# Studies of the Molecular Genetics and Epidemiology of Porcine Rubulavirus Infection

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Cover: The photo shows one of the Porcine rubulavirus infected pigs included in this study. All rights and permissions are reserved by the author.
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Abstract

Porcine rubulavirus (PorPV) infection emerged spontaneously in pigs in Mexico in the early 1980s. Since the report of the initial outbreak of the disease, only one full-length genome from a strain isolated in 1984 (PorPV-LPMV/1984) has been sequenced. There is therefore limited information about the genetic variation of this virus. The overall objective of this thesis was to develop molecular techniques to help in the diagnostic field and to investigate in greater detail the full genomes of several isolates, and if possible, gain insights into the persistence, molecular epidemiology and the possible reservoirs of PorPV. In addition, a characterisation of the immune response during acute and persistent infection was included.

A real-time RT-PCR was developed for the detection of viral RNA from PorPV in clinical samples using TaqMan technology and primers for the P gene. This assay was highly sensitive (approximately 10 copies per reaction), specific, reproducible and a very useful tool for molecular diagnostics and for enabling studies of various aspects of PorPV throughout this thesis. RT-PCRs based on the NP and P genes were used to study the tissue distribution of the virus. Viral mRNA in the lymph nodes showed that the NP gene was consistently detected in the parotid, submaxilar, cervical and mesenteric nodes and the pancreas. Full-length genomes were sequenced from new isolates obtained from clinical cases of infected swine. The genetic comparison and phylogenetic analysis indicated that three different genetic variants of PorPV had spread in the swine population and that a new generation of circulating virus with a pronounced attenuation has begun to emerge in nature.

We also report the isolation of PorPV, or a related virus, from frugivorous, insectivorous, and hematophagous bats. A partial genome sequence analysis showed a 99.97 - 100% amino acid identity to the reference strain isolated from swine. However, larger parts of the genome must be sequenced to ascertain the genetic relationship between these viruses.

The study of the immune response during acute and persistent infection revealed enhanced levels of CD8+, CD4+ and CD2+ T-cells in all infected pigs at 10 days PI. CD8+ T-cell subpopulations were significantly higher ($p<0.05$) at 10 and 250 days PI, and CD4+ T-lymphocytes were also significant at 250 days PI.

In summary, this work developed molecular techniques that can be used to study the pathogenesis and molecular epidemiology of PorPV. The knowledge of the presence of different virus variants in nature, associated with a wildlife reservoir of PorPV can provide greater knowledge regarding the molecular genetic changes and useful data to establish new strategies in the control of this virus in Mexico.

*Keywords*: Porcine rubulavirus; PorPV, PorPV-LPMV, bats; epidemiology; persistent infection, RNA

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# Dedication

The most important thing in life is that you never lose your dreams;
It doesn't matter…… when you arrive!

In memorial to my Father
*Salvador Cuevas*

To my mom, *Paula Romero*
To my husband *Miguel* and our
Dear daughters: *Sarahí, Ximena*
*Maria Fernanda and Cassandra*

# Contents

# List of Publications

This thesis is based on the work contained in the following papers and manuscripts, referred to by Roman numerals in the text:

I Sandra Cuevas-Romero, Anne-Lie Blomström, Arcelia Alvarado, Pablo Hernández-Jáuregui, Francisco Rivera-Benítez, Humberto Ramírez-Mendoza, Mikael Berg. (2013). Development of a real-time RT-PCR method for detection of Porcine rubulavirus (PorPV-LPMV). *Journal of Virological Methods*. 189, 1–6

II Sandra Cuevas-Romero, E. Hernández-Baumgarten, S. Kennedy, P. Hernández-Jáuregui, M. Berg, J. Moreno-López. (2014). Long-term RNA persistence of Porcine rubulavirus (PorPV-LPMV) after an outbreak of a natural infection: The detection of viral mRNA in sentinel pigs suggests viral transmission. *Virus Research* 188, 155–161

III Sandra Cuevas-Romero, Francisco Rivera-Benítez, Anne-Lie Blomström, Miriam Ramliden, Eliseo Hernández-Baumgarten, Pablo Hernández-Jáuregui, Humberto Ramírez-Mendoza, MikaelBerg. (2015). Molecular epidemiology of Porcine rubulavirus (PorPV) isolates from different outbreaks in Mexico). (*Manuscript*).

IV Sandra Cuevas-Romero, J.F. Rivera-Benítez, M. Ramliden, E. Hernández-Baumgarten, J.A. Guerrero, G. Santos-López, H. Ramírez-Mendoza, E. Castrejón-Espinoza, A.L Blomström and M. Berg. (2015). Detection and isolation of Porcine rubulavirus (PorPV) in Mexican bats suggests as a possible wildlife reservoir. (*Manuscript*).

V Sandra Cuevas, A. Rodríguez-Ropón, Seamus Kennedy, Jorge Moreno-López, Mikael Berg, Pablo Hernández-Jáuregui. (2009). Investigation of T-

cell responses and viral mRNA persistence in lymph nodes of pigs infected with Porcine rubulavirus. *Veterinary Immunology and Immunopathology* 127, 148–152

# Abbreviations

| | |
|---|---|
| aa | amino acid |
| bp | base pairs |
| b-ELISA | blocking ELISA |
| CNS | central nervous system |
| CPE | cytopathic effect |
| CTD | carboxy-terminal domain |
| DI | defective interfering |
| DNA | deoxyribonucleic acid |
| F | fusion protein |
| HAD | Hemadsorption |
| HA | Hemagglutination |
| HeV | Hendra virus |
| HN | hemagglutinin-neuraminidase protein |
| HP | Histopathology |
| IPMA | immunoperoxidase monolayer assay |
| IF | Immunofluorescence |
| L | large protein |
| LPMV | La Piedad-Michoacan-Mexico virus |
| M | matrix protein |
| Mabs | monoclonal antibodies |
| MASL | meters above sea level |
| MDCK | Madin-Darby canine kidney cell line |
| MenPV | Menangle virus |
| MeV | Measles virus |
| MprPV | Mapuera virus |
| mRNA | messenger-RNA |
| MuV | Mumps virus |
| NDV | Newcastle disease virus |

| | |
|---|---|
| NiV | Nipah virus |
| NP | Nucleoprotein |
| nPCR | nested-Polymerase chain reaction |
| nt | Nucleotide |
| P | Phosphoprotein |
| PCR | polymerase chain reaction |
| PI | post-infection |
| PK-15 | porcine kidney cell line |
| PorPV | Porcine rubulavirus |
| PMBC | peripheral blood mononuclear cells |
| qRT-PCR | quantitative real-time RT-PCR |
| RNA | ribonucleic acid |
| SV5 | Simian virus type 5 |
| TioPV | Tioman virus |
| VI | virus isolation |
| VN | virus neutralisation |

# 1 Introduction

Porcine rubulavirus (PorPV), which was discovered in the early 1980s, belongs to the *Rubulavirus* genus in the Paramyxoviridae family (Moreno-López et al., 1986; Stephano et al., 1988; Wang et al., 2012). It is the causative agent of a disease in pigs that is considered to be one of the four most economically important diseases affecting pigs in Mexico (Kirkland and Stephano, 2006). Currently, the disease remains endemic to the central and western-central parts of Mexico. The infection has been detected by serology in 16 out of 32 states of Mexico, whereas the disease remains unreported in other countries (Escobar-López et al., 2012).

PorPV infection is most severe in newborn piglets that usually die within 2-7 days of the appearance of clinical signs. The disease is much less severe in older pigs, and mortality rates are lower in older pigs (Stephano, 2002). Clinical signs are characterised by fever, rough coat, progressive neurological disturbance, pneumonia, and uni- or bilateral corneal opacity. In adult pigs, symptoms were mainly associated with the reproductive organs (Moreno-López et al., 1986; Ramírez-Mendoza et al., 1997; Stephano et al., 1988, 2002; Hernández-Jaúregui et al., 2004). Atypical outbreaks, including neurological signs in fattening and adult pigs, have been documented between 2000 to 2003 with changes in the clinical behaviour and increased virulence of PorPV. This change in behaviour was presumed to be associated with mutations in the hemagglutinin-neuraminidase gene (HN) (Sánchez-Betancourt et al., 2008). Pigs are the only animals known to be affected clinically by PorPV under natural conditions. A preliminary assessment suggested that the source of the virus might be subclinically infected and/or persistently infected pigs (Stephano, 1988; Wiman et al., 1998).

Early phylogenetic analyses indicated that the virus was most related to the Mumps virus (MuV) and the Simian virus (SV5) (Berg et al., 1991, 1992,

1997; Sundqvist et al., 1992; Svenda et al., 1997, 2002), although the identity to these two viruses at the amino acid level was only approximately 40%. More recent genetic studies have shown that PorPV is more closely related to the Mapuera virus (MprPV, genus *Rubulavirus*), which was isolated from the salivary glands of an apparently healthy fruit bat (*Sturnira lilium*) in Brazil in 1979 (Henderson et al., 1995; Wang et al., 2007). Related to these phylogenetic studies, it has been suggested that PorPV may have an unknown wild reservoir (Svenda, 1997).

During PorPV infection, the reported immune response in experimentally infected adult pigs displayed an immunodominance of antibodies against the HN protein, although antibodies against the matrix (M) and nucleoprotein (NP) have also been shown (Hernández et al., 1998). Pigs naturally infected with PorPV develop antibodies that usually persist throughout their lives (Stephano, 2002).

Concerning diagnostic tests, at present there are serological tests and classical virus isolation techniques available (McNeilly et al., 1997; Nordengrahn et al., 1999; Rivera-Benítez et al., 2013a). In addition, classical RT-PCR technologies have been used for various research purposes (Wiman et al., 1998; Cuevas-Romero et al., 2009; Rivera- Benítez et al., 2013a), but no real-time PCR method specific to PorPV was available prior to the one described in the present thesis. Real-time PCR has many advantages over classical PCR, such as better sensitivity and specificity (Belák and Thorén, 2001). The implementation of highly sensitive assays that yield results quickly will be of great assistance in improving the control strategies of PorPV infection in Mexico.

# 2 Background

## 2.1 General aspects of Porcine rubulavirus infection

A new porcine viral disease was recognised in the early 1980s in Mexico with respiratory, reproductive and central nervous system disorders in swine (Moreno-López et al., 1986; Stephano et al., 1988). Porcine rubulavirus was identified to be a new member of the family Paramyxoviridae, genus *Rubulavirus*, and was initially named the La-Piedad-Michoacan-Mexico virus (LPMV) after the town in which the virus was first isolated (Moreno-López et al., 1986; Stephano et al., 1988); it was later renamed Porcine rubulavirus (PorPV). As a part of the lesions, the eyes showed a uni- or bilateral corneal opacity and a blue colour. This fact was easy to recognise by veterinarians and producers, and the colloquial name of (Enfermedad del ojo azul) "blue eye disease" (BED) was coined. Since the first recognised outbreak, sporadic outbreaks of PorPV infection have continued to occur; however, the specific source of many of these outbreaks remains unknown. During the last ten years, the evolution of PorPV has been investigated mainly through phylogenetic analyses of the HN protein and studies of the clinical histories and experimental infections using different isolates.

## 2.2 Aetiology

The Paramyxoviridae family is divided into the subfamilies Paramyxovirinae and Pneumovirinae. The Paramyxovirinae subfamily is in turn divided into five genera: *Rubulavirus* (prototype: Mumps virus, MuV), *Avulavirus* (prototype: Newcastle disease virus, NDV), *Respirovirus* (prototype: Sendai virus), *Henipavirus* (prototype: Hendra virus, HeV) and *Morbillivirus* (prototype: Measles virus, MeV) (Wang et al., 2012).

### 2.2.1 Virion characteristics

Similar to other rubulaviruses, the PorPV virion consists of a lipid bilayer or envelope outer surface derived from the plasma membrane of the host cell. Into this bilayer two glycoproteins, fusion (F) and haemagglutinin/neuraminidase (HN), are inserted (Sundqvist et al., 1990, 1992). Positioned just under the envelope is the matrix (M) protein associated with part of the glycoproteins and with the nucleocapsid. The nucleocapsid is present as a tightly packed structure. Two proteins are associated with the nucleocapsid core, the phospho- (P) and the large (L) proteins. The nucleocapsid consists of a non-segmented negative-sense, single-stranded genomic RNA (approximately 15,000 nucleotides), which is tightly encapsulated by the nucleoprotein (NP). The P gene has the capacity to encode four possible polypeptides, P, V, C and I, from the same gene via editing and the alternative initiation of translation (Berg et al., 1991, 1992, 1997).

### 2.2.2 Genome organisation

The genomic structure of PorPV contains a single promoter in the untranslated 3´region, which is followed by NP, P, M, F, HN and L ORFs. All viruses belonging to the families in the order Mononegavirales require that the virion contain its own RNA-dependent RNA polymerase because the cells lack this enzyme; thus, these viruses direct the synthesis of mRNA at the start of the infectious cycle. Several viruses of this order can produce defective interfering (DI) particles including PorPV (Hjertner et al., 1997, 1998), the Measles virus (Guido Re, 1991; Schneider-Schaulies et al., 1999) and the Vesicular stomatitis virus (Sreevalsan, 1970). The presence of DI particles appears to be a very common feature in persistent paramyxovirus infections *in vitro* (Hjertner et al., 1997).

### 2.2.3 Phylogenetic analysis

Currently, several strains of PorPV have been isolated, and their HN gene has been sequenced; hence, the evolution of PorPV has so far solely been based on the HN gene. The electrophoretic protein patterns of some of the gene sequences were similar to the original reference strain of PorPV-LPMV/1984 (Sundqvist et al., 1990; Moreno-López et. al. 1996). Recent studies of PorPV isolates (PorPV-PAC-7/2002, PorPV-PAC-8/2002 and PorPV-PAC-9/2002) have identified changes in the HN structure, and it has been suggested that these mutations are associated with an increased neurovirulence of PorPV. The

amino acid sequence comparisons and phylogenetic analyses of the HN protein have revealed three genetically different lineages: Group 1 is comprised of PorPV-LPMV/1984 and PorPV-PAC-4/1993 and is characterised exclusively by the presence of neurological signs in piglets; group 2 is comprised of PorPV-PAC-2/1990, PorPV-PAC-3/1992, and PorPV-CI, II, III, IV and is characterised by neurological signs, high mortality in piglets and older pigs (3-4 months) and lesions in the reproductive tract of adult pigs; and group 3 is comprised of PorPV-PAC-6/2001, PorPV-PAC-7/2002, PorPV-PAC-8/2002 and PorPV-PAC-9/2003 and is associated with clinical signs of neurological involvement in adult animals and in commercial fattening lines (Reyes-Leyva et al., 2002; Sánchez-Betancourt et al., 2008).

The PorPV proteins (i.e., NP, M, F, and L) have indicated an association with the human mumps virus with an amino acid identity ranging from 45 to 55% (Berg et al., 1991, 1992, 1997; Sundqvist et al., 1992; Svenda et al., 1997, 2002). The relatively far distance shows that PorPV is a unique virus of pigs. These viruses probably share a common ancestor, but the divergence must have occurred in the distant past (Berg et al., 1997, Svenda et al., 1997, 2002).

## 2.3 Origin and epidemiological distribution

The source of PorPV that is responsible for the disease outbreaks in Mexico has been suggested to be subclinically infected and/or persistently infected pigs (Wiman et al., 1998; Stephano, 2002). However, since the first recognised outbreak in the 1980s, sporadic outbreaks have continued to occur, and the specific source of many of these outbreaks remains unknown.

Molecular studies of PorPV, with an emphasis on proteins of the replicative complex, suggest that this virus has existed as a separate species for a long time in nature and that it could have been transmitted from a natural wildlife reservoir to domesticated pigs (Berg et al., 1997; Svenda et al., 1997, 2002). More recent studies comparing the completed genome sequence and organisation indicate that PorPV is more closely related to MprPV than to other members of the genus *Rubulavirus*, suggesting that PorPV may possibly originate from bats (Wang et al., 2007). In addition, a serological analysis in 108 non-hematophagous bats from the Central Pacific coast of Mexico showed the presence of antibodies against PorPV in an insectivorous bat (*Rhogeessa parvula major*). However, because only one bat was seropositive, the authors suggested that bats in the surveyed localities do not play a role in the epidemiology of PorPV (Salas-Rojas et al., 2004). Nevertheless, because fruit

bats are considered to be the natural host of not only MprPV but also of other related paramyxoviruses (HeV, NiV, MenPV and TioPV) (Chant et al., 1998; Philbey et al., 1998; Halpin et al., 1999, 2000; Chua et al., 2001; Wang et al., 2007; Barr et al., 2012), it has been suggested that bats could be the original natural host of the PorPV strain responsible for the outbreak in pigs (Wang et al., 2007). Furthermore, other natural reservoirs are also possible. Although very unlikely, it may be that bats were infected from pigs.

PorPV is now endemic in pigs in central parts of Mexico and is thus a major problem for pig health in the region. Porcine rubulavirus infection has only been diagnosed in Mexico and is considered one of the four most important diseases affecting the Mexican swine industry. It is an endemic disease in the central and western-central parts of Mexico with a sero-prevalence ranging from 9 to 23.7%. The disease has been serologically diagnosed in at least 16 states. In this respect, an important point is the difference of antigenic variants that have been identified in non-vaccinated swine from four states in Mexico (Guanajuato, Jalisco, Michoacan and Estado de Mexico) where there was not complete antibody cross-antigenicity among three isolates of PorPV that were evaluated (Escobar-López et al., 2011). These findings suggest that genetically and antigenically different PorPV strains are circulating in the swine population, imposing challenges to diagnostic and vaccination efforts.

## 2.4 Pathogenesis

The morphological characteristics of the virions and the distribution patterns of the HN and NP proteins in infected PK-15 cells indicate that the mechanisms of PorPV replication are generally similar to those of other members of the Paramyxoviridae family (Hernández-Jáuregui et al., 2001). The pathogenesis of PorPV depends on a positive organotropism, which is determined by specific interactions between the receptors of sialic acid-expressing cells and viral adhesion proteins. The virus recognises neuraminic 5 acid α-2,3 galactose (Neu5Ac α-2,3Gal) oligosaccharides, which are required for the infection process. These receptors are modified during the maturation of porcine tissues in relation to the age of the pigs. There is a high expression of Neu5Ac α-2,3Gal in the CNS and in respiratory tissues of newborn pigs, principally in the olfactory bulb, hippocampus, brain cortex, cerebellum and medulla oblongata, with less expression in urogenital tissues. The expression of the specific oligosaccharide Neu5Ac α-2,3Gal sequence appears to be correlated with tissue susceptibility to PorPV (Reyes-Leyva et al., 1997; Vallejo et al., 2000).

It has been suggested, based on experimental intratracheal or intranasal exposure by either instillation or aerosol, that these are effective routes of infection and result in clinical signs and lesions similar to those observed in natural PorPV infection (Stephano, 2002). A sequential study of PorPV distribution in tissues and body fluids in experimentally infected pigs by intranasal and eye drop routes showed that all pigs infected at 3 days of age were either dead or moribund after 8 days PI, whereas only 30% of the pigs infected at 17 days of age were affected. The distribution of the virus in different tissues was shown to be very localised. In the pigs inoculated at day 17, the isolation of the virus was restricted to the olfactory bulb and midbrain. By contrast, the pigs inoculated at 3 days of age showed a dual mode of spread to the brain. After primary replication in the respiratory tract and tonsils, the virus spread throughout the brain via the trigeminal and olfactory nerves combined with a low-level viremia and passage through the immature blood-brain barrier. Excretion of the virus occurred mainly via the respiratory tract and urine (Allan et al., 1996). Recent studies on the pathogenesis and distribution in the respiratory tract of experimentally PorPV infected young pigs (6-week-old pigs) inoculated with PorPV-PAC-3/1992 (a low virulence strain) showed prolonged viral excretion of this virus from nasal fluid samples for up to 23 days PI and from the respiratory tract for up to 28 days PI. The distribution in the soft palate tonsil and lymph nodes exhibited high viral loads. The major microscopic lesions observed in the lungs corresponded to interstitial pneumonia and hyperplasia of the associated lymphoid tissue (Rivera-Benítez et al., 2013b).

Similar to these results, the presence of antigen was demonstrated by IF in the head of the epididymis of experimentally infected boars at 15, 30, 45 or 70 days PI (Ramírez-Mendoza et al., 1997). Additionally, isolation of the virus was detected in semen samples of experimentally infected boars between 5 and 48 days PI and in the testis and epididymis between 64 and 142 days PI. However, no evidence of infective virus was detected in the semen of three of the nine infected boars. In other studies of experimental inoculation of young hybrid boars, inflammation and oedema of the testis and epididymis was shown 15 days after inoculation. Boars sacrificed 80 days after infection showed fibrosis and granuloma formation in the epididymis and testicular atrophy (Ramírez-Mendoza et al., 1997).

## 2.5 Immunology

All viruses of the Paramyxoviridae family are extremely infectious. Because of their common labile structure, this family is dependent on transmission by the close association of hosts. Infections occur through aerosols; direct physical contact between hosts is not required (Black, 1991).

Evidence from previous studies has shown that during PorPV infection, antibodies are produced against internal and external proteins. The reported immune response to PorPV in experimentally infected adult pigs indicates an immunodominance of specific antibodies against the HN protein, but antibodies against the M and NP proteins have also been demonstrated (Hernández et al., 1998). Pigs naturally infected with PorPV develop antibodies that usually persist throughout their lives (Stephano, 2002). In this respect, it is important to mention that antibodies to the HN and F proteins are vital for eliciting a virus-neutralising response (Randall and Russell, 1991).

Clinical reports have indicated that the virulence of PorPV has increased (Stephano, 2002). Sánchez-Betancourt (2008) identified changes in HN protein structure, and it was suggested that these changes were associated with an increased neurovirulence of PorPV infection. However, some variants of this virus were able to preserve the HN sequence for long periods of time. Amino acid sequence comparisons and phylogenetic analyses of the HN protein have revealed genetically different lineages, as mentioned earlier (Sánchez-Betancourt et al., 2008, 2012).

The cellular immune response has been characterised in experimentally infected pigs in which an increased number of CD2+ T-lymphocytes was demonstrated during the early stages of PorPV infection in adult pigs, whereas the proportion of B-cells was reduced in these animals at 4 weeks PI. A decrease in the proportion of CD4+ T-cells was observed at 3 weeks PI, whereas both CD4- CD8+ and CD4+CD8+ T-cell proportions were increased at 1 and 4 weeks PI (Hernández et al., 1998). Similar studies in 5- and 17-day-old piglets showed significantly increased relative levels of CD2+ and CD8+ T-cells (Rodríguez-Ropón et al., 2003).
The changes in the T-cell populations during acute and persistent paramyxovirus infections are poorly understood, and only limited information is available on the cellular immune response to PorPV infection.

## 2.6 Viral persistence

Many members of the Paramyxoviridae family can establish low-grade persistent infections both *in vivo* and *in vitro* that are possibly mediated by the production of DI particles or mutants (Randall and Russell; 1991Ahmed et al., 1996; Hjertner et al, 1997, 1998); however, the exact mechanism of persistence is not fully understood. For example, Sendai viral RNA can persist in the olfactory bulb of intranasally infected mice for at least 168 days PI (Mori et al., 1995). Simian virus 5 can establish a quiescent infection in murine fibroblastic cells. It has been proposed that in persistently infected cells, the majority of virus genomes reside in an inactive form within cytoplasmic inclusion bodies, from which the virus may occasionally be reactivated (Fearns et al., 1994). Hjertner (1997, 1998) reported that PorPV establishes persistent infection in PK-15 cells, which can be maintained for 65 cell passages over at least one year and is associated with the presence of DI particles and of several subgenomic RNAs. In another study, Wiman (1998) used a nested reverse transcriptase-polymerase chain reaction (nRT-PCR) at 53 days PI and detected RNA of the nucleoprotein (NP) and phosphoprotein (P) genes in the midbrain and forebrain samples in experimentally PorPV infected pigs that had recovered from an acute infection. After immune suppression, RNA of the NP and P genes was also detected in lung tissue. In this respect, it is known that the major histocompatibility complex (MHC) proteins are expressed at comparatively low levels within the central nervous system (CNS), and it has been proposed that virus-infected neurons avoid the immune response by failing to express MHC class I molecules. This mechanism may explain the ability of some viruses to persist in these cells (Joly et al., 1991). Further evidence of PorPV persistence was observed in experimentally infected boars: the viral RNA of the nucleoprotein was detected in semen samples for up to 142 days PI (Rivera-Benítez et al., 2013a).

Persistent paramyxovirus infection may induce a restricted T-cell response, which is mainly directed at the NP, P and L proteins. The changes in viral protein levels in cells persistently infected with PorPV could be associated with a reduction in the amount of mRNA of the L protein gene and a shift in editing of the P gene, the latter of which leads to decreased expression of the P protein (Hjertner et al., 1997). It has been suggested that the P protein may severely depress viral gene expression and therefore plays an important role in the maintenance of persistence (Garcin et al., 1994; Hjertner et al., 1997).

There are some similarities between persistence described *in vivo* and that described *in vitro* after the establishment of persistent PorPV infection in PK-

15 cell cultures. These cells produced viral antigens and virus particles but were unable to cause a lytic infection in PK-15 cells, indicating that a selection of virus and cell variants may occur (Hjertner et al., 1997, 1998). Similar findings have been reported in other persistent paramyxovirus infections, such as in cell cultures persistently infected with MeV. The infection differs from the classic definition of a chronic infection because the virus slowly replicates in the cultures, with little or no production of extracellular virus. Restricted expression of the viral genome may occur through the reduced synthesis of one or more structural components or the selection of a defective virus (Ahmed et al., 1996).

The establishment of viral persistence could have an important effect on the pathogenesis of PorPV infection because of the risk of reactivation of the virus from persistently infected pigs. It is possible that there is transmission of the reactivated virus to susceptible pigs on farms with production systems affected by multifactorial immunosuppressive factors and other disease pressures (Stephano, 1998).

## 2.7 Diagnosis

Because of the variability in symptoms associated with PorPV infection and the possibility of co-infections with other agents, PorPV is difficult to diagnose clinically. Clinical symptoms, necropsy findings and histopathological changes can often provide an insight into the aetiology of the disease. Methods currently in use for the diagnosis of PorPV infection in Mexico include serological tests and virus isolation (McNeilly et al., 1997; Nordengrahn et al., 1999; Rivera-Benítez et al., 2013ab). The virus has been shown to be able to grow in many different cells including pig kidney cells with typical syncytial formation, demonstrating fusion activity (Moreno-López et al., 1986). Virus isolation, electron microscopy, direct immunofluorescence and classical RT-PCR have been used to detect infectious virus or viral RNA of PorPV for various research purposes (Berg et al., 1992; Wiman et al., 1998).

For serological diagnosis, the most commonly used test is hemagglutination inhibition. Other serological techniques frequently used are indirect fluorescent antibody, serum-virus neutralisation (VN) (McNeilly et al. 1997; Rivera-Benítez et al., 2013ab) and a blocking ELISA (b-ELISA) (Nordengrahn et al., 1999).

Currently, the disease is considered a commercial barrier that affects the commercialisation of Mexican swine to other countries; therefore, an effective and rapid diagnosis method to confirm PorPV as the causal agent of a disease outbreak is indispensable.

# 3 Aims

The overall objective of this thesis was to develop molecular techniques to help with diagnostics in the field and to investigate different aspects of the persistence and molecular epidemiology of PorPV in naturally infected pigs. The studies of Mexican bats as possible reservoirs of PorPV in nature and the characterisation of the immune response in experimentally infected pigs were included. The specific aims were as follows:

- To develop a rapid, sensitive and quantitative method based on a specific real-time reverse transcriptase polymerase chain reaction assay (qRT-PCR) to enable both a rapid detection of the virus and studies of various aspects of PorPV infection (Papers I and II).

- To investigate the distribution of viral mRNA after a natural outbreak in domestic pigs (Paper II).

- To study the expression of viral proteins in PorPV infection that are related to the tissue tropism and pathogenicity of this virus (Paper III).

- To increase the current knowledge of genetic variation from different clinical cases of infected swine with neurological or respiratory disease collected at different years (Paper III).

- To analyse the full-length genes and phylogenetic characterisation of current PorPV isolates (Paper III).

- To evaluate bats as a possible reservoir of PorPV in nature (Paper IV).

- To characterise the immune response during acute and viral PorPV persistent infection in Vietnamese pot-bellied pigs (Paper V).

# 4 Materials and Methods

This section briefly describes the research methods used in this thesis. More detailed descriptions are provided in each individual paper (I-V).

## 4.1 Animals, experimental design and sample collection

### 4.1.1 Natural Porcine rubulavirus infected pigs

Studies of naturally PorPV infected pigs were used to investigate the persistent viral infection in convalescent pigs after recovery from a natural infection (Paper II). An outbreak, which was confirmed by virus isolation and ELISA serology of PorPV infection, was recorded on a commercial farm in Mexico State, Mexico. Veterinarians followed the development of the outbreak, and after the mortality in piglets subsided, five recently weaned pigs were selected for this study on the basis of having suffered the infection at early age, as evidenced by their corneal opacity and other clinical evidence. The selected pigs remained in the same farm until complete recovery (three months old) and were then housed in individual isolation pens. Six other 2-month-old pigs were obtained from a farm certifiably free from PorPV infection by means of an ELISA test and clinical data. Five of these animals served as sentinel pigs, whereas the sixth was housed separately from the other pigs and served as a control for the entire duration of the experiment. Five groups of animals, each consisting of one convalescent pig together with one sentinel pig, were housed in isolation pens. The experimental protocol and housing conditions were approved by the Animal Care and Ethical Committee of the National Microbiology Research Centre, INIFAP, in Mexico City.

Serum samples from all the pigs were periodically obtained at the beginning of and throughout the experiment at days 0, 14, 30, 60, 75, 90, 120, 150, 190, 210, 225, 240, and 270 (Table 1 of Paper II)). Tissue samples (midbrain, olfactory bulb, *corpus callosum*, cerebellum, medulla oblongata, choroid plexus,

trigeminal nerve, optic nerve, cervical spinal cord, lumbar spinal cord, lungs, tonsil, thymus, and pancreas salivary gland) were obtained for histopathology (HP), virus isolation (VI), hemagglutination (HA), hemadsorption (HAD), immunofluorescence (IF), virus neutralisation and blocking ELISA testing and for the extraction of RNA for nRT-PCR of the NP and P genes of PorPV. The tissue samples were immediately frozen in liquid nitrogen, fixed in 10% formalin or maintained in Trizol LS for RNA extraction. The serum samples were frozen at -20°C until use.

### 4.1.2 Experimental Porcine rubulavirus infected pigs

To investigate the T-cell response and persistence of the PorPV infection, nine 17-day-old Vietnamese pot-bellied piglets were selected (Paper V). Six piglets, two males and four females, were inoculated by intranasal instillation into each nostril with 1 ml of 107 TCID50/ml of the reference strain (PorPV-LPMV/1984). The three remaining piglets were used as uninfected controls. The experimental procedures and housing conditions were approved by the Ethical Committee for the Mexican Institute of Social Security (IMSS), based on the guidelines in the Mexican Sanitary Federal Rules. Blood samples from all animals were taken at the following intervals; 0, 10, 17, 24, 31, 72, 161, 168, 250 and 277 days PI; these samples were analysed for lymphocyte subpopulation profiles (Cuevas et al., 2009). Serum samples were obtained at days 0, 10, 17, 24, 250 and 277 for testing by a competitive antibody b-ELISA (Nordengrahn et al., 1999). The animals were killed at 277 days PI, and tissue samples from the CNS (olfactory bulb, medulla oblongata, and cerebellum), the tonsil, cervical, parotid, mesenteric and submaxilar lymph nodes, the pancreas and the epididymis were collected and frozen at -70°C until use. The tissue samples were thawed for virus isolation and testing by a nested reverse transcriptase-polymerase chain reaction (nRT-PCR) specific to segments of the NP and P genes (Wiman et al., 1998).

### 4.1.3 Bats (Porcine rubulavirus in nature)

To study different aspects of PorPV in nature, a total of 59 bats were caught from two different refuges (N. 18° 42′ 32.4” W 99° 14′ 14.1” to 1004 MASL and N. 18° 46′ 14.33” W 98° 51′ 55.95” 1403 MASL) at locations in a central zone of Mexico in which spontaneous outbreaks of PorPV infection occur (Table 1 of Paper IV). The climate is warm and sub humid all the year. The captured bats were collected under SEMARNAT DGVS permit FAUT-0211 following the recommendations of the Animal Care and Ethical Committee of the National Microbiology Research Centre, INIFAP, in Mexico City. The collected specimens were deposited at “The Colección de Mamíferos del

Centro de Investigación e Biodiversidad y Conservación", UAEM. Blood samples were collected by cardiac puncture, and serum samples were tested by serum-virus neutralising antibodies to PorPV and an immunoperoxidase monolayer assay (IPMA) (Rivera-Benítez et al., 2013a; see section 4.2.3 in the thesis). Un-inoculated cultures were included as negative controls, and inoculated PorPV cultures served as positive controls in the various experiments. At sacrifice, the brain tissues were processed according to standard procedures for virus isolation and RT-PCR amplification and sequencing (Berg et al., 1991; McNeilly et al., 1997; Wiman et al., 1998).

#### 4.1.4 Sample collection for molecular epidemiology

Seven outbreaks of PorPV infection were recorded on different commercial farms from the central part of Mexico (Paper III). The outbreaks were confirmed by virus isolation and ELISA serology. One infected pig from each farm with typical clinical signs of PorPV infection was selected and placed under light anaesthesia and bled to death; a necropsy was conducted to collect brain tissue samples, which were immediately frozen in liquid nitrogen for virus isolation and RT-PCR. One sample was recovered from the peripheral blood mononuclear cells (PBMC) of a clinically healthy persistently infected pig, with corneal opacity as the only clinical sign. The genome sequences of these viruses isolated in different years were examined to determine the genetic variations among them.

## 4.2 Serology

#### 4.2.1 Serum-virus neutralisation

A serum-virus neutralisation assay was performed with the PorPV-LPMV/1984 reference strain on porcine kidney (PK-15) cells (Paper II) and with the PorPV-PAC3/1992 isolate on Madin-Darby canine kidney (MDCK) cell lines (Paper IV). Double serial dilutions of the sera were performed with concentrations ranging from 1:10 to 1:1028 (Papers II and IV). The neutralising antibody titration was expressed as the maximum dilution at which the serum was able to neutralise the replication of the virus in the cell cultures (Rivera-Benítez et al., 2013ab).

#### 4.2.2 Blocking ELISA

Antibodies to PorPV were detected using a commercial blocking ELISA kit (Svanova Biotech, Uppsala, Sweden) (Paper II and V). This test was based on a monoclonal antibody against the hemagglutinin-neuraminidase protein of PorPV (Nordengrahn et al., 1999).

### 4.2.3 Immunoperoxidase monolayer assay

An immunoperoxidase monolayer assay test was conducted on 96-well plates using low-passage MDCK cells infected by the PorPV-PAC3/1992 isolate with a 0.1 MOI (Paper IV). The test plates were incubated for 48 hrs in 5% $CO_2$ at 37°C. The plates were fixed and stained with immunoperoxidase using standard procedures (Rivera-Benítez et al., 2013ab). The reaction was "visualised" by applying AEC substrate (AEC Substrate Kit, Invitrogen, USA) for 10 to 15 min. Finally, the plates were washed in PBS and observed with an inverted microscope. The presence of red-brown cytoplasmic staining in any of the wells denoted a positive result. Positive and negative control sera were run on each plate.

## 4.3 Virus isolation and indirect immunofluorescence test

For virus isolation attempts, approximately 10% (w/v) of the brain tissue samples were suspended in minimal essential medium containing antibiotics, clarified by centrifugation at 400x g for 30 min and inoculated onto PK-15 (Paper II) and MDCK monolayer cultures (Paper IV) in 25 cm flasks with a 1 ml inoculum (1:10 dilution of the original samples); the total volume was 5 ml. The MDCK cell cultures were observed for 96 hrs for cytopathic effect (CPE). If no CPE was observed, the samples were blind-passaged three times. After each passage, the cultures were frozen at -70°C and thawed, and the supernatants were collected after centrifugation. The cultures and supernatants were assayed for HA activity using chicken erythrocytes (0.5%) and IF according to standard procedures (Rivera-Benítez et al., 2013ab). Briefly, for the IF assay, the MDCK cell cultures that were inoculated as described above were fixed with 4% paraformaldehyde, washed with phosphate-buffered saline (PBS) and incubated with the pig polyclonal antibodies against PorPV, followed by another incubation with a fluorescein isothiocyanate-labelled anti-pig IgG. The cells were then observed with a fluorescence microscope (Allan et al., 1996, Rivera-Benítez et al., 2013a).

## 4.4 Lymphocyte subpopulation analysis

T-cell subpopulations were determined in the peripheral blood of all the experimentally infected pigs (Paper V) by an indirect immunofluorescence method as described by Lunney and Pescovitz (1987). The monoclonal antibodies 76.2.11, 74.12.4 and MSA 4 (VMRD, Washington, D.C.) were used

to label the CD8+, CD4+ and CD2+ T-cells, respectively. The labelled cells were analysed by flow cytometry on a FACSCALIBUR flow cytometer (Becton Dickinson) using the Cell Quest program (version 2.0). A Student's t-test was employed for the statistical analysis ($p<0.05$).

## 4.5 RT-PCR

### 4.5.1 RNA extraction and cDNA synthesis

Total RNA was extracted from 100 mg of tissue using Trizol LS reagent (Gibco-BRL, Life Technologies) according to the manufacturer's protocol (Papers I to V). The synthesis of cDNA was performed using a random hexamer primer or an oligo-dT primer (mRNA) and was reverse-transcribed in a final volume of 20 µl, using 1 µl of 200 u/µl Moloney murine leukaemia virus reverse transcriptase (M-MLV-RT) (Gibco, BRL). The cDNAs were used as templates for PCR (Papers II to V). Briefly, the primers for PCR amplification were designed to amplify a specific region from different genes of PorPV. The PCR assay was performed using a Master Mix PCR Assay Kit (Promega) according to the manufacturer's instructions. The PCR was run in a total volume of 50 µl of reaction mixture containing 1 µl of 10 pmol of each primer and 5 µl of cDNA as described above.

### 4.5.2 Quantitative real-time RT-PCR (qRT-PCR)

In the first study (Paper I), a quantitative real-time RT-PCR (qRT-PCR) method was developed to identify and quantify viral RNA of PorPV in infected pigs. The system was based on the P-gene of the PorPV-LPMV/1984 reference strain (GenBank accession number AF416650) because of the limited sequence data concerning PorPV and because the P gene is one of the most expressed during infection (Berg et al., 1992). To evaluate the assay, clinical samples were recovered from experimentally and naturally infected pigs (Table 1 of Paper I).

For optimisation of the qRT-PCR procedure (Figure 1 of Paper I), a plasmid constructed for the P-gene as described previously was initially used (Berg et al., 1992). Different concentrations of each primer (50-900 µM) and of the fluorogenic probe (100-300 µM) were tested. Final concentrations of 400 µM for the primers, 3.5 µM of Mn (OAc)2 and 100 µM of the fluorogenic probe provided the optimal sensitivity with the target plasmid. All the reactions were performed in triplicate, and 100 ng of total RNA in a volume of 2 µl was included in the assay. RNA from PK-15 infected cells was used to optimise the final protocol. The rTth enzyme was used throughout the optimisation. For this

latter procedure, 50-100 ng of total cellular RNA from uninfected and infected cell cultures was used, and the reaction was conducted using one-step real-time RT-PCR.

The implementation of the highly sensitive qRT-PCR assays developed was used to detect persistent PorPV infected pigs with low concentrations of viral RNA in their tissue samples (Paper II). The evaluation of the convalescent and sentinel pig groups using the qRT-PCR was performed as confirmation of the results from the nested PCR. One hundred nanograms of total RNA extracted from the mid brain, olfactory bulb, cerebellum, choroid plexus, tonsil, lung and pancreas was used in the quantification of viral copies/µg of total RNA of the P gene from the convalescent and sentinel pigs, respectively (Table 2 of Paper II).

A second real-time RT-PCR was used as an internal control to avoid apparent false-negative results. The quality of the RNA was tested by evaluation of the expression of the housekeeping gene cyclophilin, which was used as an internal control in the quantitative real-time PCR assays, as described by Duvigneau, 2005. Each sample was tested for the expression of the housekeeping gene before being used in the qRT-PCR for PorPV (Papers I, II, and V).

### 4.5.3 Nested-PCR (nPCR)

For nPCR (Papers II and V), several cDNA were used in separate nested PCR reactions. Briefly, the cDNA was amplified by the nPCR technique specific for the NP and P genes as described (Wiman et al., 1998). The final nested products were 375 and 298 base pairs (bp) in length for the NP and P genes, respectively. The PCR products were analysed by electrophoresis using 1.5% agarose gel and were visualised using ethidium bromide staining. The nPCR products of the P gene from the sentinel pigs (Paper II) were purified using the QIAquick PCR purification system (Qiagen) and directly sequenced.

## 4.6 Sequencing and sequence analysis

### 4.6.1 Sequencing of PCR products (Paper II)

Two nPCR products of the PorPV-NP gene were sequenced for confirmation of PCR data (Paper II). The nPCR products from the sentinel pigs were purified using the QIAquick PCR purification system (Qiagen) and directly sequenced. One amplicon was selected for topo cloning into the pCR2.1 vector (Invitrogen) according to the manufacturer's protocol and was purified using

QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). At least two clones were sequenced in both directions using an ABI Prism sequencing device based on automated cycle sequencing with the incorporation of fluorescent labelled dideoxynucleotide terminators.
The sequencing was performed with the same primers that were used to generate the nPCR products. The consensus sequences determined for each clone or amplicon were compared using the BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) from the National Centre for Biotechnology Information (NCBI) (Morgulis et al., 2008) with the published sequence of the genomic region.

### 4.6.2 Sequencing of full-length PorPV genes

The PCR products (Paper III) were purified prior to sequencing with the GeneJET PCR Purification Kit or with the GeneJET Gel Extraction Kit (Thermo Scientific, USA). Briefly, the purification was performed according to the instructions of the manufacturer by binding the PCR product to a silica membrane, washing it, and finally eluting it in 25 µl of elution buffer. The samples were prepared for sequencing by combining 5 µl of purified PCR product and 5 µl (10 pmol/µl) of each primer in separate 1.5 ml tubes, respectively (Table 2 of Paper III). The samples were sequenced by Macrogen Europe, The Netherlands. The nucleotide sequences were assembled with SeqMan (Lasergene 9.1, DNASTAR), and the consensus sequences were determined for each amplicon. The consensus sequences from the NP, P, M, F, HN and L genes of the PorPV isolates were compared with those deposited in the GenBank database using the BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) at the National Centre for Biotechnology Information (NCBI) (Zhang et al., 2000; Morgulis et al., 2008).

### 4.6.3 Sequence analysis

Phylogenetic studies comparing current PorPV isolates with the reference strain PorPV-LPMV/1984 were performed within this thesis to establish the genetic evolution and molecular epidemiology of this virus in pigs (Paper III). Phylogenetic trees (Papers III and IV) were constructed using the neighbour-joining algorithm (Saitou and Nei, 1987) with bootstrap values determined by 1000 replicates (Felsenstein, 1985). For this genetic analysis, Mega V 5.0 and 6.0 (Tamura et al., 2011, 2012) software were used.

# 5 Results and Discussion

## 5.1 Development of a rapid and sensitive method based on a specific real-time reverse transcriptase polymerase chain reaction assay (real-time RT-PCR) for the detection of PorPV (Paper I)

The detection of PorPV represents a diagnostic challenge because the viral RNA is present in very small amounts in tissue samples. Until now, it has been impossible to study the epidemiology of PorPV because no suitable diagnostic method has been available. Moreover, to be able to investigate the spread of PorPV in nature and in domestic pigs, in which the virus now is endemic, a new detection tool was necessary. Therefore, a qRT-PCR assay was developed for PorPV to study these and other aspects of the viral disease throughout this thesis.

Real-time PCR assays have many advantages over other diagnostic methods, such as better sensitivity and specificity (Belák and Thorén, 2001). Additionally, it is possible to quantify the exact levels of viral RNA if required, and the procedure limits possible cross-contamination. For the assay presented here, the P-gene was used because this gene is highly expressed (Hjertner et al., 1998), its sequence was available (Berg et al., 1992) and the genetic variations of this gene among different isolates were likely limited. In this study, a TaqMan® real-time PCR assay was designed based on the P-gene of PorPV to allow for the specific amplification and detection of viral RNA in clinical samples. The assay conditions for the primers and probe were optimised using infected PK15 cells and ten-fold serial dilutions of a plasmid containing the whole P-gene. The sensitivity of the developed TaqMan® assay was approximately 10 copies per reaction and was shown to be 1000 fold better than a conventional nested RT-PCR (Figure 1 of Paper I). The assay detects current known variants of the virus that have been reported (Sánchez-

Betancourt et al., 2008), which was an important point to increase the knowledge of molecular epidemiology and continue with studies of various aspects of PorPV infection (Paper II, III, IV, V). A housekeeping gene was used as an internal control to avoid apparent false-negative results.

## 5.2 Long-term persistence of Porcine rubulavirus (PorPV) after an outbreak of a natural infection and transmission of the virus to susceptible sentinel pigs (Paper II)

The detection of viral mRNA of the NP or P genes in different tissues from convalescent pigs may be an indication that viral transcription occurs and that the expression of the NP and P proteins is maintained during persistent PorPV infection, as has been described in persistent measles virus infection (Guido Re, 1991; Schneider-Schaulies et al., 1999) and in the persistent PorPV infection in experimentally infected boars (Rivera-Benítez et al., 2013a).
Additionally, the absence of the infectious virus in the samples of the sentinel pigs suggests that these animals could have been infected by direct physical contact with the virus produced during the establishment of the persistent PorPV infection in the convalescent pigs or that, more likely, the sentinel pigs could have been infected by viral particles already eliminated as defective viruses. There are some similarities between persistence described *in vivo* and *in vitro* that were observed after the establishment of persistent PorPV infection in PK-15 cell cultures. These cells produce viral antigens and virus particles unable to cause a lytic infection in PK-15 cells involving a selection of virus and cell variants (Hjertner et al., 1997, 1998). It has been suggested that the P protein may severely depress viral gene expression and may therefore play an important role in the maintenance of persistence (Garcin et al., 1994; Hjertner et al., 1997). The changes in viral protein levels in persistently infected cells could be associated with a reduction in the amount of mRNA of the L protein gene and a shift in editing of the P gene, together with a decrease in the amount of P protein (Hjertner et al., 1997). Further studies of mutations of the V protein encoded from the P gene related to the persistence of PorPV infection will be described later (Paper III).

The suspected transmission of PorPV to sentinel pigs without causing any clinical signs or lesions can also be explained by the fact that the severity of the disease is age-related and is most severe in piglets less than 3 weeks of age. In contrast, by 17 days of age, the clinical signs are mild, and as the animals become older, disturbance of the CNS is rare or atypical (Allan et al., 1996; Ramírez-Mendoza et al., 1997; Sánchez-Betancourt et al., 2008). The question

consequently arises of whether the age of non-infected pigs in this study may be considered a crucial requirement when a reactivation of PorPV in long-term persistently infected pigs is expected.

In conclusion, these results indicate that PorPV persists in naturally infected pigs for more than 10 months after infection, and specific antibodies against the HN protein were detected during the persistent infection. This virus induces a durable humoral immune response in pigs that have recovered from a natural infection. After a possible reactivation of the virus, it was transmitted to sentinel pigs that were in contact with the convalescent pigs. Therefore, the establishment of viral persistence could have an important effect on the pathogenesis of PorPV because of the risk of reactivation of the virus from persistently infected pigs in which the source of PorPV responsible for the disease outbreaks in Mexico was suggested to be subclinically infected and/or persistently infected pigs, as mentioned previously (Wiman et al., 1998; Stephano, 2002).

## 5.3 Molecular epidemiology of current PorPV isolates collected from different clinical cases of infected swine that occurred during the last ten years (Paper III)

Until now, only one full-length genome of PorPV has been sequenced; sequence data are scare from other isolates, and only a few individual genes have been sequenced. During the last ten years, the evolution of PorPV has been studied based on the phylogenetic analysis of the HN protein, and there is therefore limited information about other genetic variations of this virus. In this study of current PorPV isolates from different clinical cases of infected swine, full-length (or almost complete) genes were sequenced to perform genetic comparisons and phylogenetic analyses. The aligned sequences of the NP protein indicated some amino acid changes of the N-terminal and C-terminal tails, whereas the middle part of the protein revealed high sequence conservation throughout the years compared to the PorPV-LPMV/1984 reference strain. All the isolates showed a well conserved editing site of the P gene, allowing the expression of the P and V proteins. Nevertheless, the V protein of one PorPV isolate from 2013 that was recovered from a healthy persistently infected pig displayed a mutation at the arginine residue ($R_{182}$) of the C-terminal domain, which is required to antagonise the antiviral immune responses of the host. It also showed an inability to encode the C protein because it was an early stop-codon in the reading frame. These two features may restrict the capacity of this virus to escape host defences; additionally, the

V protein has been reported as an important virulence factor that can interfere with host immunity (Ramachandran and Horvath, 2010). However, it might also be a strategy of the new generation of viruses that currently have remained in the pig population with a gradual attenuation to adapt to the host.
The analysis of the M protein indicated that all the new isolate sequences presented a variation in a short region of six amino acid residues between $I_{187}$ to $T_{192}$ (IQGRQT), which could affect the electrostatic charge and the interaction between the M protein and the membrane, particularly the substitution of $Q_{188}K$ and $Q_{191}R$, and increase the potential of the positively-charged area that would extend the contact area for protein–protein and protein–membrane interactions.

Furthermore, one PorPV isolate (PorPV/1/Jalisco/2007) presented the highest conserved sequence related to the PorPV-LPMV/1984 reference strain in all the analyses (approximately 99.9% identity); however, this isolate was recovered from the lungs but not from the brain and showed a mutation at the cleavage site (HRKKR) that could represent a factor to determine the tissue tropism and pathogenicity of PorPV related to the cleavability of the F protein. However, the change led to another basic amino acid and may be irrelevant.

Additionally, the alignment comparison of the HN amino acid sequences of the new isolates showed high homology and conserved sites through the years (up to 2013); approximately 22 nucleotide positions differed of the 1768 nucleotides, which corresponds to approximately one percent of the HN protein. At this point, we have identified some of the blocks of the highest amino acid conservation among the PorPV isolates important for polymerase activity, which has already been reported in the L protein of PorPV-LPMV/1984.

The relatively high sequence identity among all the isolates over a long time period of the P, F and HN genes indicates that the immunological pressure is limited. This may suggest that PorPV has been adapted to the pig population for a long time. It could reflect the movement of PorPV throughout the endemic area with a high association between the geographic areas and ecology on virus diversity.

In conclusion, based on genome organisation, sequence homologies of the encoded proteins (NP, P, M, F, HN, L) and phylogenetic analysis, we can identify three different genetic variants that have spread into the swine population throughout the geographical region in which the first outbreaks

occurred. We suggest that a new generation of circulating virus has begun to emerge in nature during the last ten years. This could be related to the presence of different virus strains in nature that are associated with a wildlife reservoir. It could be considered an additional criterion in the evolutionary relationship of this virus. This information was used to evaluate bats as a possible reservoir of PorPV in nature (Paper IV).

## 5.4 Studies of Mexican bats as possible reservoirs of PorPV in nature (Paper IV)

Porcine rubulavirus has been endemic in Mexico since the early 1980s, and it remains a problem for the pig-farming industry in Mexico (Moreno-López et al., 1986; Stephano et al., 1988; Escobar-López et al., 2011). However, where the virus came from and how it was introduced to pigs is unknown. It was discovered that PorPV is relatively closely related to the Mapuera virus, which was isolated from a fruit bat in Brazil in 1979 (Wang et al., 2007). However, the viruses were quite divergent on the nucleotide level, with similarity ranging from 40-60%. In addition, in 2004, the presence of antibodies to PorPV from a non-hematophagous bat on the subtropical Pacific coast of Mexico was reported (Salas-Rojas et al., 2004). It was therefore conceivable that bats introduced the virus to pigs. However, other natural reservoirs are also possible. At this point, we report the first isolation of PorPV from frugivorous, insectivorous, and hematophagous bats. Serological evidence revealed the presence of PorPV antibodies in 19% (by serum-virus neutralising analysis) and 39% (by immunoperoxidase monolayer assay) of the investigated bats. PCR analysis, using an RT-PCR targeting the HN gene of the virus, gave positive results for 18 of the 59 bats. The sequence analysis of the RT-PCR positive samples showed that, over the 600-nucleotide region analysed, the sequences displayed a 99.97 - 100% amino acid sequence identity to the PorPV-LPMV reference strain, which was isolated from swine in 1984, and the PorPV-PAC-6/swine/2001 strain. The high sequence similarity was surprising, but larger parts of the genome must be analysed before the true relationship between the bat virus and the virus isolated from pigs can be established.

Furthermore, PorPV was isolated in cell cultures from four of the PCR positive bats. These findings support the hypothesis that bats could act as a wildlife reservoir in nature. The isolation of this virus in different bat species will be important for our understanding of its ecology, evolution and mechanism of cross-species transmission, as has been reported in other emerging paramyxoviruses (Hendra in 1994, Menangle in 1997, Nipah in 1999 and

Tioman viruses in 2001) (Chant et al., 1998; Chua et al., 2000; Halpin et al., 2000; Calisher et al., 2006; Barr et al., 2012). This information will help to establish new strategies for the control of this virus in the country.

## 5.5 Characterisation of the immune response and viral persistence in pigs after experimental and natural infection by PorPV; transmission of the virus to susceptible sentinel pigs (Paper V)

Additionally, selected lymphocyte subpopulations were studied, and the distribution of viral mRNA in lymph nodes was investigated during acute and persistent infection in six Vietnamese pot-bellied pigs. The six inoculated piglets exhibited sneezing, coughing and slight conjunctivitis from day 7 to 10 PI. These mild signs were considered characteristic of PorPV infection. One infected piglet died 11 days PI; moderate pneumonia and encephalitis were observed at necropsy of this animal, whereas the other five piglets recovered around day 13 PI and survived until euthanasia on day 277 PI. No macroscopic alterations were observed at necropsy on day 277 PI of these pigs. No clinical signs or macroscopic lesions were observed in the uninfected control animal. These data were consistent with a previous study in which the experimental inoculation of 3 and 17-day-old piglets with PorPV resulted in the death of all the 3-day-old piglets 6–8 days PI, whereas 60% of the 17-day-old piglets survived (Allan et al., 1996). In our work, the piglet survival rate was 90%, although the number of animals in our case was very low.

T-lymphocytes play a central role in the antigen-specific immune response in acute and persistent viral infections. Enhanced levels of CD8+, CD4+ and CD2+ T-cells were detected in all infected animals at 10 days PI. The increased levels of the CD8+ and CD2+ T-cells at 10 days PI may participate in the clearance of the virus, which has been proposed for infection by the related paramyxovirus SV5 (Young et al., 1990; Gray et al., 2001). Particularly notable were the increased levels of CD8+ T-cells throughout the entire period of observation. The CD8+ T-cell subpopulations of the infected pigs were significantly greater ($p<0.05$) than the lymphocyte subpopulations of the uninfected controls at 10 and 250 days PI. The CD4+ T-lymphocytes in the infected pigs were also significantly greater ($p<0.05$) than in the control animals at 250 days PI. These cells may correspond to ‘‘double-positive’’ T-lymphocytes, which have been associated with immune memory and are unique in the porcine immune system (Zuckerman and Husmann, 1996).

The NP gene product was consistently detected in the lymph nodes (parotid, submaxilar, cervical and mesenteric) and pancreas, whereas P gene expression was detected in all tissues except the epididymis (Table 1, Figures 1 and 2 of Paper V). This finding is in line with other negative-stranded RNA viruses in which more transcripts of the NP gene are present and therefore accessible for RT-PCR detection (Hjertner et al., 1998). All the tissue samples from the uninfected piglets tested negative for PorPV RNA.

The presence of viral mRNA in the epididymis tissue of pigs 277 days PI confirms previous results and supports the possibility of the transmission of PorPV to the female reproductive tract and foetuses via infected semen (Solís et al., 2007). It has been reported that in experimentally infected boars, the isolation of the virus from semen has been achieved between 5 and 48 days PI and from the testicles and epididymis between 64 and 142 days PI (Rivera-Benítez et al., 2013a). PorPV has a tropism for the epididymis, as demonstrated in experimentally infected mature male pigs (Ramírez-Mendoza et al., 1997).

The persistence of PorPV viral mRNA in lymph nodes is not unexpected because of the relatively high concentrations of receptors for this virus in cells of the lymphoid, nervous, respiratory and genitourinary tissues (Reyes-Leyva et al., 1997; Vallejo et al., 2000). The presence of viral mRNA in the lymph nodes supports the possibility that continuing lymphocyte interaction with persisting viral antigens could be a factor in promoting cellular and humoral immune responses, despite an absence of detectable infectious viral particles.

# 6 Concluding Remarks

- The development of an efficient TaqMan® real-time PCR assay provides a useful tool for the rapid detection and molecular identification of all known PorPV variants. The ability to detect low concentrations of viral RNA will be of great assistance to identify persistently infected pigs and thereby improve the control strategies of PorPV associated disease in Mexico.
- After recovery from infection, convalescent pigs could establish a long-term persistence in the CNS and organs other than the primary site of replication. The establishment of viral persistence could have an important effect on the pathogenesis of PorPV because of the risk of reactivation of the virus from persistently infected pigs. After a possible reactivation of the virus, it can be transmitted to sentinel pigs in contact with the convalescent naturally infected pigs. This is a novel finding of significance to the epidemiology and pathogenesis of this viral infection.

- This work includes the detection of the "newly emerging" genetic variants of PorPV associated with mutations in different gene sequences.

- One PorPV isolate (PorPV/1/Jalisco/2007) showed 100% protein identity for the NP and P (V/C) proteins compared to the reference strain PorPV-LPMV/1984. This shows that these proteins have been conserved during the evolution of more than 20 years. The NP protein was also shown to be conserved, with three amino acid substitutions in five of six PorPV isolates evaluated. Similarly, the HN protein analysis revealed only one amino acid substitution in two of the isolates (PorPV/1/Jalisco/2007 and PorPV/1/Guanajuato/2008), indicating that the HN gene appears to be rather stable and well conserved.

- A mutation in the V protein in one PorPV isolate (PorPV/3/Michoacan/2013) could possibly be involved in modifying the virus's ability to evade the interferon response of the host and affect the ability of the virus to adapt to the host, which is particularly related to the virus-host interaction. This mutation in the V protein can have effects on many aspects of the replicative cycle of the virus and may be important in establishing persistent infection in naturally infected pigs. Perhaps it is a new strategy to select different cells or viruses, which support persistent infection by the negative effect on RNA synthesis associated with the degree of the host antiviral response. Additionally, this isolate showed the absence of the C protein, and together with the mutation of the V protein in the region corresponding to antagonise the antiviral immune defence of the host, this may restrict the capacity of this virus to escape host defences. It might also be a strategy of the new genetic variant virus to remain silent in the pig population to induce an adaptive immune response, which would allow the generation of new viruses with a gradual attenuation.

- Unexpectedly, one of the isolates (PorPV/1/Jalisco/2007) that was obtained from lung tissue, in contrast with the ones recovered from brain tissue, showed an amino acid substitution located at the cleavage site (RRRKR instead of HRKKR) of the fusion protein. These changes in the basic amino acid composition could be a factor determining the tissue tropism and pathogenicity of the PorPV infection. These findings could possibly be used to aid an initial biological characterisation of the PorPV isolates related to the viral tropism and pathogenicity (neurotropic or pneumotropic). For example, a single amino acid change ($Q_{114}R$) in the cleavage site of the NDV F protein attenuates viral replication and pathogenicity (Samal et al., 2011).
  The highest genetic variation was observed in the different regions of the L gene, and because of the complexity of the mechanisms involved in polymerisation, we would expect some changes in the protein structure or enzymatic function. It will be a good starting point to investigate in greater detail the 3D-model of the PorPV-L protein to help establish any change in shape or function as a result of these mutations.

- The phylogenetic studies showed a genetic variation of different PorPV isolates that are currently spread throughout the major swine-producing zones in Mexico. We have observed that at least three different genetic virus strains are circulating in the pig populations in these regions. One

group contains strains closely related to the reference strain PorPV-LPMV/1984, which implies that some PorPV isolates remain stable for many years in the pig population; a second group includes new PorPV isolates with possible changes in their abilities to regulate viral transcription/replication and to antagonise the antiviral responses of the host; this group is related to the absence of expression of the C protein; finally, a last group of new variant viruses has the ability to infect pigs and spread into the farms with a graded attenuation.

- Serological evidence in bat populations revealed the presence of neutralising antibodies to PorPV in 19% (by serum-virus neutralising analysis) and 39% (by immunoperoxidase monolayer assay) of the investigated bats. A specific fragment (600 nt) of the HN gene was analysed from 59 brain tissue samples by RT-PCR; from four of these samples, a virus was isolated in the cell cultures. The sequence analysis displayed a 99.97-100% amino acid sequence identity to swine PorPV-LPMV/1984. These findings support the hypothesis that bats could act as reservoirs of PorPV in nature, which is related with the presence of this virus that sporadically emerged in pigs in the early 80s. The isolation of this virus in different specimens of bats will be highly important to our further understanding of viral evolution, the mechanism of cross-species transmission, and pathogenesis in different animal species.

- The knowledge of the presence of different virus variants in nature, associated with a wildlife reservoir of PorPV can provide greater knowledge regarding the molecular epidemiology and useful data to establish new strategies regarding the control of this virus in Mexico.

- The persistence of PorPV in the lymph nodes is not unexpected because of the relatively high concentrations of receptors for this virus in cells of the lymphoid, nervous, respiratory and genitourinary tissues (Reyes-Leyva et al., 1997; Vallejo et al., 2000). The detection of PorPV mRNA of the NP or P genes in the lymph nodes and reproductive tract of boars as long as 277 days after the experimental challenge suggested that viral replication occurs after the synthesis of mRNAs and that the expression of the NP and P proteins is maintained during persistent PorPV infection. It could be associated with a durable immune response induced by a continued lymphocyte interaction with viral RNA, viral

proteins or defective particles as an important factor in promoting cellular and humoral responses during persistent infection.

# 7 Future research

- The work presented in this thesis contributes to a better understanding of the pathogenesis of PorPV infection in persistently infected pigs; however, further investigations are necessary to understand the pathogenesis, reactivation and shedding of this virus from persistently infected pigs to susceptible ones and to elucidate whether recovered animals can act as a reservoir under natural conditions.

- Further studies related to the cleavability of the F protein of the PorPV isolates could be an important factor to determine the tissue tropism and pathogenicity of this virus to clarify the virulence and pathogenicity associated with the mutation of the viral protein envelopes (M, F and HN).

- This work shows that PorPV, or a related virus, is circulating in the bat populations. Therefore, future studies are required to investigate the infection in bats, to determine its exact role in the epidemiology, the behaviour of this virus in nature and to elucidate whether interspecies transmission occurs because PorPV may have jumped from bats to pigs, which has been reported in other emerging paramyxoviruses (Hendra in 1994, Menangle in 1997, Nipah in 1999 and Tioman viruses in 2001) (Chant et al., 1998; Chua et al., 2000; Halpin et al., 2000; Calisher et al., 2006; Barr et al., 2012).

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