then refolded using the refolding buffer to bring the protein to its native functional conformation. After refolding the small sized contaminant proteins and salt (urea) was removed from the refolded protein by dialysis using Snake Skin Pleated dialysis bag (3.5kDa) against PBS (pH 7.4) followed by concentration using PEG8000.

4.12 Characterisation of the insect cell expressed recombinant PCV-2 ORF-2 protein by dot blot analysis

Dot blot was performed for the characterization of the dialyzed recombinant capsid protein using anti-His tag protein mouse antibody at 1µg/ml concentration as primary antibody and anti-mouse IgG-HRP at 1µg/ml concentration as the secondary antibody. In dot blot, a brown colured spot appeared on the site where ORF-2 protein was spotted on the NCM comb while no colour appeared on the control sites on the NCM comb (Fig. 24).

Liu *et al* 2004 cloned the capsid protein coding sequence into the baculovirus transfer vector and expressed the recombinant capsid protein as a complete fusion protein in frame with a C-terminal peptide of six histidine tags. They also purified the protein using Ni-NTA affinity chromatography and used as an antigen in ELISA to detect the PCV-2 specific antibodies in the swine herd. Lee *et al* 2012 generated recombinant baculovirus containing *ORF-2* gene insert of PCV-2 and analyzed the optimal conditions for the production of capsid protein in insect cell. The expression of the recombinant capsid protein in insect cell was then confirmed by SDS-PAGE and western blot analysis using His tag antibody and anti-PCV-2 serum. They concluded that recombinant protein expressed in the infected Sf21 cells was only observed in cell culture supernatant not in the cell lysate pellet, indicating the secretory expression of the recombinant protein.

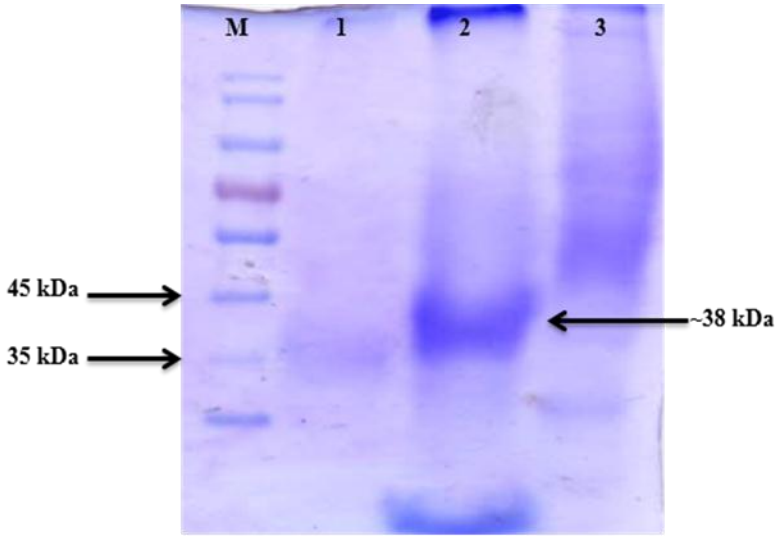


Fig. 23: SDS-PAGE analysis of the Ni-NTA purified recombinant ORF-2protein

M : Prestained protein ladder (Thermo scientific)
Lane 1 : Purified protein (Denaturing conditions) (72 hrs)
Lane 2 : Purified protein Denaturing conditions) (96 hrs)
Lane 3 : Non-infected cell lysate

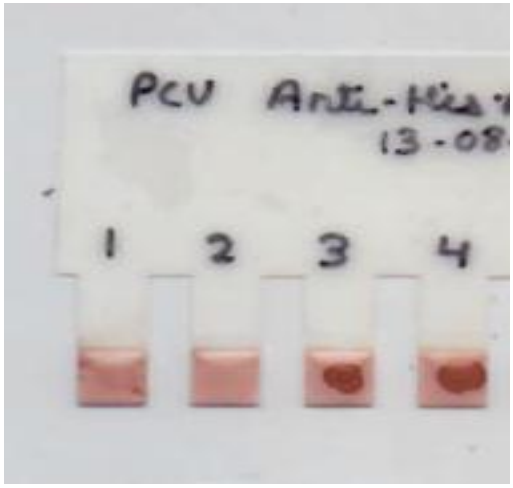


Fig.24: Dot blot analysis of the Hi- Five cell expressed, purified recombinant ORF-2 protein with Anti-His antibody as primary antibody and anti-mouse IgG-HRP as secondary antibody

Lane 1 : Non –infected cell lysate
Lane 2 : Serum free media control
Lane 3 : Cell lysate of PCV-2 ORF-2 protein
Lane 4 : Purified ORF-2 proteins (denaturing conditions)

CHAPTER V

SUMMARY AND CONCLUSIONS

Porcine circovirus type 2 (PCV-2) is associated with post-weaning multisystemic wasting syndrome (PMWS), an emerging swine disease that causes progressive weight loss, dyspnea, tachypnea, anemia, jaundice, and diarrhea in piglets. Simple and reliable diagnostic methods are needed for detecting antibodies to PCV-2 for monitoring of PCV infection and efforts need to be made for development of simple, rapid, non invasive tests that can be performed without expensive laboratory equipment. The present study was conducted to produce a recombinant ORF-2 protein and to characterize its potential as a diagnostic antigen.

For expression of PCV-2 *ORF-2* gene in the bacterial expression system, codon optimization for the PCV-2 *ORF-2* coding sequences was done and synthesized commercially in pUC57 plasmid. The codon optimized *ORF-2* gene insert (~702 bp) from the recombinant pUC57 plasmid was then released by using *Eco* RI and *Bam* HI enzymes, gel purified and further cloned in to pET302 NT His vector linearized with the same RE's. The recombinant pET302 NT His clones were screened by colony PCR using the sequencing primers which showed an amplicon size of ~952 bp indicating the presence of the desired gene insert in the recombinant plasmid. The purified plasmid carrying the PCV-2 *ORF-2* gene in pET302 NT His vector was used to transform the competent BL21 *E. coli* cells for protein expression purpose. The protein expression level was found to be highest at 6 hrs post induction at 1mM IPTG concentration following SDS-PAGE analysis as a protein band of ~28 kDa was detected in the induced culture. The initial attempts to purify the recombinant capsid protein under native conditions using the Ni-NTA affinity chromatography was not satisfactory as the expressed protein was mostly present in insoluble form rather than the soluble form. Protein expression was found to be drastically reduced when attempts were carried out to express the protein by growing the culture at lower temperature (25-30ºC) for harvesting the protein under native conditions. Therefore to enable the purification of the capsid protein, denaturing conditions in the presence of 6M urea were utilized. The purified protein was then refolded using the refolding buffer.

The purified, refolded capsid protein showed a good reactivity with anti-PCV-2 hyperimmune sera and the anti-His antibody in dot blot and the western blot. Further, this purified, refolded protein when used to develop indirect ELISA, the standard curve between different antigen and antibody concentrations and corresponding O.D. values at 492 nm was plotted, which yielded a significant value of coefficient of determination indicating the accuracy and significance of this assay. The antigen reactivity was also checked with the anti-PCV-2 antibody positive and the negative pig sera samples from the field.

For the expression of PCV-2 *ORF-2* gene in the baculovirus expression system (pFastBacHBM TOPO vector), codon optimized for the PCV-2 *ORF-2* coding sequences were commercially synthesized in pUC57 plasmids. The codon optimized *ORF-2* gene was then PCR amplified using the newly designed primers and an amplified product of ~699 bp was obtained. The amplified *ORF-2* fragment was then gel purified and ligated into the pFastBacHBM TOPO entry/cloning vector to generate a recombinant pFastBacHBM-TOPO construct by blunt end cloning. The recombinant clones were screened by colony PCR using different primer combinations. The PCR amplification with the gene specific forward primer and the SV 40 PolyA reverse sequencing primer resulted in an amplicon size of ~800 bp and with the gene specific reverse primer and polyhedrin forward sequencing primer resulted in a amplicon size of ~880 bp, indicating the correct orientation of the cloned *ORF-2* gene in the vector. The recombinant pFastBacHBM TOPO plasmid was then transformed into DH10Bac cells to produce the recombinant bacmid. The recombinant DH10Bac colonies were screened by blue white selection as well as by colony PCR using the pUCM13 forward and the reverse sequencing primers which in an amplicon size of ~3300 bp (699 bp of *ORF-2* gene + 2500 bp of vector) indicating the presence of desired gene insert in the recombinant bacmid. The recombinant bacmid was then used to transfect the Sf-9 insect cells with the help of cellfectin reagent to produce recombinant P-1 baculovirus stock. The P-1 baculovirus stock was then used to obtain next passage high titre baculovirus stocks P-2 and P-3. The High-Five cells grown in suspension culture were then infected with the high titre P-3 baculovirus stock, and was harvested at different time intervals (72 and 96 hrs) post infection and the protein expression in both the culture supernatant and the cell lysate was analysed by SDS-PAGE. A protein band of ~38 kDa was observed in the cell

lysate prepared under denaturing conditions using 6M urea. The recombinant protein was purified using the Ni-NTA affinity chromatography, which showed a very good reactivity with the anti-His antibody in the dot blot. The size of the insect cell expressed protein (~38 kDa) was slightly larger than the bacterial expressed protein (~28 kDa), which might be due post translational modifications like glycosylation.

Further work is required to evaluate the efficacy of bacterial expressed recombinant protein as a diagnostic antigen using more number of field pig sera samples from the field and also to purify the insect cell expressed recombinant protein in large quantities and to characterize it as potential diagnostic antigen by dot blot, western blot and indirect ELISA using the anti-PCV-2 hyper immune sera and the field pig sera samples.

CONCLUSIONS:

- PCV-2 ORF-2 protein was successfully expressed in bacterial as well as baculovirus mediated insect cell expression system.
- The expressed ORF-2 capsid protein showed good reactivity with anti-PCV-2 hyperimmune sera, anti-PCV-2 positive and negative pig sera samples in dot Blot, Western Blot and indirect ELISA indicating its potential use as a diagnostic antigen.

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VITA

Name of the student	**:**	Rupinder Kaur
Father's name	**:**	Mr. Nirmal Singh
Mother's name	**:**	Mrs. Narinder Kaur
Nationality	**:**	Indian
Date of birth	**:**	14.10.1991
Permanent home address	**:**	V.P.O Hambran, Distt. Ludhiana, Pin 141110, Punjab.

EDUCATIONAL QUALIFICATIONS

Bachelor degree	**:**	B.Sc. (Hons). Biotechnology
University	**:**	Punjab Agricultural University, Ludhiana, Punjab
Year of award	**:**	2013
OGPA/OCPA/% marks	**:**	8.04/10.00
Master's degree	**:**	M.Sc.
OCPA	**:**	8.56/10.00
Award/ Distinction/Fellowship	**:**	University Merit Scholarship