From the Institute of Animal Hygiene and Veterinary Public Health
Faculty of Veterinary Medicine
University of Leipzig

Studies on genetic properties of porcine parvoviruses

Inaugural-Dissertation
to obtain the degree of
Doctor medicinae veterinariae (Dr.med.vet.)
by the Faculty of Veterinary Medicine
of the University of Leipzig

Submitted by
André Felipe Streck
from São Leopoldo, Brazil

Leipzig, 2013

With the approval of the Veterinary Faculty of the University of Leipzig

Dean:	**Prof. Dr. Uwe Truyen**
Supervisor:	**Prof. Dr. Uwe Truyen**
Reviewers:	**Prof. Dr. Uwe Truyen,** Institute of Animal Hygiene and Veterinary Public Health, Faculty of Veterinary Medicine, University of Leipzig
	Prof. Dr. Susanne Modrow, Department of Medical Microbiology and Hygiene, University Hospital of Regensburg

Date of defense: 02.04.2013

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AA	-	Amino acid
AB	-	Antibody
BS	-	Bayesian skyline
CPV-2	-	Canine parvovirus 2
CTL	-	Cytotoxic T cell
DMEM	-	Dulbecco's Modified Eagle Medium
DNA	-	Deoxyribonucleic acid
dNTP	-	Deoxyribonucleotide triphosphate
EDTA	-	Ethylenediaminetetraacetic acid
ELISA	-	Enzyme-linked immunosorbent assay
FBS	-	Fetal bovine serum
FPLV	-	Feline panleukopenia virus
GTR	-	General time reversible
HA	-	Haemagglutination test
HI	-	Haemagglutination inhibition test
HKY	-	Hasegawa-Kishino-Yano
HPD	-	Higher posterior density
ICNV	-	International Committee of Nomenclature of Viruses
ICTV	-	International Committee of Taxonomy of Viruses
LB	-	Luria Bertani
MCMC	-	Markov chain Monte Carlo
ML	-	Maximum likelihhod
MLV	-	Modified-live virus vaccine
NCBI	-	National Center for Biotechnology Information
Nuc	-	Nucleotide
NS	-	Non-structural protein
NS1	-	Non-structural protein 1
NS2	-	Non-structural protein 2
NS3	-	Non-structural protein 3
ORF	-	Open reading frame
PBoV	-	Porcine bocavirus
PBS	-	Phosphate-buffered saline solution
PCR	-	Polymerase chain reaction
PMWS	-	Postweaning multisystemic wasting syndrome
PRRS	-	Porcine reproductive and respiratory syndrome
PPV	-	Porcine parvovirus

LIST OF ABBREVIATIONS

PPV2	-	Porcine parvovirus 2
PPV3	-	Porcine parvovirus 3
PPV4	-	Porcine parvovirus 4
RNA	-	Ribonucleic acid
RPMI	-	Roswell Park Memorial Institute Medium
ss	-	Single strand
SMEDI	-	Stillbirth, mummification, embryonic death and infertility
TCID	-	Tissue culture infective dose
UCED	-	uncorrelated exponential distribution of rates
UCLN	-	uncorrelated lognormal distribution of rates
VP	-	Viral protein
VP1	-	Viral protein 1
VP2	-	Viral protein 2
VP3	-	Viral protein 3

PREFACE

This dissertation represents a cumulative study, composed by the following manuscripts:

Title: **High rate of viral evolution in the capsid protein of porcine parvovirus**

Authors: André Felipe Streck, Sandro Luis Bonatto, Timo Homeier, Carine Kunzler Souza, Karla Rathje Gonçalves, Danielle Gava, Cláudio Wageck Canal, Uwe Truyen

Published in: Journal of General Virology, 2011, 92(11):2628-2636.

Title: **Population dynamics and *in vitro* antibody pressure of porcine parvovirus (PPV) indicate a decrease of variability**

Authors: André Felipe Streck, Timo Homeier, Tessa Foerster, Uwe Truyen

Submitted to: Journal of General Virology

Title: **Analysis of porcine parvoviruses in tonsils and hearts from healthy pigs revealed high prevalence and genetic diversity in Germany**

Authors: André Felipe Streck, Timo Homeier, Tessa Foerster, Stefan Fischer, Uwe Truyen

Accepted in: Archives of Virology, December 2012

Leipzig, 19.12.2012

CHAPTER 1 - INTRODUCTION

Porcine parvovirus (PPV) is a small non-enveloped virus considered to be one of the major causes of reproductive failure in swine. The clinical signs of PPV infections can be characterized by the reoccurring of estrus, abortion and the delivery of mummified or stillborn fetuses (MENGELING et al. 2000) and are commonly described with the acronym SMEDI (stillbirth, mummification, embryonic death and infertility). The virus is considered to be endemic in most areas of the world and can be found in all pig herd categories (TRUYEN and STRECK 2012).

The PPV genome is a single stranded DNA molecule of about 5.2 kilobases. Two large open reading frames (ORFs) can be found in the genome, one coding for the non-structural proteins (3´ side), and the other coding for the structural virus proteins (5´ side). Sixty copies of these structural proteins assemble the icosahedral viral capsid. In each of these copies (capsid subunit), a spike on the 3-fold axis, a depression or canyon around the 5-fold axis and a dimple on the 2-fold axis can be observed.

These structural proteins appear to be responsible for the virulence. A sequence comparison between the non-virulent NADL2 and the virulent Kresse strains revealed that the non-coding regions of these viruses are almost identical. However, in the structural protein genes eight differences are present and six of those led to amino acid substitutions. Five of these amino acids present in the Kresse strains were also observed in virulent field isolates (I-215-T, D-378-G, H-383-Q, S-436-P and R-565-K), while three of these substitutions (D-378-G, H-383-Q and S-436-P) were considered to be responsible for the different tissue tropism (BERGERON et al. 1996).

Despite the several amino acids substitutions observed in the last 10 years (SOARES et al. 2003; ZIMMERMANN et al. 2006), it has been commonly assumed that PPV has a low rate of nucleotide substitution close to that found in its mammalian host (SOARES et al. 2003). It was shown that autonomous parvoviruses, including canine parvoviruses and human B19 erythrovirus, have a nucleotide substitution rate of approximately 1×10^{-4} substitutions per site per year, similar to that observed for RNA viruses (SHACKELTON et al. 2005; SHACKELTON and HOLMES 2006).

In this scenario, the continuous use of the same inactivated vaccine in swine herds during the last three decades, and the remaining occurrences and reports of reproductive failures caused by PPV, highlights the importance of a monitoring of PPV isolates and put in question the genome conservative genome of this virus.

In addition, by mean of more sensitive molecular techniques, several novel viruses have been identified for the first time. In the swine host, porcine parvovirus 2 (PPV2), porcine parvovirus 3 (PPV3) and porcine parvovirus 4 (PPV4) were recently identified (HIJISAKA et al. 2001; LAU et al. 2008; CHEUNG et al. 2010), but the veterinary importance remain unknown.

In order to address these questions, the present dissertation includes three manuscripts. In the first study, recent PPV isolates were analyzed by determining nucleotide substitutions, phylogenetic analysis and estimation of the molecular evolutionary rate. In the second, the population dynamics of the PPV was performed by using an *in vitro* and an *in silico* models. In the last manuscript, distribution of novel parvoviruses (PPV2, PPV3 and PPV4) in hearts and tonsils of clinically healthy swine from Germany was evaluated.

CHAPTER 2 - LITERATURE REVIEW

Classification and history

The first report of a parvovirus dates back to the year 1959 (KILHAM and OLIVIER 1959) with the description of viruses causing disease in newborn hamsters, called “hamster-osteolytic viruses”. One of these hamster-osteolytic agents, the Kilham rat virus, was identified as the first parvovirus (SIEGL. 1976). In the subsequent years, parvoviruses were found in more than five different hosts in several countries (HALLAUER and KRONAUER 1960; BURGER et al. 1963; BERNHARD et al. 1963; HAMPTON. 1964; CARTWRIGHT and HULK 1967; ROSE et al. 1969) and the size, structure and biological properties were defined (PAYNE. 1964; KARASAKI. 1966; HOGGAN. 1971; TINSLEY and LONGWORTH 1973).

Initially, these viruses were classified as picodnaviruses (MAYOR and MELNICK 1966), due to their similar size and morphology to the picornaviruses. After the identification of their single-stranded properties of the their genome, the name parvovirus was proposed (ANDREWS. 1970) and accepted by the International Committee on the Nomenclature of Viruses (ICNV). Currently, according to the last release of the taxonomical classification of the International Committee on Taxonomy of Viruses (TIJSSEN et al. 2011) the parvoviruses comprise the family *Parvoviridae*, divided in the subfamilies *Densovirinae* and *Parvovirinae*. The viruses of the subfamily *Densovirinae* are divided in the genus *Brevidensovirus*, *Densovirus*, *Iteravirus* and *Pefudensovirus*, and infect mostly insects; the viruses of the subfamily *Parvovirinae* consist of the genera *Amdovirus*, *Bocavirus*, *Dependovirus*, *Erythrovirus* and *Parvovirus*, and infect a wide variety of animals.

In pigs, the reproductive losses in commercial herds associated to unknown causes were high in the 1960s, and, in that time, considered to be associated with environmental, nutritional, genetic and toxicological problems (LAWSON. 1961; RASBECH. 1969). The first evidence of porcine parvovirus was recognized in primary and secondary cell cultures from porcine kidney and testicle used to cultivate hog cholera virus, where persistent contaminant small particles (22-23 nm size) were found (MAYR and MAHNEL 1964). These particles were found to be similar to the Kilham rat virus (MAHNEL. 1965). Due to the replication ability of the virus in cell lines from swine, it was possible to isolate and classified as a parvovirus (SIEGL. 1976).

The occurrence of the PPV in pigs was first described by CARTWRIGHT and HUCK (1967) and associated with herd infertility, abortion and stillbirth. In the following years, PPV was identified as the main agent for causing the SMEDI syndrome in the pig industry (S: stillbirth,

M: mummification, ED: embryonary death and I: infertility) (THOMPSON and PROZESKY 1994).

Genome

The genome of parvoviruses is a single stranded DNA molecule of about 4.5 kilobases to 5.5 kilobases. In both terminal sequences of the virus, a complex palindromic hairpin structure (in a similar “Y” or a “T”-shape) of about 120 to 200 bases is located (BERNS and HAUSWIRTH 1983). These structures can serve as primers during the viral DNA replication and probably play an important role for the viruses’ genome integrity. Two large open reading frames (ORFs) are present in the parvovirus genome, one coding for the non-structural proteins, and the other coding for the structural virus proteins (Figure 1). For PPV, the ORF located at the 5´end of the genome encodes the non-structural protein 1 (NS1), and by means of alternative splicing also encodes the NS2 and NS3 non-structural proteins 2 and 3 (NS2 and NS3) (BERGERON et al. 1996). The NS1 protein has a helicase and nickase function, being responsible for viral replication and packaging. In contrast, the exact functions of NS2 and NS3 proteins are still unknown (NUESCH et al. 1995). At the 3´end, one small ORF and the large ORF encodes for the viral protein 1 (VP1) by using a splicing, the viral protein 2 (VP2) is transcribed from the same ORF and the viral protein 3 is an enzymatic cleavage product of the VP2 (BERGUERON et al. 1996). Additionally, an accessory protein for replication (SAT) is codified by another small ORF located in the middle of the genome, close to the VP2 start codon (ZADORI et al. 2005). The entire genome transcription is guided by only two promoters: P4, located at the 5´ extremity of the genome, initiates the transcription of the non-structural proteins; and the P40, located at the middle of the genome, initiates the transcription of the viral proteins and SAT protein (BERGERON et al. 1996).

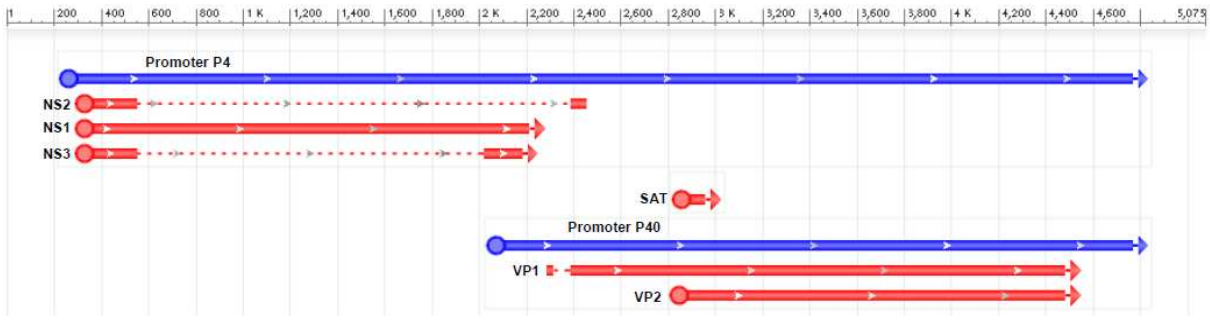
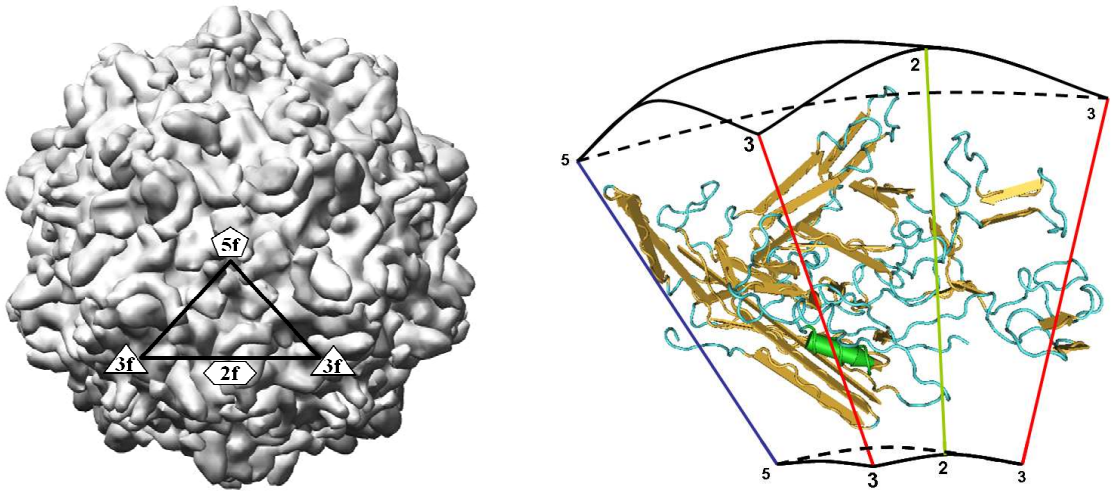


Figure 1. Coding map of the PPV genome. Blue arrow indicates mRNA precursor and red arrows indicate gene products. Nucleotide numbers are indicated above. The graphic was generated on the NCBI homepage (http://www.ncbi.nlm.nih.gov/) using the NADL2 strain (GenBank accession number NC_001718).

Structural characteristics

The capsid of PPV is a spherical shell consisting of 60 identical copies of the viral proteins arranged in an icosahedral symmetry (Figure 2) (CHAPMAN and ROSMANN 1993). Each of the 60 copies is built by the amino acids located between site 37 and 584 of the VP2 protein and is called “capsid subunit” (TSAO et al. 1992). The subunit consists of eight antiparallel β-strands, a common structure for viral capsids, together with one &-helice and four loops (Figure 2) (CHAPMAN and ROSMANN 1993). On the surface, a projection at the 3-fold axis, a depression or canyon around the 5-fold axis and a dimple on the 2-fold axis of symmetry can be observed. The 2- and 3-fold axes consist mainly of amino acids located in the subunit loops. These regions are considered to be important for the viral infection and immunogenicity (SIMPSON et al. 2002).

On the internal surface of the PPV capsid several neutral amino acids are located. This is unusual for viruses, which usually harbor basic amino acids on their internal virus surface, which are supposed to interact with the DNA molecule phosphates (XIE and CHAPMAN 1996). As hypothesis, the presence of several basic residues (in the neutral amino acids) among the parvoviruses binding sites could result in a high electrostatic enthalpy reaction with the DNA molecules (STEITZ et al. 1990) ensuring, this way, an efficient packaging of the viral genome.



A B

Figure 2. The capsid structure of the PPV. A: Surface representations of the capsid calculated from X-ray coordinates with a low pass filter at 17 Å in a temperature factor of 500 Å. B: 3D model of the PPV VP2 proteins using the cartoon technique, with a rocket (&-helice) and arrows (β-strands) representing the secondary structure. The image was generated with

the software Cn3D version 4.1. The coordinates of both figures were retrieved from the NCBI Structure Database (http://www.ncbi.nlm.nih.gov/Structure/index.shtml). Accession number: 1K3V (SIMPSON et al. 2002). The neighboring five- (in blue), three- (in red) and twofold (in green) axes of the capsid subunit are shown.

Epidemiology

Porcine parvovirus is considered to be endemic in most areas of the world. The virus can be found in all pig herd categories, including in boars and fattening pigs. In the first studies about the topic, it was observed that PPV had a high infection level in pigs, with a sero-conversion rate of 30% to 90% among animals tested (BACHMAN. 1969; JONHSON and COLLINGS 1969; MENGELING. 1972). This lead to the development and common use of inactivated vaccines since the 1980s (TRUYEN and STRECK 2012). It is not unusual to find vaccinated sows with high antibody titers (> 1:512), indicative of viral activity, and even outbreaks in regularly vaccinated animals (DIAS et al. 2012). Furthermore, PPV DNA can also be demonstrated in those animals and their appear independent of the observed the antibody titers (STRECK et al. 2011).

The epidemiology of porcine parvovirus is primarily marked by the high stability of the virus in the environment. PPV can remain infectious for months, and contaminated instruments or stables may therefore be a constant source of infection. The virus can be transported by the clothing of farmers from one herd to another, and it is also speculated that rodents can mechanically introduce the virus into new herds (TRUYEN and STRECK 2012).

The virus spreads in a herd usually through feces and other secretions from acutely infected pigs. The introduction of the virus does not necessarily cause clinical problems. If sows are regularly vaccinated or naturally immunized, the viruses' infections may be asymptomatic (TRUYEN and STRECK 2012). Separation or acquisition of gilts for breeding could result in the selection of naïve animals and when these gilts are introduced into an infected herd, it may lead to acute outbreaks of infection (SZELEI et al. 2006).

The virus can also be introduced into susceptible herds by infected boars. Seronegative boars could become infected by the oronasal route or during coitus, if virus is in the vaginal mucus (SZELEI et al. 2006). According to several reports, PPV was already observed in semen of naturally infected boars (CARTWRIGHT and HULK 1967; CARTWRIGHT et al. 1969; RUCKERBAUER et al. 1978); however, it is controversially discussed whether the virus could reach the testis during infection, or if the positive results represent secondary contaminations.

Pathogenesis

The primary replication of the PPV is suggested to occur in lymphoid tissues. After that, the virus is distributed systemically via viremia (PAUL et al. 1980). In the porcine epitheliochorial placenta, six tissue layers completely separate the sow and the fetal blood circulation. Since these cells are closely connected, not allowing the passage of even small molecules as antibodies, it is still not understood how the PPV crosses this placental barrier and reaches the fetus (MENGELING. 2000). Probably, as the virus was already detected in lymphoid tissue from pigs (LUCAS et al. 1974), in fetal lymphocytes (RUDEK and KWIATKOWSKA 1983) and is able to remain infectious after phagocytosis by macrophages (PAUL et al. 1979), PPV could use them to infect the fetus. After fetal infection, the PPV has an environment particularly susceptible to infection and to replication due to the high mitotic activities present in the fetuses' tissues (TRUYEN and STRECK 2012).

Parvoviruses use a variety of glycoproteins, glycans and glycolipids to attach to cells (HARBINSON et al. 2008). The entry mechanisms of PPV are still unclear, but seem to include clathrin-mediated endocytosis, or macropinocytosis, followed by transportation through the endosomal pathway (BOISVERT et al. 2010). The consequent endosome acidification results in reversible modifications in the virus' capsid, which may allow the viral escape from the endosome (VIHINEN-RANTA et al. 2002; FARR et al. 2005). Probably, acidification can externalize the phospholipase A2 motif (PLA2) from the capsid, which is supposed to be essential to break the vesicular membranes (GIROD et al. 2002), and to enter the cellular nucleus (VENDEVILLE et al. 2009). Once in the nucleus, the parvoviruses' replication is conducted by the cell DNA polymerase complex.

The virulence properties of the PPV appear to be related to the viral protein gene. A genetic comparison between the non-virulent NADL2 and the virulent Kresse strains revealed that the non-coding regions of these viruses are nearly identical. For the non-structural gene region (NS1/NS2), all differences found are silent (synonymous), while in the structural genes (VP1/VP2) six of eight differences led amino acid substitutions (non-synonymous). Five of these different amino acids present in the Kresse strains were also observed in virulent field isolates (I-215-T, D-378-G, H-383-Q, S-436-P and R-565-K), and three of these amino acid substitutions (D-378-G, H-383-Q and S-436-P) were considered to be responsible for the different tissue tropism (BERGERON et al. 1996). In the capsid subunit, the amino acid positions 378, 383 and 565 are located next to the 2-fold axe and these locations have been suggested to be important for immune response and hemagglutination activity (BERGERON et al. 1996). The amino acid position 436 is located right in the 3-fold spike center of the capsid subunit and the amino acid position 215 at its base. This location in the 3-fold spike has been considered to be an important antigenic surface region in various parvoviruses (CHAPMAN and ROSSMANN 1993).

Clinical signs

The major clinical sign of PPV infections are reproductive failures in sows (Figure 3). Even after experimental inoculation, other clinical signs in boars and gilts could not be demonstrated (MENGELING and CUTLIP 1976; THACKER et al. 1987). Subclinically, a moderate and transient lymphopenia, independent of sex and age, can be observed between 5 and 10 days after initial infection (JOO et al. 1976b; MENGELING and CUTLIP 1976; ZEEUW et al. 2007). Reports identifying PPV in the feces of pigs with diarrhea were published as well. However, whether the virus replicates in the intestinal epithelium and thereby causes enteric diseases, as observed in parvovirus infections in other animals, is uncertain (DEA et al. 1985; YASAHURA et al. 1989). Additionally, PPV was also isolated in vesicular lesions, but without defined etiological role (KRESSE et al. 1985).

Figure 3. Litters of inoculated pregnant sows (with 27a and NADL2 viruses) at the 90th gestation day displaying distinct levels of lesions. The fetuses' position correspond to their position in the uterus (the most cervical-positioned fetuses at the top) (ZEEUW et al. 2007).

For the female, the pathological sequela caused by PPV is related mainly to the gestational period in which infection occurs (Figure 4). At the gestational beginning, the conceptus is protected by the zona pellucida and is therefore resistant to infection. During the stage of the embryo, infection with PPV results in embryonic death and resorption. From the 35th day on, i.e. the beginning of the ossification, the infection results in death and subsequent mummification. Finally, around the 70th day from gestation, the fetus is already immune competent and may resist viral infection. An infection in this stage is then usually controlled and the piglet is born with anti PPV antibodies (JOO et al. 1976a; LENGHAUS et al. 1978).

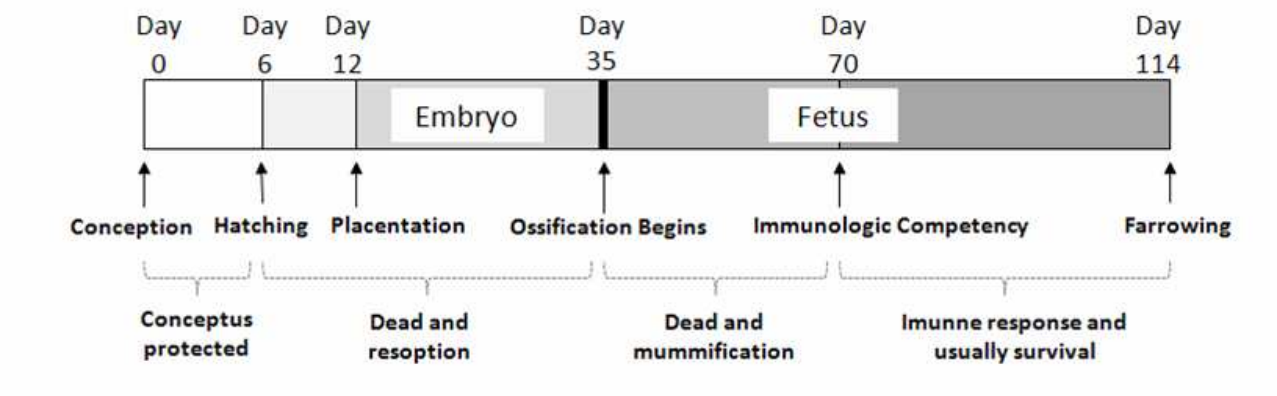


Figure 4. Clinical consequences of PPV infection observed in various time-points of gestation. Adapted from MENGELING et al. (2000).

Diagnosis

A possible PPV infection should be investigated always when one of the following signs can be identified: increasing of the return-to-estrus index; abortions associated with mummified fetuses; failure to farrow; stillbirth; weak piglets and small litters. These symptoms mainly occur in first or second parity order females and are usually not followed by other clinical manifestations (SZELEI et al. 2006; TRUYEN and STRECK 2012). As differential diagnosis, reproductive diseases like Aujeszky's disease, brucellosis, leptospirosis, porcine reproductive and respiratory syndrome (PRRS), toxoplasmosis and unspecific bacterial uterine infections should be excluded.

The material submitted to laboratory for direct diagnosis should include frozen mummified fetuses and fetal remains. Traditionally, the virus detection and titration can be performed by the hemagglutination technique (HA), based on the hemagglutinating activity of PPV against erythrocytes of certain species, e.g.: chickens, humans, guinea pigs (JOO et al. 1976b).

Since the virus has a high replication efficiency in renal or testicular swine cells, virus propagation in cell-lines such as ESK (embryonic swine kidney), PK-15 (pig kidney), SK6 (swine kidney), STE (swine testicular epitheloid) and SPEV (swine embrio kidney) is routinelly used. Once in these cells, the PPV replication usually causes cytopathic effects, including granulations, irregular shape, slow replication, intra-nuclear inclusions, pyknotic nucleus and consequently cell death (CARTWRIGHT et al. 1969; MENGELING. 1972). Due to possible similar cytophatic effects of other viruses or enzymatic effects, virus isolation is often associated with immunofluorescence microscopy (Figure 5) (CARTWRIGHT et al. 1971; JONHSON. 1973; MENGELING et al. 1978).

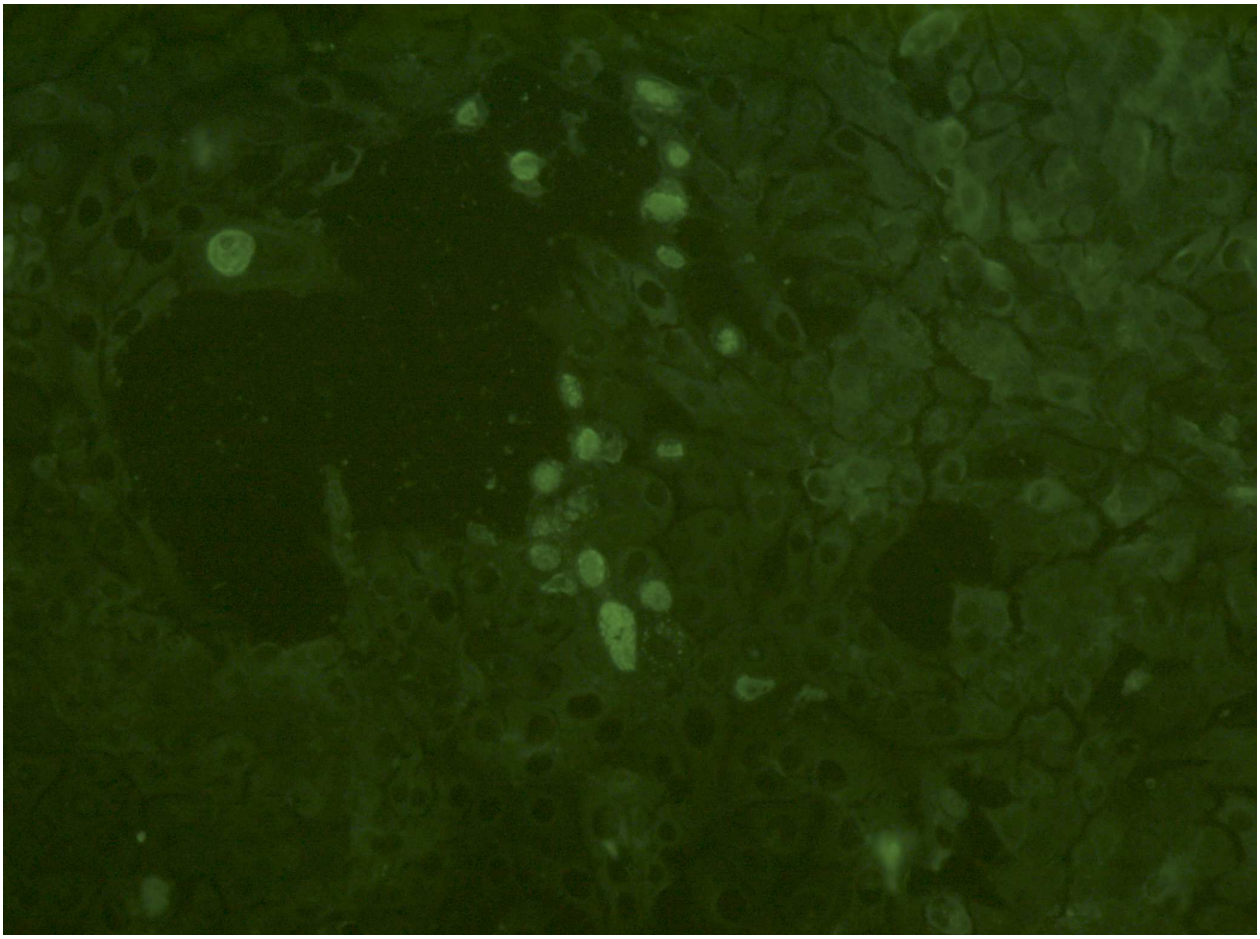


Figure 5. Indirect immunofluorescence of PK-15 cells infected with PPV. Positive nuclear fluorescence (green) is evidenced five days post-infection. Magnification of 400x.

More recently, nucleic acid-based techniques can be used for viral detection in clinical samples with a better sensitivity. The PCR, a useful technique in fetal tissues, possesses higher sensibility and even specificity than the HA (SOARES et al. 1999). Additionally, several real-time PCR protocols have been described, using Taqman probes (CHEN et al.

2009) or SYBR Green (MIAO et al. 2009; WILHELM et al. 2006) which can be used for viral DNA detection and quantification.

Alternatively, serum from gilts and sows can be tested at the time of reproductive failure, with a second sampling performed 2-4 weeks later. As standard method, the hemagglutination inhibition (HI) assay is frequently used for the quantification of PPV specific antibodies. Usually, the serum examined by HI test needs to be pre-treated by heat inactivation followed by adsorption with kaolin (MORIMOTO et al. 1972). Another technique, the enzyme-linked immunosorbent assay (ELISA) can be standardized more easily and is suited to automatization. Moreover, serum does not have to be pre-treated before testing in the ELISA (HOHDATSU et al. 1988; WESTENBRINK et al. 1989). The ELISA can potentially differentiate vaccinated animals from animals having been infected with PPV. As the currently used inactivated vaccines induce antibodies only against PPV VP proteins and not against the NS proteins, ELISAs that differentiate these two proteins could identify an field virus infection (MADSEN et al. 1997; QING et al. 2006).

Immunity

The first protection of the piglet against PPV takes place via colostral antibodies consumed in the first day of life. The piglet itself starts to produce antibodies in the second week of live; meanwhile the maternal antibody titer decreases gradually, with antibodies being still detectable until 4-6 months of live (JONHSON et al. 1976). If the animal is vaccinated in the presence of a high maternal antibody titer, these antibodies can interfere with vaccination. On the other hand, if the antibody levels decrease swiftly the animal will be unprotected for several weeks until vaccination. Therefore, the time point of the first vaccination is important for a successful vaccination program.

The antibody titers raised by commercial vaccines and those raised by natural infections are markedly different. By HI test, the vaccine antibody titers are commonly lower than 1:512 (ORAVAINEN et al. 2005), whereas the antibody titers after a field infection can regularly exceed 1:2000 (van LEENGOED et al. 1983). Usually, the animal produces titers 6-9 days after vaccination/infection, with the peak between day 14 and 21 (JONHSON and COLLING 1969; CARTWRIGHT et al. 1971). The titers can remain high in sows for about four years after a natural infection (JONHSON et al. 1976). However, it is also suggested that a latent PPV infection is able to cause persistence of detectable antibodies (SZELEI et al. 2006).

The use of the inactive vaccine against PPV reduced the reproductive problems associated with PPV in herds. As inactive vaccines are expected to produce mainly humoral response, it is usually agreed that the protection against the PPV is mediated mainly through protective

antibodies. Cellular immunity is also described, mainly based on cytotoxic T lymphocytes (CTL). In experimental infections with naïve animals, the CTL response was weak in animals that received only one viral inoculation. This is suggested as a result of the rapid response of the humoral immunity, clearing the infection before CTL activation. However, CTL response was markedly evident in a second infection, indicating that CTLs may play a role in controlling PPV re-infections (LADERKJÆR-MIKKELSEN and NIELSEN 2002).

Prevention and control

Currently, there is no specific treatment against PPV. Additionally, the widespread and resistant characteristics of this virus lead to difficulties to control the virus. Practices that ensure the biosafety and herd´s hygiene are of major importance to reduce contamination. However, the most efficient measure is the regular vaccination of the breeding stock (TRUYEN and STRECK 2012).

Before the worldwide dissemination of the commercial vaccine, the “feed-back” technique was usually used in herds. This technique was based on an induced infection of the gilts approximately one month before the insemination. The immunization was performed through direct contact of the sows with feces and abortions´ remains (TRUYEN and STRECK 2012). The technique provided immunization with a high antibody titer, but as viruses were not eliminated or reduced, a potential risk of infection outbreaks was always present.

The first vaccines were experimentally developed in the end of the 1970s (JOO and JOHNSON 1977; MENGELING et al. 1979). A few years later, regular vaccination of breeding sows with inactivated vaccines became a worldwide practice. Currently, PPV vaccines are a cell culture derived virus (usually the non-pathogenic NADL2 strain) which is chemically inactivated (by formalin, beta-propiolactone or binary ethyleneimine), mixed with oil or aluminium hydroxid as adjuvants and administrated parenterally (TRUYEN and STRECK 2012). The use of these vaccines induces antibody titers that can reduce clinical manifestations, but cannot prevent infection (JÓZWIK et al. 2009). The vaccination schedules can be adjusted to the animal category. Gilts receive the first dose at the age of 170-180 days, or 30 days before insemination. The second dose is usually administrated 15 days later. Sows are boosted usually 10-15 days after each farrowing. Boars can be vaccinated as well and boosted yearly.

Modified-live virus vaccines (MLV) could be an alternative for PPV. Reports on PPV MLVs were made in the 1980s using the NADL2 virus (PAUL and MENGELING 1984). In all cases transplacental transmission was prevented, but viremia and shedding of the vaccine strain after vaccination was observed. More recently, an experimental infection of pregnant sows

with the strains 143a, IDT, NADL2 and Stendal (strains considered to have low virulence) also revealed no transmission of these viruses to the fetuses. Additionally, these strains induced a very strong humoral immune response (ZEEUW et al. 2007).

The emergence of novel phenotypes

Genetic variability of PPV and the emergence of new phenotypes are a very recent topic. The first study focusing on genetic variability of PPV field strains was published in 2002, with strains isolated in Brazil from 1994 to 2000 (SOARES et al. 2003). In this study, a partial fragment of the VP2 gene was analyzed and at least eighteen different phenotypes (differing at least in one amino acid) were identified and disposed in two phylogenetic groups. Additionally, a similar study analyzing samples from Germany, isolated in 2001 and 2002, observed six different phenotypes which could be phylogenetically distinguished in two clusters (ZIMMERMANN et al. 2006). Thereafter, studies with field samples from China and Romania were published describing several new phenotypes as well (SHANGJIN et al. 2009; HAO et al. 2011; CADAR et al. 2012).

The report of these several PPV phenotypes raises questions about the conservative properties of the PPV genome. Currently, it is not clear whether these new PPV phenotypes represent a viral emergence or only viruses that are now detectable due to more sensitive techniques. Furthermore, it was observed that new phenotypes with amino acid substitutions in the capsid protein have modified their antigenic properties, and differences in the cross neutralization of sera raised against NADL2 and IDT viruses (used in the commercial vaccines) have been demonstrated. Those findings lead to the hypothesis that the emergence of new mutations could be a viral adaptation to the largely used vaccines resulting in “escape mutants” (ZEEUW et al. 2007).

The emergence of novel parvoviruses

In the last decade, with the development of novel molecular techniques, like the next generation sequencing, several viruses have been identified for the first time. For porcine parvoviruses, the virus H-1 in sera from pigs in Myanmar was the first newly identified virus (HIJISAKA et al. 2001), originally termed as porcine parvovirus 2 (PPV2). The virus was accidentally PCR amplified during an attempt to identify hepatitis E virus. Recently, in the USA, a high prevalence of this virus was found in lung samples (20.7%) and a moderate prevalence in feces (7.6%). Also in thoracic fluids the virus could be detected (1.6%).

In 2008, a virus closely related to the human parvovirus 4 was found in slaughtered pigs in Hong Kong (LAU et al. 2008). Currently, several sequences with high homology (> 98 %

DNA similarity) exist in the DNA databanks under different names, including porcine hokovirus, PARV4-like and porcine parvovirus 4. The virus has already been detected in several countries (CADAR et al 2011; PAN et al. 2012; XIAO et al. 2012), including Germany, where a high DNA detection rate (37.7%) was observed in hunted wild boars (ADLHOCH et al. 2010).

In 2010, another parvovirus was found in lungs from a diseased pig co-infected with porcine circovirus type 2 and designated as porcine parvovirus 4 (PPV4) (CHEUNG et al. 2010). After that, the virus was found in diseased animals in China (HUANG et al. 2011). Finally, in pigs with postweaning multisystemic wasting syndrome (PMWS), a porcine bocavirus (PBoV) was identified in lymph nodes (BLOMSTRÖM et al. 2009). No correlation with clinical signs could be observed, which is also true for the other novel parvoviruses as well.

Probably, the novel parvoviruses may have a limited clinical implication on swine health; however, they may possess a potential public health interest due to their genetic similarity with human parvoviruses.

CHAPTER 3 – HIGH RATE OF VIRAL EVOLUTION IN THE CAPSID PROTEIN OF PORCINE PARVOVIRUS

André Felipe Streck[1], Sandro Luis Bonatto[2], Timo Homeier[1], Carine Kunzler Souza[3], Karla Rathje Gonçalves[3], Danielle Gava[3], Cláudio Wageck Canal[3], Uwe Truyen[1]

Author's affiliations:

1. Institute for Animal Hygiene and Veterinary Public Health, Faculty of Veterinary Medicine, University of Leipzig, An den Tierkliniken 1, 04103 Leipzig, Germany.

2. Laboratório de Biologia Genômica e Molecular, Pontifícia Universidade Católica, Av. Ipiranga, 6681, Prédio12, bloco C, sala 172, 90619-900, Porto Alegre, Brazil.

3. Laboratório de Virologia, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves, 9090, Prédio 42.602, CEP 91540-000, Porto Alegre, Brazil.

Journal of General Virology, 2011, 92(11):2628-2636.

ABSTRACT

In the last years, it has been shown that some parvoviruses exhibit high substitution rates, close to those of RNA viruses. In order to monitor and determine new mutations of the porcine parvovirus (PPV), recent PPV field isolates from Austria, Brazil, Germany and Switzerland were sequenced and analyzed. These samples, together with sequences retrieved from GenBank, were included in three datasets (consisting of a VP1 complete gene, VP1 partial gene and NS1 complete gene). For each dataset, the nucleotide substitution rate and the molecular clock were determined. The analysis of the PPV field isolates revealed that a recently described amino acid substitution, S-436-T, appears to be common in the VP2 protein in the Austrian, Brazilian and German virus population. Furthermore, new amino acid substitutions were identified, being mainly located in the viral capsid loops. By inferring the evolutionary dynamics of the PPV sequences, nucleotide substitution rates of approximately 10^{-5} nucleotide substitutions per site per year for the non-structural protein gene and 10^{-4} for the capsid protein gene (for both viral protein datasets) were found. The latter rate is similar to that commonly found in RNA viruses. An association of the phylogenetic tree with the molecular clock analysis revealed, that the mutations, on which the divergence for both capsid proteins is based, occurred in the last 30 years. Based upon these findings, one

can conclude that PPV variants are continuously evolving and that vaccines, which are mainly based on strains isolated about thirty years ago, should possibly be updated.

INTRODUCTION

The porcine parvovirus (PPV) was first isolated in Germany and in the United States in 1965 and later found worldwide (CARTWRIGHT and HUCK 1967; JOHNSON. 1973; JOO et al. 1976; SIEGL. 1976). PPV is considered to be a major cause of reproductive failure in swine. Clinical signs of PPV infections are characterized by the reoccurring of estrus, abortion and the delivery of mummified and stillborn fetuses (MENGELING et al. 2000) commonly described with the acronym SMEDI (stillbirth, mummification, embryonic death and infertility).

PPV is a small, single-stranded (ss) DNA virus. The genome has a length of about 5000 nucleotides encoding four proteins transcribed from two promoters and the coding capacity is extended through alternative splicing. Two nonstructural proteins, NS1 and NS2, are transcribed and translated from the 5´ ORF and are important for DNA replication. Additionally, two structural proteins are transcribed and translated from the 3´ ORF (VP1 and VP2). The smaller protein, VP2, is produced by splicing from the same RNA template as the larger protein (VP1). The whole VP2 sequence is therefore present in the VP1, which has a unique amino terminus of about 120 amino acids. A third protein, VP3, is a post-translational modification product of VP2 (Simpson *et al*., 2002). Together, these three viral proteins assemble to form the icosaedral capsid (CHAPMAN and ROSSMANN 1993; PARRISH 2010). Various biotypes of PPV with different pathogenic properties are known. The genetic basis of the pathogenicity has not yet been defined, but the structural protein appears to play a major role. A genomic comparison between the non-pathogenic strain NADL2 (GenBank accession number L23427) and the virulent strain Kresse (U44978) revealed that the non-coding regions of the genome are nearly identical (BERGERON et al. 1996). All nucleotide substitutions found in the NS genes are silent, while six out of eight substitutions located in the structural genes (VP1/VP2) change the transcribed amino acid. In detail, among the VP2 amino acids, five changes are consistent in comparison with the field isolates (I-215-T, D-378-G, H-383-Q, S-436-P and R-565-K) and three of these amino acid changes (D-378-G, H-383-Q and S-436-P) are considered to be responsible for the different tissue tropism (BERGERON et al. 1996). Recently, genetic variation and the possible emergence of a new antigenic type of PPV has been described (ZIMMERMANN et al. 2006), although its importance in the field is still unclear.

As PPV replicates by using the host DNA replication machinery, it is generally assumed that the virus has a low rate of nucleotide substitution close to that found in its mammalian host. In the last years, it was shown that canine parvoviruses and human B19 erythrovirus, both autonomous parvoviruses, have a nucleotide substitution rate of approximately 1 x 10^{-4} substitutions per site per year (SHACKELTON et al. 2005; SHACKELTON and HOLMES 2006). This rate is similar to that known for RNA viruses.

The continuous use of an inactivated vaccine in swine herds in the last three decades, and the remaining occurrences and reports of reproductive failures caused by PPV, highlights the importance of a continuous monitoring of PPV isolates. To address these questions, recent PPV isolates were analyzed by determining nucleotide substitutions, phylogenetic analysis and the estimation of the molecular evolutionary rate of PPV.

RESULTS

GENETIC ANALYSIS OF THE NEWLY IDENTIFIED SEQUENCES

In order to identify new mutations, recent isolates from Austrian, Brazil, Germany and Switzerland were analyzed. The sequence analysis of the structural gene (VP2) of these isolates (n=9) revealed nucleotide substitutions at 32 sites. Seventeen substitutions were synonymous and fifteen substitutions were non-synonymous. In the region previously considered as highly variable [located between the nucleotides 3889 to 4239 of the full PPV genome (ZIMMERMANN et al. 2006)], three synonymous substitutions and five non-synonymous substitutions were found. Two conserved regions (with no nucleotide substitution) could be defined between the nucleotides 3507-3718 and 4251-4425.

The analysis of the nonstructural genes (NS1) of the new isolates (n=9) revealed nucleotide substitutions in 44 sites. Twenty-seven substitutions were synonymous and seventeen substitutions were non-synonymous. Conserved regions (with no nucleotide substitution) were identified in sites 568-781, 847-1022, 1072-1362 and 1504-1793.

At the protein level, conserved regions are located in the structural gene between amino acids 83-225 and 437-579. Two variable regions were found: 226-233 and 365-436. For the nonstructural gene, conserved regions (with no amino acids substitution) were located between the amino acids 83-163, 165-357 and 368-561. A region with a higher variability was observed at the end of the gene, between the amino acids 562-658.

The amino acid substitutions in the structural gene per isolate displayed per isolate in Table 1. In the sites that differ between the NADL2 and the Kresse strain, the recent isolates appear to be nearly homologous to the Kresse sequence. Unique amino acids substitutions could be identified in the sites 436 and 565. In all the new German and Austrian strains, substitutions were located in sites 228, 414 and 419. In the site 436, all German strains contained the amino acid Threonine, which was also observed in the Brazilian strain S30. A higher number of amino acid substitutions, in comparison to the other isolates, could be shown in strain 15425 with six unique amino acids changes.

Table 1. Amino acid substitution within PPV VP2 sequences.

	VP isolate											
Amino acid VP2	Kresse	NADL2	27a	15425	7a	8a	14a	693a	Embrapa	PA	PB	S30
20	T	T	T	A	T	T	T	T	ND	ND	ND	ND
45	S	T	S	S	S	S	S	S	ND	ND	ND	ND
82	R	R	R	K	R	R	R	R	ND	ND	ND	ND
215	T	I	T	T	T	T	T	T	ND	ND	ND	ND
226	Q	Q	Q	E	Q	Q	Q	Q	ND	ND	ND	ND
228	Q	Q	E	Q	E	E	E	E	ND	ND	ND	ND
233	S	S	T	S	S	S	S	S	ND	ND	ND	ND
304	P	P	P	T	P	P	P	P	ND	ND	ND	ND
320	I	I	I	T	I	I	I	I	I	I	I	I
366	D	D	D	N	D	D	D	D	D	D	D	D
378*	G	D	G	G	G	G	G	G	D	D	D	G
383*	Q	H	Q	Q	Q	Q	Q	Q	H	H	H	Q
407	K	K	K	N	K	K	K	K	K	K	K	K
414	A	A	S	A	S	S	S	S	A	A	A	A
419	E	E	Q	E	Q	Q	Q	Q	E	E	E	E
423	N	N	N	N	N	N	N	N	N	K	N	N
436*	P	S	T	P	T	T	T	T	S	S	S	T
565	K	R	K	K	K	K	K	K	R	R	R	K

*Site considered responsible for the different tissue tropism (BERGERON et al. 1996).
ND: not determined

SUBSTITUTION RATES AND SELECTION PRESSURES

Only the MaxChi method (from the five method tested) estimated a recombination event in the samples 7a (this study), 143a (AY684867) and 32260005_1d (GQ884037) in the NS1 dataset. Since no consensus was obtained with three or more methods, all samples were used for further analysis.

The root-to-tip analysis was constructed to examine whether the samples exhibited adequate temporal structure for a substitution rate analysis. The first plot of the genetic distance versus the isolation year of the sample displayed an outlier sequence [IDT (AY684872)]. This sequence was excluded from the further analysis [according to the methodology adopted by SHACKELTON and HOLMES (2006)] and a new ML tree and root-to-tip plot was calculated (Figure 6). The R-squared obtained by the linear regression for the VP1 complete gene, VP1 partial gene and NS1 complete gene were 0.3828, 0.4438 and 0.2984, respectively.

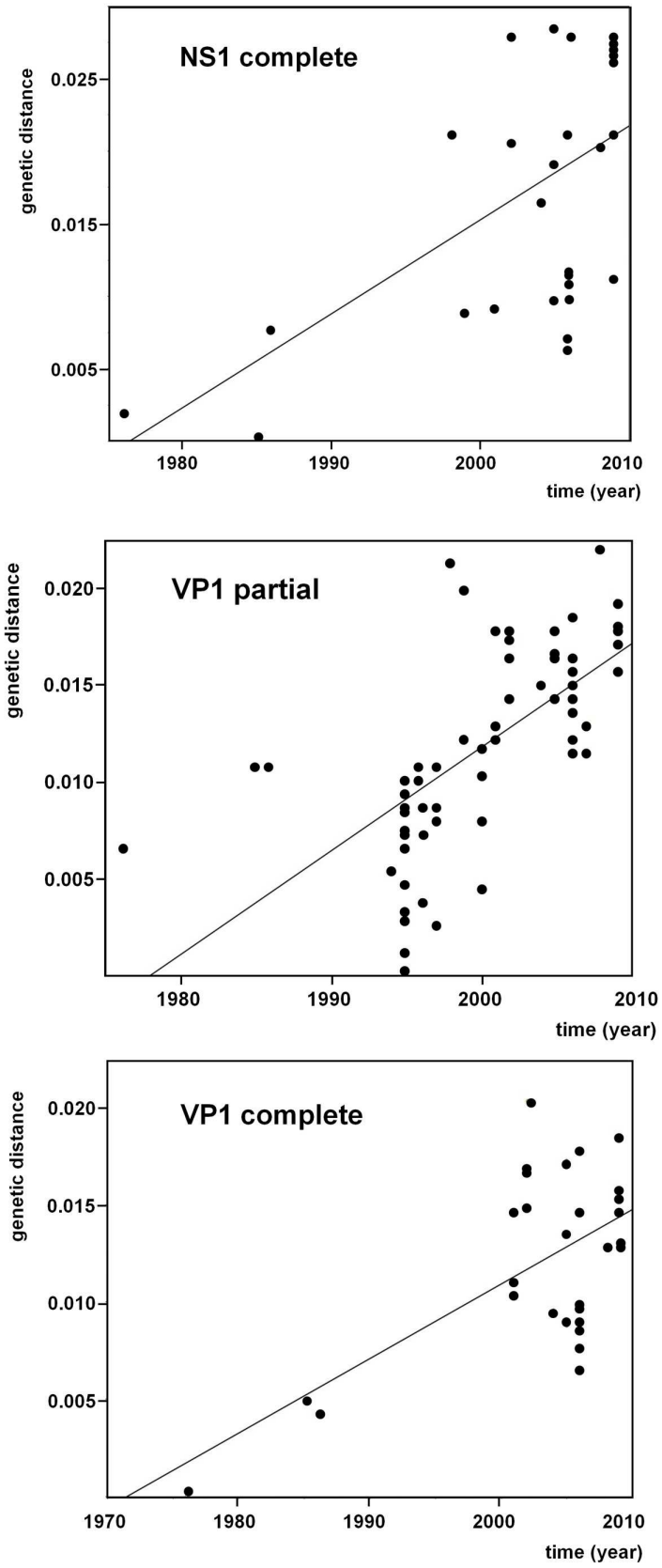


Figure 6. Linear regression plot graphic between the genetic distances (number of substitution per site) versus the isolation year for each sample in the distinct datasets.

The mean evolutionary rate estimated for the three data sets, using the BEAST approach, ranged from 10^{-5} to 10^{-4}. According to the model best fitting the data, the dataset of the nonstructural genes, VP1 complete gene and the VP1 partial gene presented a mean rates (substitution/site/year) of 5.39 x 10^{-5}, 3.02 x 10^{-4} and 4.04 x 10^{-4} (respectively) (Table 2). According to the methodology, a mean variation of between 4.00 - 7.73 x 10^{5} for the nonstructural dataset, a mean variations of between 1.74 - 3.02 x 10^{4} for the VP1 complete dataset and a mean variation of between 3.93 - 4.45 x 10^{4} for the VP1 partial datasets were obtained.

Table 2. Sequence information, clock model, demographic model, marginal Likelihood (*ml*), mean of nucleotide substitution rate (mean rate in subs/site/year) and the corresponding 95% Higher Posterior Density (HPD) lower and upper for the three data sets.

Data set	Sequences length (nt)	Sequences number	Clock model	Dem. model	*ml*	Mean rate	HPD
VP1 complete	2187	31	UCED	Constant	-4022.3	3.02 x 10^{-4}	4.86 x 10^{-4}, 1.43 x 10^{-4}
VP1 partial	739	65	UCED	BS	-1762.7	4.04 x 10^{-4}	5.72 x 10^{-4}, 2.56 x 10^{-4}
NS1 complete	1989	31	UCED	BS	-3867.2	5.39 x 10^{-5}	1.97x 10^{-7}, 1.37 x 10^{-4}

The difference between non-synonymous and synonymous substitution rates (d_N/d_S) for the NS1 and VP1 datasets presented variable results according to the methodology. A chi-square test comparison between the models M7 and M8 revealed that both complete genes are under purifying selection [NS (p = 0.3146) and VP (p = 0.0407)]. Furthermore, no positive selection sites were found in the M8 model. On the other hand, evidence of a positive selection for the VP partial dataset was found with the M7 and M8 models (p = 0.0006) and in the MEGA analysis (d_N/d_S = 1.610). For the VP1 (complete and partial), no evidence could be identified in the branch-site model test (p = 0.0786 and 0.9972, respectively) for the case that different selective pressures would drive the evolution of the distinct lineages/branches. In contrast, branches characterized by different selective pressures were present within the NS1 dataset (p = 0.0001).

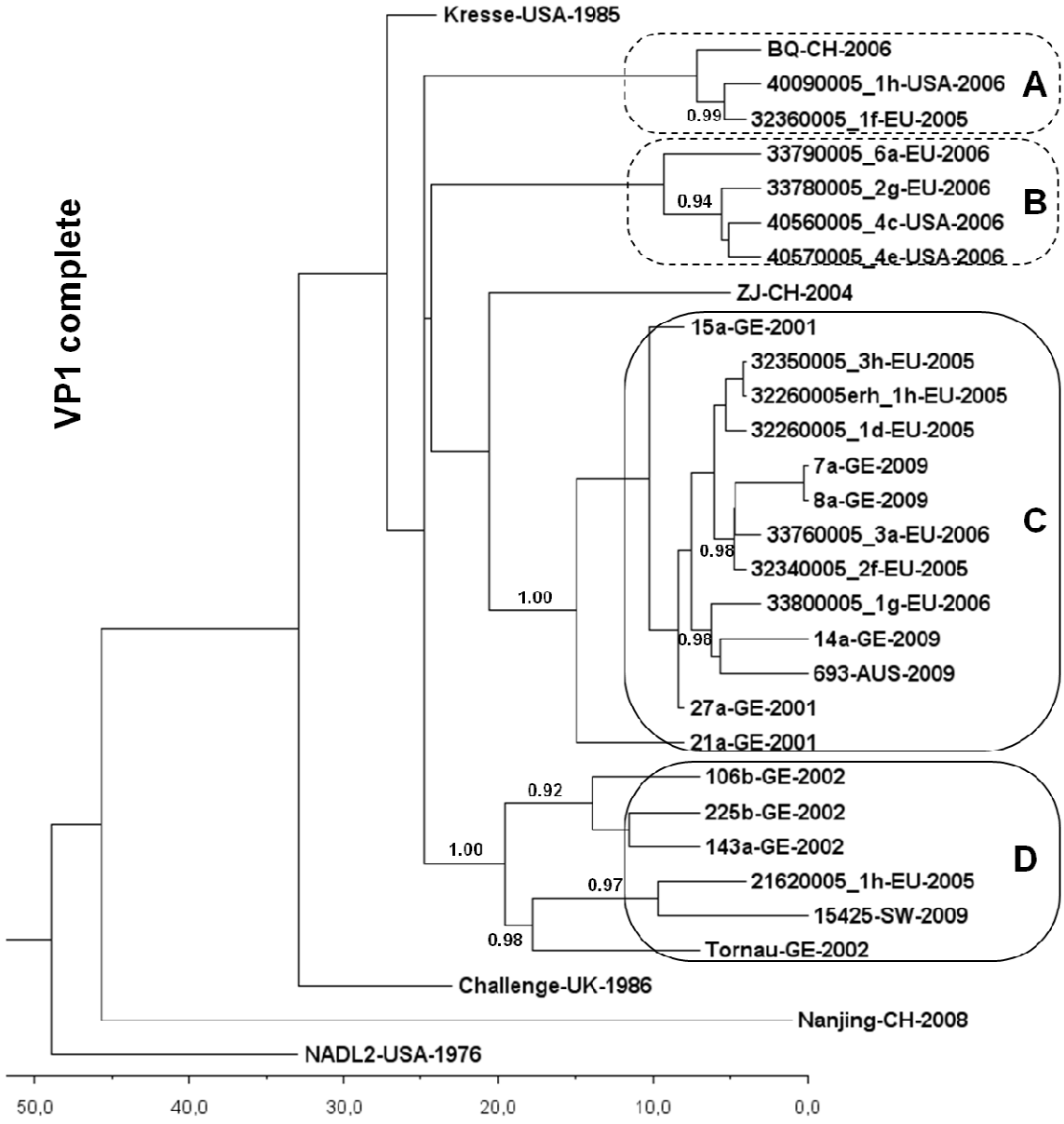
PHYLOGENETIC ANALYSIS

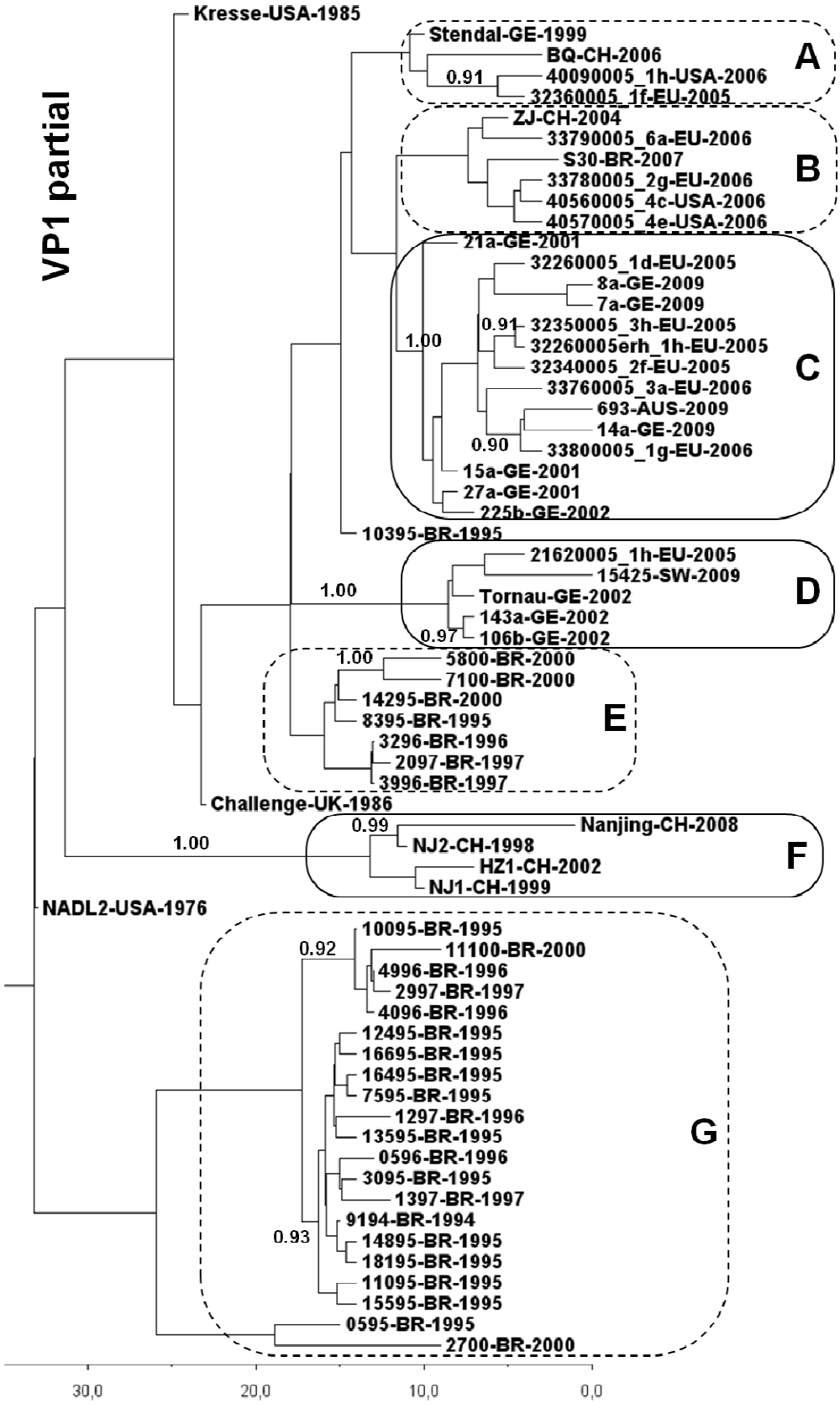
As shown in Figure 7, the Bayesian maximum clade credibility tree generated for the VP1 complete gene revealed two different clades [here called A and B (posterior

probability < 0.90)] and two clusters [C and D (posterior probability = 1.0)]. The new German and Austrian strains were located in Cluster C, and the Swiss strain was located in Cluster D.

In the tree that is based upon the partial VP1 gene sequence, clades and clusters A-D were also present and additionally, two other clades [E and G (posterior probability < 0.90)] and one other cluster [F (posterior probability = 1.0)] could be identified. The new German and Austrian strains remained in the same cluster, while the new Brazilian and the Swiss strains were located in Clade B and cluster D, respectively. On the contrary, except for ZJ (EU790642) [with no clade (VP partial) ↔ clade B (VP complete)] and 225b (AY684864) [cluster C (VP partial) ↔ cluster D (VP complete)] all the sequences were affiliated with the same clade/cluster in the VP1 complete and the VP1 partial tree. In the NS gene tree, almost all sequences formed distinct clusterizations and the only clusters in agreement with the VP1 trees were D and F.

The chronological analysis of the NS and VP1 datasets demonstrated two distinct temporal periods. In the structural genes, the main divergences occurred in the last 30 years for both datasets, and the events resulting in the outcome of new strains were dated back to the last 20 years. In the nonstructural dataset, the divergences were estimated to be formed in the former time period of between 10 to 125 years ago, being mainly concentrated in the last 30-60 years.


VP1 complete
Kresse-USA-1985
BQ-CH-2006
40090005_1h-USA-2006
32360005_1f-EU-2005
A
0.99
33790005_6a-EU-2006
33780005_2g-EU-2006
40560005_4c-USA-2006
40570005_4e-USA-2006
B
0.94
ZJ-CH-2004
15a-GE-2001
32350005_3h-EU-2005
32260005erh_1h-EU-2005
32260005_1d-EU-2005
7a-GE-2009
8a-GE-2009
33760005_3a-EU-2006
32340005_2f-EU-2005
C
0.98
1.00
33800005_1g-EU-2006
14a-GE-2009
693-AUS-2009
0.98
27a-GE-2001
21a-GE-2001
106b-GE-2002
225b-GE-2002
143a-GE-2002
21620005_1h-EU-2005
15425-SW-2009
Tornau-GE-2002
D
0.92
1.00
0.97
0.98
Challenge-UK-1986
Nanjing-CH-2008
NADL2-USA-1976
50,0
40,0
30,0
20,0
10,0
0,0


VP1 partial
Kresse-USA-1985
Stendal-GE-1999
BQ-CH-2006
40090005_1h-USA-2006
32360005_1f-EU-2005
0.91
A
ZJ-CH-2004
33790005_6a-EU-2006
S30-BR-2007
33780005_2g-EU-2006
40560005_4c-USA-2006
40570005_4e-USA-2006
B
21a-GE-2001
32260005_1d-EU-2005
8a-GE-2009
7a-GE-2009
0.91
32350005_3h-EU-2005
1.00
32260005erh_1h-EU-2005
32340005_2f-EU-2005
33760005_3a-EU-2006
693-AUS-2009
14a-GE-2009
0.90
33800005_1g-EU-2006
15a-GE-2001
27a-GE-2001
225b-GE-2002
C
10395-BR-1995
21620005_1h-EU-2005
15425-SW-2009
1.00
Tornau-GE-2002
143a-GE-2002
0.97
106b-GE-2002
D
1.00
5800-BR-2000
7100-BR-2000
14295-BR-2000
8395-BR-1995
3296-BR-1996
2097-BR-1997
3996-BR-1997
E
Challenge-UK-1986
0.99
Nanjing-CH-2008
1.00
NJ2-CH-1998
HZ1-CH-2002
NJ1-CH-1999
F
NADL2-USA-1976
10095-BR-1995
0.92
11100-BR-2000
4996-BR-1996
2997-BR-1997
4096-BR-1996
12495-BR-1995
16695-BR-1995
16495-BR-1995
7595-BR-1995
1297-BR-1996
13595-BR-1995
0596-BR-1996
3095-BR-1995
1397-BR-1997
9194-BR-1994
0.93
14895-BR-1995
18195-BR-1995
11095-BR-1995
15595-BR-1995
0595-BR-1995
2700-BR-2000
G
30,0
20,0
10,0
0,0

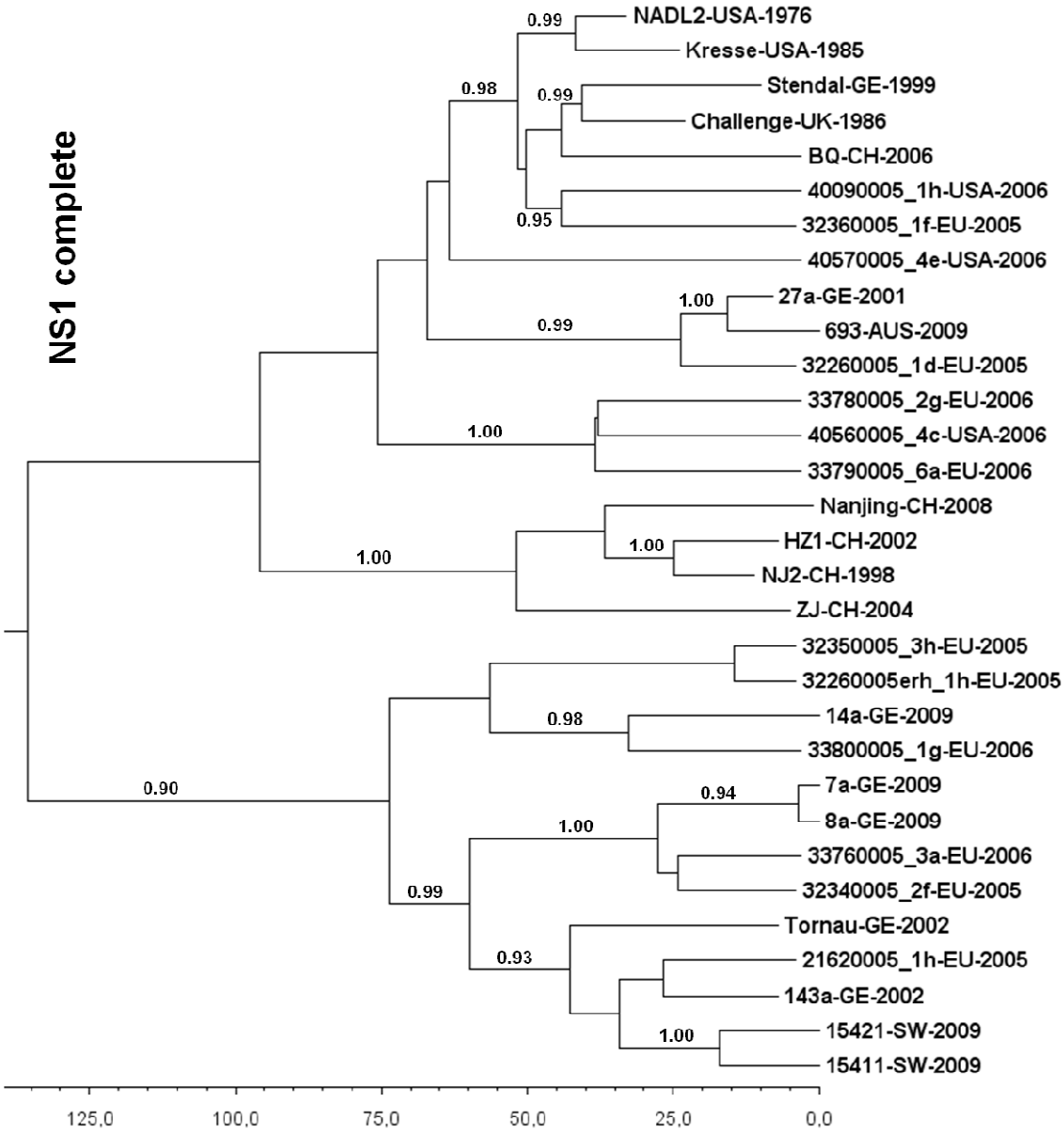


Figure 7. Phylogenetic tree based on BI analysis of PPV for the three datasets. The scale axis indicates the distance in years. Posterior probabilities (≥0.90) are indicated above the branches. The name (identification) of each sequence is followed by the location and year of isolation. The location is coded as: AUS (Austrian), BR (Brazil), CH (China), EU [Europe (no precise location available)], GE (Germany), SW (Switzerland), UK (United Kingdom) and USA (United States). For both VP trees, clades/clusters are delimitated and indicated with the letters A-G (continue line = cluster; discontinue line = clade).

DISCUSSION

A possible antigenic selection of PPV mutations, as a consequence of the current vaccination schedules, have been discussed in the past years (SOARES et al. 2003; ZEEUW et al. 2007; ZIMMERMANN et al. 2006). In the present paper, new virus sequences from Austria, Brazil, Germany and Switzerland, together with the available PPV sequences from GenBank/Embl/DDBJ, were analyzed and the evolutionary rate of this virus was calculated.

The analysis was performed using three different datasets. The VP1 partial dataset represents a small region of the VP1 gene; however, it enabled us to analyze a higher number of sequences over a broad range of years. The phylogenetic trees generated for the VP1 complete and partial genome contained four (A-D) and seven clades/clusters (A-G), respectively, and the majority of the sequences located in the A-D Clades/Clusters matched in both datasets (Figure 7). This suggests that the analyses of this short region of the VP1 gene (e.g.: SOARES et al. 2003) can be used as a representation of the full-length gene.

In a previous study that analyzed the complete VP1 gene, the authors discussed that the recent German strains 15a (AY684865), 21a (AY684868) and 27a (AY684871) formed a new phylogenetic cluster (ZIMMERMANN et al. 2006). The new German and Austrian strains were located in this cluster (Cluster C) present in both VP trees (complete and partial gene), and together with European sequences isolated in 2005-2006. The Swiss strain was located in Cluster D together with German and other European strains. Both of the clusters containing mainly German sequences and a cluster with Chinese sequences were characterized by high phylogenetic confidence, indicating that these clusters could have emerged from a well established ancestor. The Brazilian strain S30 was located in the Clade B together with two United States strains and one European strain. One other cluster (F) and two other clades (E and G) were seen in the partial dataset phylogeny. Cluster F was formed by Chinese strains, and Clades E and G only clustered in Brazilian strains. The two clades mainly formed by Brazilian strains have already been described in a previous study, where Maximum-parsimony analysis was carried out on a similar dataset (SOARES et al. 2003).

A comparison of both VP1 datasets with the NS1 dataset on the phylogenetic level was difficult. Only two VP clusters could be observed (D and F) in the tree generated for the NS1 gene. According to our temporal analyses, two distinct evolutionary rates between these genes were found. The phylogenetic incongruencies between both genes could reflect the different evolutionary development of these genes.

At the protein level, amino acids differing in the VP2 protein, when regarding the NADL2 and Kresse strains, were equally distributed between loops and β-strands. Here, we observed that the substitutions in the new strains were mainly located in the protein loops (Figure 8). The only exception was the amino acid 82 (R → K) substitution at the site number 82 in the Swiss strain 15425. Several substitutions in these loops could also be observed in the canine parvovirus and are reported to be involved in cell specificity and hemagglutination activity (CHAPMAN and ROSSMANN 1993). The substitutions 226 (Q → E), 228 (Q → E), 320 (I → T), 419 (E → Q) and 423 (N → K) were located near the 3-fold shoulder of the capsid subunit. This location has been considered to be a common antigenic surface region in distinct parvoviruses (CHAPMAN and ROSSMANN 1993). These sites are also further displaced from the center of the PPV capsid in comparison with other capsid protein amino acids (SIMPSON et al. 2002), suggesting that these sites may interact with the host immune system.

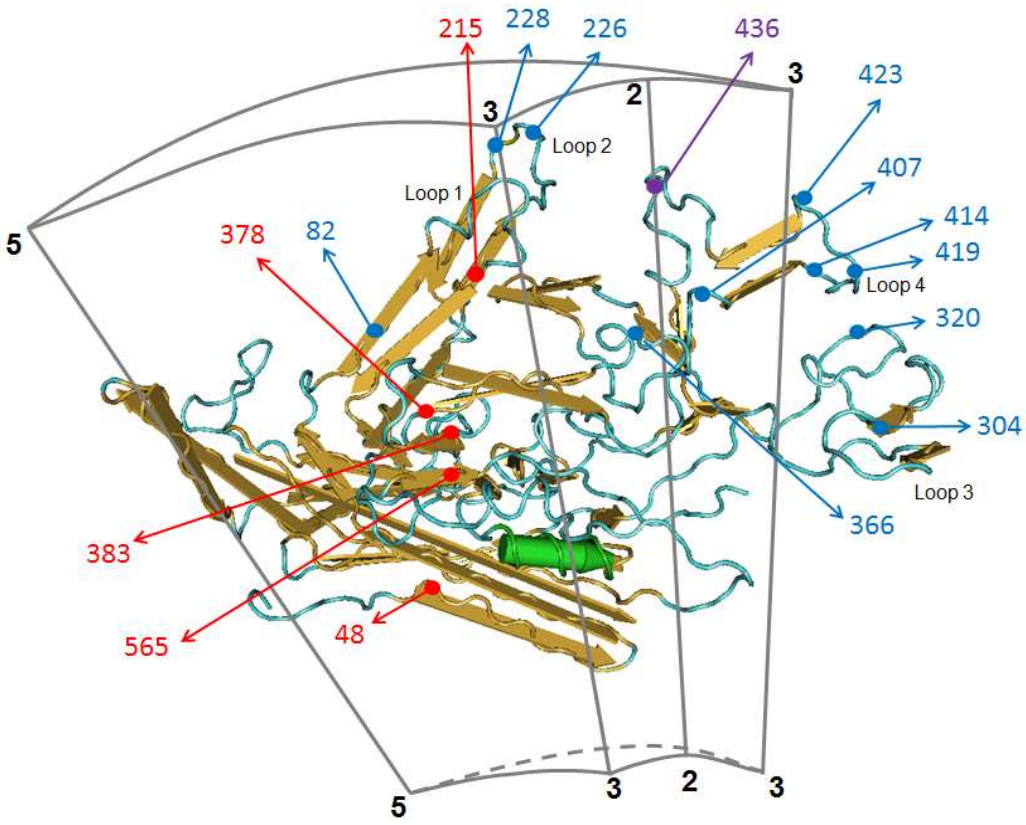


Figure 8. A 3D model of the PPV VP2 proteins using the cartoon technique, with rockets (α-helices) and arrows (β-strands) representing the secondary structure [adapted from CHAPMAN and ROSSMANN (1993)]. The neighboring five-, three- and

twofold axes of the capsid subunit are shown. The sites indicated with red circles represent the differences between NADL2 and Kresse strains, exception for the 436 site, in violet. The sites in blue represent the substitution in the new strains. The image was generated through the software Cn3D version 4.1. (available in http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml). The coordinates were retrieved from the NCBI Structure database (http://www.ncbi.nlm.nih.gov/Structure/index.shtml). Accession number: 1K3V (SIMPSON et al. 2002).

The Swiss strain 15425 displayed several unique amino acid substitutions and appears to be distant from the other samples. These unique substitutions were also found in the PPV sequence 21620005_1h (GQ884035) from Europe. The relationship between these two samples can be observed by the proximity in the phylogenetic trees.

In all new German and Austrian strains and in the Brazilian strain S30 the amino acid Threonine was found in position 436, an amino acid site located right in the 3 fold spike center of the capsid subunit. The higher incidence of a Threonine in the recent samples suggests that this amino acid is providing some adaptive advantage to the virus. According to the M8 model in the VP1 complete dataset, a higher selective pressure at this site in comparison with the whole virus genome was detected (0.998). The presence of this amino acid in this site has also been related to a possible decrease in the affinity of neutralizing antibodies (ZEEUW et al. 2007). To contribute to this discussion, future studies introducing mutations in these sites using the recent virulent strains aren need to be performed in order to determine whether these mutations are influencing/mediating the pathogenicity.

On the other hand, a high identity between the β-strands within the VP sequences was observed. That was expected, as these regions have important functions in maintaining the capsid integrity or in the capsid-DNA interaction (CHAPMAN and ROSSMANN 1993).

In this study, a high evolutionary rate was found for PPV capsid genes (~3-4 x 10^{-4}) and only a moderate evolution rate was found for PPV non-structural genes (~5 x 10^{-5}). The Higher Posterior Density (HPD) data reflects the uncertainty in the analysis. In the capsid genes, the lower and higher HPD were quite similar to the mean rate, providing support to the data. The root-to-tip analysis also supports the temporal structure for both VP1 datasets. In contrast, the NS1 dataset revealed that a lower R-squared and temporal analysis could only be suggested (FIRTH et al. 2010). The HPD for this

dataset revealed a larger substitution rate range (10^{-4} to 10^{-7}), indicating that this parameter could not be estimated with confidence. A similar HPD range for the substitution rate could also be seen in the NS1 gene for the FPLV (Figure 9), highlighting an uncertainty in the temporal analysis for the NS1 gene of the parvovirus (SHACKELTON et al. 2005).

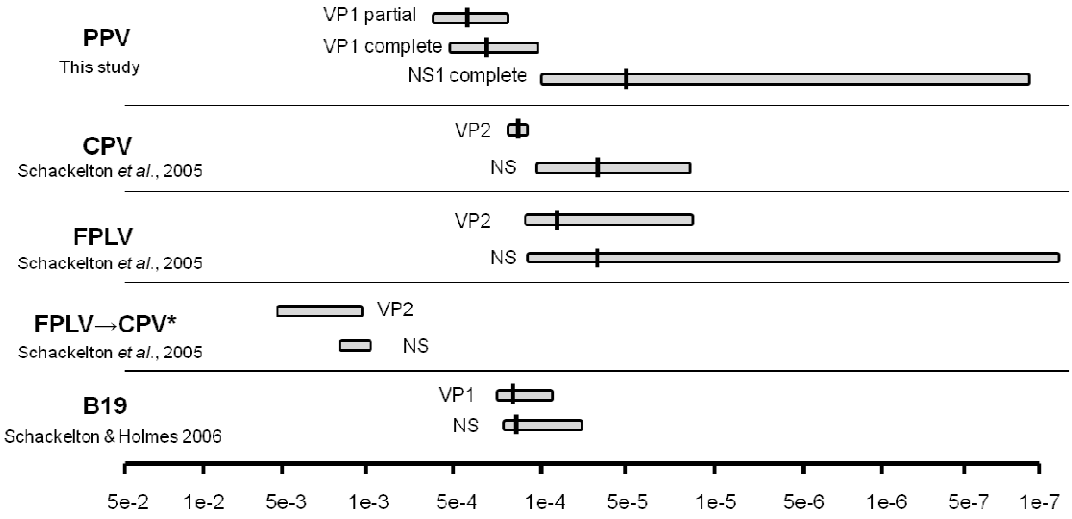


Figure 9. Nucleotide substitution rate per site per year of the PPV, CPV, FPLV and B19 viruses. The gray bars represent the interval between higher and lower HPD and the middle line represent the mean rate.
*HPD not determined. The bars represent the mean rate variation.

It is expected that the selective pressure and the evolutionary rate should exhibit similar levels. In previous parvovirus studies, a strong corelation between the selective pressure and the substitution rate for distinct viruses or genes has been observed (SHACKELTON et al. 2005, SHACKELTON and HOLMES 2006). In the present study, the mean rate of d_N/d_S substitution per site data suggests that the NS1 and VP1 genes were under purifying selection, while the partial VP dataset was under positive pressure (PAML and MEGA analysis). According to all the models tested, a pressure order of VP1 partial > VP1 complete > NS1 complete was verified. The same relation was demonstrated in the PPV evolution rate.

Recombination is one of the main diversity forces and allows genomic regions to have independent histories. Recombination events complicate the use of phylogenetic parameters such as timing events and selection pressures (AWADALLA. 2003). The

ubiquitous character of the PPV in association with an immune pressure could result in recombination events, as has been suggested by SHACKELTON et al. (2007). In our dataset alignments, no consensus for recombination events was found while applying several methods. However, incongruence in the partial structural protein dataset tree could be observed in comparison with the complete structural gene tree, e.g.: 225b (AY684864) and ZJ (EU790642) strains. The strain 225b was previously reported to be a possible recombinant strain originated from two distinct phylogenetic clusters (SHACKELTON et al. 2007). In order to avoid any false result in our timing analysis, the substitution rate was re-calculated without the incongruent sequences. No significant differences were observed.

Traditionally, PPV is considered to be a virus with a more conservative genome than other parvoviruses and viruses with single-strand DNA (LUKASHOV and GOUDSMIT 2001; LÓPEZ-BUENO et al. 2006; DUFFY et al. 2008). In a previous study, surface amino acid substitutions were found in German strains from 2001-2002 (ZIMMERMANN et al. 2006). Furthermore, it was demonstrated that antibodies raised against the vaccine strains (strain NADL2 and IDT) have a lower neutralization activity against field strains (27a and 143a) with these substitutions (ZEEUW et al. 2007; JÓŹWIK et al. 2009). In the present study, the modifications found in these two strains could also be observed for the current PPV-isolates in the population, indicating that the vaccines may no longer be fully protective. Additionally, new surface amino acid substitutions were also observed. These structural modifications found in strains collected over the last years, may represent an escape mechanism from the inactivated PPV vaccine, often used in the last 30 years. Rapid evolution is already known among ssDNA viruses, including canine parvovirus and human B19 erythrovirus (Figure 9) (SHACKELTON et al. 2005; SHACKELTON and HOLMES 2006). Reasons for the observed high substitution rates in parvoviruses and other ssDNA viruses remain unclear. Unlike RNA viruses, which use their own error-prone polymerases, PPV replicates using the cellular DNA polymerase of the swine host, implying that they should have the same replication fidelity as its host. Previous studies suggest that the proofreading or repair mechanism may not be efficient or accurate enough in these genomes or in cells with an active viral infection (SHACKELTON et al. 2005; SHACKELTON and HOLMES 2006). Alternatively, processes like the ssDNA deamination due to the lack of the double helix or non-functional host exonucleases (resulting from the absence of proper methylation patterns) could also lead to a mutational vulnerability (SANZ et al. 1999; DUFFY and HOLMES 2008).

In agreement with our report, previous studies could also define new virus profiles with new amino acid substitutions (SOARES et al. 2003; ZIMMERMANN et al. 2006; SHANGJIN et al. 2009). These substitutions are mainly located at the capsid surface and the development of a distinct surface profile from the vaccine strains (mainly based on the NADL2 strains) can be clearly noticed. In addition, the phylogenetic tree associated with a molecular clock analysis revealed that the divergences between the main isolates were introduced for the nonstructural gene dataset in the last 100 years and for both structural gene datasets in the last 10-30 years. Therefore, our data indicates that the currently used immunization schedules with inactivated whole-virus vaccines may need to be discussed. In the closely related canine and feline parvoviruses, inactivated full virus vaccines are no longer common, and have been fully replaced by modified-live virus vaccines (MLV). MLVs in these animals induces a long-lasting immune response that provides protection for at least several years and could also be an attractive alternative for PPV vaccination.

METHODS

PPV SAMPLES

A total of eleven recent isolates from Austrian, Brazil, Germany and Switzerland were analyzed. The Austrian (693a), German (7a, 8a and 14a), Swiss (15411, 15421 and 15425) and the Brazilian strains (PA and PB) were originated from clinical cases involving reproductive losses. The Brazilian strain (S30) were obtained from 55-day old (mean age) wasting piglets, and one (Embrapa) was isolated from an unknown source in Brazil.

AMPLIFICATION

The total DNA was purified from the clinical samples and the cell culture supernatant using the QIAamp DNA Mini kit (Qiagen, Germany), in accordance with the manufacturer's instructions. The DNA was stored at -22℃ until further analysis. The NS1 and VP1/VP2 genes amplification was performed using the primer pairs listed in the supplementary data. PCR was performed using ReddyMix PCR Master Mix (Abgene, United Kingdom) in a final volume of 50 µL. Amplification by PCR was performed under the following conditions: 1 cycle at 94℃ for 2 min, 35 cycles of 94℃ for 30 sec, 55℃ for 40 sec and 72℃ for 1 min and 1 cycle at 72℃ for 5 min. PCR amplification products were purified using the NucleoSpin purification kit (Macherey-Nagel, Germany) and sequenced using a BigDye terminator v 3.1 cycle sequencing kit

(Applied Biosystems, USA) and analized on an ABI3100 genetic analyzer (Applied Biosystems, USA) by Zentraler Funktionsbereich DNA-Sequenzierung (University of Leipzig, Germany). The DNA from the Brazilian strains was extracted using a silica-based protocol (BOOM et al. 1990). The nested-PCR reactions were performed as previously described (SOARES et al. 2003) and custom sequenced as above described.

The assembly of the obtained sequences to a full length sequence was performed using the SeqMan program of the Lasergene software (DNASTAR, USA). The sequences were deposited in GenBank with the accession numbers FJ643427-FJ643430 and JN400516-JN400526.

All nucleotide numbers used in the present study refered to the Kresse strain (GenBank accession number U44978). The amino acid numbers are according to the VP2 protein of the same strain.

PPV DATASET

All NS1 and VP gene sequences deposited in GenBank (up to September 2010), containing the isolation year, were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/) using “porcine parvovirus” as keyword. These sequences, taken together with the new sequences described here totaled 31 complete sequences of the NS1 gene and 31 complete sequences of the VP2 gene, which were analyzed separately. In order to increase the number of sequences in the analysis, a third dataset containing 65 partial sequences of VP2 gene was also constructed. The latter dataset consisted of 739 nucleotides (between nucleotide position 3701 and 4439). The year of isolation from the Brazilian samples PA, PB and Embrapa are unknown; therefore, these samples were excluded from further analyses. The datasets were aligned by applying the ClustalW method using MEGA 4 software (TAMURA et al. 2007). Possible genetic recombination events between virus strains were examined using the Bootscan, Chimaera, GENECONV, MaxChi and SisScan methods with default parameters, implemented in the RPD3 software package (MARTIN et al. 2010). Details of these methods are given in MARTIN et al. (2010) and program manual. Recombination events were assumed when a consensus between three or more methods was found.

PHYLOGENETIC ANALYSIS AND SELECTION PRESSURES

To perform the Maximum likelihood (ML) phylogenies, the DNA substitution model was obtained using the software ModelTest v.3.7 (POSADA and CRANDALL 1998). The analysis was performed with the software PAUP v.4.0.b (SWOFFORD. 2002). The heuristic search was performed using the Tree-Bisection-Reconnection method of branch swapping. Support was obtained using 100 bootstrap repetitions. The clock-like behavior of each data set was visualized using the regression of the root-to-tip genetic distance inferred from the ML trees against the sampling time in the software Path-O-Gen v.1 (DRUMMOND et al. 2003).

The rates of nucleotide substitution per site per year were estimated with a Bayesian Markov chain Monte Carlo method, using the software BEAST v.1.5.4 (DRUMMOND and RAMBAUT 2007). These analyses were run using the Hasegawa-Kishino-Yano (HKY) DNA substitution model with partitions into codon positions, performing 100 million generations through the Markov Chain Monte Carlo (MCMC) and sub-sampling each 10,000 generations. The population dynamic models tested were i.: constant population size and ii.: Bayesian skyline coalescent model. The molecular clock models tested were i.: strict molecular clock, ii.: relaxed clock with an uncorrelated lognormal distribution of rates (UCLN) and iii.: relaxed clock with an uncorrelated exponential distribution of rates (UCED). Multiple runs were performed for each data set to find the best model fitting the data [i.e. with a higher marginal Likelihood score (SUCHARD et al. 2001)]. The resulting data were visually analyzed using Tracer software (http://tree.bio.ed.ac.uk/software/tracer/) after removing a 10% “burn in” for each data. A “consensus” tree for each dataset was generated by the software TreeAnnotator v.1.5.4 (BEAST package). Phylogenetic trees were visualized with the software FigTree v.1.3.1 (http://tree.bio.ed.ac.uk/). The resulting trees were sorted to facilitate the comparison with the previously described VP1 and VP2 gene clusters. Groupings with posterior probabilities ≥ 0.90 were considered to be clusters and those with posterior probabilities ≤ 0.90 were considered to be clades.

The ratio of non-synonymous to synonymous nucleotide changes per site (d_N/d_S) for each branch of the phylogenetic tree was estimated for the NS1, VP1 complete and VP1 partial data sets using the PALM 4.2b package (YANG. 1997) following SHACKELTON et al. (2005) with few adjustments and using the ML tree as described above. A comparison of the M7 and M8 models was used to analyze selection pressures at specific codons in the codeml option ($p > 0.001$). The branch-site model was used to detect the positive selection acting in particular lineages/branches and compared with a null ω^2 fixed model ($p > 0.001$). In addition, the modified Nei &

Gojobori method (NEI and GOJOBORI 1986) applied with the MEGA 4 software was also used to find the ratio of non-synonymous to synonymous nucleotide changes.

ACKNOWLEDGEMENTS

This research was supported by the Deutscher Akademischer Austausch Dienst (DAAD, Germany), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil) and Coordenacão de Aperfeicoamento Pessoal de Nível Superior (CAPES, Brazil). We thank Prof. Ritzmann, Vienna, Prof. Sidler, Zürich, Dr. Ohlinger, Münster, and Dr. Ciacci-Zanella, Concórdia, for providing parvovirus positive samples and Prof. Pfeffer for critically reading the manuscript.

REFERENCES

Awadalla, P. The evolutionary genomics of pathogen recombination. Nat Rev Genet. 2003; 4:50-60.

Bergeron J, Hérbert B, Tijssen P. Genome organization of the Kresse strain of porcine parvovirus: identification of the allotropic determinant and comparison with those of NADL-2 and field isolates. J Virol. 1996; 70:2508-2515.

Boom R, Sol CJA, Salimans MMM, Jansen CL, Wertheim-Van Dillen PME, Van Der Noordaa J. Rapid and simple method for purification of nucleic acids. J Clin Microbiol. 1990; 28:495-503.

Cartwright SF, Huck RA. Viruses isolated in association with herd infertility, abortions and stillbirths in pigs. Vet Rec. 1967; 81:196-197.

Chapman MS, Rossmann MG. Structure, sequence and function correlations among parvoviruses. Virology. 1993; 194:491-508.

Drummond AJ, Pybus OG, Rambaut A. Inference of evolutionary rates from molecular sequences. Adv Parasitol. 2003; 54:331-358.

Drummond AJ, Rambaut A. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol. 2007; 7:214.

Duffy S, Holmes EC. Phylogenetic evidence for rapid rates of molecular evolution in the single-stranded DNA begomovirus tomato yellow leaf curl virus. J Virol. 2008; 82(2):957-965.

Duffy S, Shackelton LA, Holmes EC. Rates of evolutionary change in viruses: patterns and determinants. Nat Genet. 2008; 9:267-276.

Firth C, Kitchen A, Shapiro B, Suchard MA, Holmes EC, Rambaut A. Using time-structurated data to estimate evolutionary rates of double-stranded DNA viruses. Mol Biol Evol. 2010; 27(9):2038-2051.

Johnson RH. Isolation of swine parvovirus in Queensland. Aust Vet J. 1973; 49:257-259.

Joo HS, Donaldson-Wood CD, Johnson RH. Observation on the pathogenesis of the porcine parvovirus infection. Arch Virol. 1976; 51:123-129.

Jóźwik A, Manteufel J, Selbitz HJ, Truyen U. Vaccination against porcine parvovirus protects against disease, but does not prevent infection and virus shedding after challenge infection with a heterologous virus strain. J Gen Virol. 2009; 90:2437-2441.

López-Bueno A, Villarreal LP, Almendral JM. Parvovirus variation for disease: a difference with RNA viruses? Curr Topics Microbiol Immunol. 2006; 299:349-370.

Lukashov VV, Goudsmit J. Evolutionary relationships among parvoviruses: virus-host coevolution among autonomous primate parvoviruses and links between adeno-associated and avian parvoviruses. J Virol. 2001; 75:2729-2740.

Martin DP, Lemey P, Lott M, Moulton V, Posada D. RDP3: a flexible and fast computer program for analyzing recombination. Bioinformatics. 2010; 26:2462-2463.

Mengeling WL, Lager KM, Vorwald AC. The effect of porcine parvovirus and porcine reproductive and respiratory syndrome virus on porcine reproductive performance. Anim Reprod Sci. 2000; 60-61:199-210.

Nei M, Gojobori T. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol Biol Evol. 1986; 3:418-426.

Parrish CR. Structures and functions of parvovirus capsids and the process of cell infection. Curr Topics Microbiol Immunol. 2010; 343:149-176.

Posada D, Crandall KA. Modeltest: testing the model of DNA substitution. Bioinformatics. 1998; 14:817-818.

Sanz AI, Fraile A, Gallego JM, Malpica JM, Garcia-Arenal F. Genetic variability of natural populations of cotton leaf curl geminivirus, a single-stranded DNA virus. J Mol Evol. 1999; 49:49672-49681.

Simpson AA, Hébert B, Sullivan GM, Parrish CR, Zádori Z, Tijssen P, Rossmann M. The structure of porcine parvovirus: comparison with related viruses. J Mol Biol. 2002; 315:1189-1198.

Shackelton LA, Hoelzer K, Parrish CP, Holmes EC. Comparative analysis reveals frequent recombination in the parvoviruses. J Gen Virol. 2007; 88:3294-3301.

Shackelton LA, Holmes EC. (2006). Phylogenetic evidence for the rapid evolution of human B19 erythrovirus. J Virol. 2006; 80:3666-3669.

Shackelton LA, Parrish CR, Truyen U, Holmes EC. High rate of viral evolution associated with the emergence of carnivore parvovirus. Proc Natl Acad Sci USA. 2005; 102:379-384.

Shangjin C, Cortey M, Segalés J. Phylogeny and evolution of the NS1 and VP1/VP2 gene sequences from porcine parvovirus. Virus Res. 2009; 140(1-2):209-215.

Siegl G. The Parvoviruses. In: Gard S, and Hallauer C, Eds. Virology Monographs. 15. Edn. Wien, New York: Springer-Verlag; 1976. p. 47-52.

Soares RM, Cortez A, Heinemann MB, Sakamoto SM, Martins VG, Bacci M, De Campos FM, Richtzenhain LJ. Genetic variability of porcine parvovirus isolates revealed by analysis of partial sequences of the structural coding gene VP2. J Gen Virol. 2003; 84:1505-1515.

Suchard MA, Weiss RE, Sinsheimer JS. Bayesian selection of continuous-time Markov chain evolutionary models. Mol Biol Evol. 2001; 16:1001-1013.

Swofford DL. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. 2002. Sunderland: Sinauer Associates.

Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol. 2007; 24:1596-1599.

Yang Z. PAML: a program package for phylogenetic analysis by maximum likelihood. Comput Appl Biosci. 1997; 13:555-556.

Zeeuw EJL, Leinecker N, Herwig V, Selbitz HJ, Truyen U. Study of the virulence and cross-neutralization capability of recent porcine parvovirus field isolates and vaccine viruses in experimentally infected pregnant gilts. J Gen Virol. 2007; 88: 420-427.

Zimmermann P, Ritzmann M, Selbitz HJ, Heinritzi K, Truyen U. VP1 sequences of German porcine parvovirus isolates define two genetic lineages. J Gen Virol. 2006; 87:295-301.

CHAPTER 4 - POPULATION DYNAMICS AND *IN VITRO* ANTIBODY PRESSURE OF PORCINE PARVOVIRUS (PPV) INDICATE A DECREASE OF VARIABILITY

André Felipe Streck[1,2], Timo Homeier[1], Tessa Foerster[1], Uwe Truyen[1]

Author's affiliations:

1. Institute of Animal Hygiene and Veterinary Public Health, Faculty of Veterinary Medicine, University of Leipzig, An den Tierkliniken 1, 04103, Leipzig, Germany.

2. CAPES Foundation, Ministry of Education of Brazil, Setor Bancário Norte, Quadra 2, Bloco L, Lote 06, 70040-020, Brasília, Brazil.

ABSTRACT

To estimate the impact of PPV vaccines on the emergence of new phenotypes, the population dynamic of PPV was calculated, and an *in vitro*-model was designed to reproduce a possible immune selection. A decrease in genetic diversity was observed in the presence of antibodies *in vitro* or after vaccination. Since antibodies have reduced neutral selection, then the vaccine failures and infections in non-vaccinated populations were most likely responsible for the emergence of new PPV phenotypes.

Submitted

MAIN TEXT

Porcine parvovirus (PPV) infections are characterized by the reoccurrence of estrus, abortion and the delivery of mummified and stillborn fetuses [summarized in TRUYEN and STRECK (2012)]. The resulting economic losses for the pig industry led to the global use of inactivated vaccines after the 1980s (TRUYEN and STRECK 2012).

In the last 15 years, several reports have been published about new PPV-phenotypes with amino acid changes resulting in variations on the capsid surface (SOARES et al. 2003; ZIMMERMANN et al. 2006; STRECK et al. 2011). These amino acid substitutions may be responsible for the dramatically different pathogenic properties of the virus (BERGERON et al. 1996). In addition, there is evidence that antibodies raised against most of the PPV vaccine strains displayed a low heterologous neutralizing activity against these new phenotypes. This led to the hypothesis that the vaccines may force a selective pressure on PPV field strains resulting in "escape mutants", which could have supported the emergence of new phenotypes (ZEEUW et al. 2007).

To address the question whether PPV vaccines have influence on the emergence of new phenotypes, we analyzed the population dynamics of the virus *in silico* and the antibody pressure was evaluated with an *in vitro*-model.

To test the hypothesis of an antigenic selection we designed an *in vitro* experiment in which polyclonal serum generated from an experimentally NADL2-infected sow was used to neutralize one homologous and one heterologous PPV virus isolates. The homologous strain used was the NADL-2 (USA), a tissue culture-adapted vaccine virus (MENGELING. 1975) and the heterologous strain used was the strain called "Challenge" (England), a virulent strain that shows only a limited crossneutralization with the NADL-2 strain (ZEEUW et al. 2007). The neutralization capacity of the polyclonal antibodies is 1:5120 for the NADL2 strain and 1:640 for the Challenge strain (minimal serum dilution able to neutralize 50 TCID mL^{-1}). Both strains without antibodies were used as negative controls.

The first inoculations with Challenge and NADL-2 viruses were performed in PK-15 cell-lines ($3x10^5$ cells/mL) with a minimal concentration of virus able to produce a $TCID_{50}$ (in 96h) and maintained in Dulbecco's Modified Eagle Medium (DMEM, Sigma–Aldrich, Germany) supplemented with 5% (v/v) fetal bovine serum (FBS) at 37℃ in 5% CO_2. Additionally, different dilutions of polyclonal serum were added to the inoculated cells (hemagglutination-inhibition titer of 1:500 to 1:1.72 per 25 µL). After five days, the mixture with the minimal dilution of serum, but still displaying signs of CPE was collected and frozen. A subsequent re-inoculation of cells with different serum dilutions was performed with the collected viruses. After another five days, cells with the minimal dilution of serum and still presenting signs of CPE were collected again. This process was repeated for 21 passages. In the control, both viruses were inoculated in cells without polyclonal serum, collected, frozen and further passed this way for 21 times. A resistance was observed in the homologous NADL2 and heterologous Challenge strains inoculated with the antibodies in the last two passages, and a cytopathic effect was evident even in the most concentrated antibody dilution (1:500). Thus, no further passages were performed. The DNA amplification, sequencing, and assembly were performed as previously described (STRECK et al. 2011). The sequences were deposited in GenBank under the accession numbers (*waiting for approval*).

The VP gene sequence analysis revealed that the viruses grown without antibodies had more substitutions than the viruses cultivated with antibodies after 21 passages (Table 3). That was more evident in the homologous NADL2 strain without antibody pressure (with one synonymous and six non-synonymous substitutions) compared to the NADL2 submitted to antibody pressure (two synonymous substitutions). For the heterologous Challenge, the differences were blander: three synonymous and three

non-synonymous substitutions for the virus without the antibody pressure and three synonymous and two non-synonymous substitutions for the virus submitted to the antibody pressure.

Table 3. Nucleotide (nuc.) and amino acid (aa.) substitution for the homologous NADL2 and heterologous Challenge strains after 21 passages with and without antibody (AB) pressure.

Nuc. position number	2551	2746	2943	3163	3242	3383	3522	3635	3768	3942	3958	4030	4115	4183	4474	4503
NADL2 original	***GAA***	***AGA***	***ACT***	***GGA***	***ATA***	***AGT***	***CTA***	***TTA***	***ATT***	***GAT***	***CAC***	***AAG***	***TCT***	***CCT***	***AAC***	***AGA***
NADL2 with AB									AGT		CAA					
NADL2 without AB								CTA	ACT	GGT	CAA	AAC			AAA	AAA
AA. position number	63[A]	128[A]	45[B]	118[B]	145[B]	192[B]	238[B]	276[B]	320[B]	378[B]	383[B]	407[B]	436[B]	458[B]	555[B]	565[B]
NADL2 original	***E***	***R***	***T***	***G***	***I***	***S***	***L***	***L***	***I***	***D***	***H***	***K***	***S***	***P***	***N***	***R***
NADL2 with AB									S		Q					
NADL2 without AB									T	G	Q	N			K	K
Nuc. position number	2551	2746	2943	3163	3242	3383	3522	3635	3768	3942	3958	4030	4115	4183	4474	4503
Challenge original	***GAG***	***AGA***	***AGT***	***GGG***	***ATA***	***AGT***	***CTA***	***TTA***	***ATT***	***GGT***	***CAA***	***AAG***	***CCT***	***CCC***	***AAC***	***AAA***
Challenge with AB	GAA		ACT	GGA									TCT	CCT		
Challenge without AB	GAA	AGG			CTA	GGT	CCA							CCT		
AA. position number	63[A]	128[A]	45[B]	118[B]	145[B]	192[B]	238[B]	276[B]	320[B]	378[B]	383[B]	407[B]	436[B]	458[B]	555[B]	565[B]
Challenge original	***E***	***R***	***S***	***G***	***I***	***S***	***L***	***L***	***I***	***G***	***Q***	***K***	***P***	***P***	***N***	***K***
Challenge with AB			T										S			
Challenge without Ab					L	G	P									

[A]Amino acids numbers from VP1 protein

[B]Amino acids numbers from VP2 protein

Most of the changes were observed in positions known to be under a high selective pressure (SHANGJIN et al. 2009). In the amino acid position 320 (isoleucine), a serine was inserted in the NADL2 virus submitted to the antibody pressure and a threonine in the NADL2 virus grown without the antibody pressure, indicating that this site is highly variable. Additionally, in site 436 of the Challenge virus grown with antibody pressure, a serine was inserted (presented also in the original NADL2 strain). This site is located at the top of the 3-fold spike and is considered to have a high binding potential to antibodies (SIMPSON et al. 2002). Since antibodies raised against the NADL2 virus should now be able to strongly bind the Challenge virus with this substitution, this change was not expected. Despite the antibody pressure, the P436S substitution may have been selected because of replication advantages. A previous study with different chimaeras between the virus Kresse and NADL2 revealed that genetic elements of the NADL2 virus (including the site 436) are responsible for the replication efficiency in cell lines (FERNANDES et al. 2011).

For the *in silico* analysis, all VP gene sequences deposited in the GenBank (up to May 2012) were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/). Only viruses originally isolated from swine were considered. The dataset consisted of 739 nucleotides (between nucleotide position 3701 and 4439) from 74 samples. The nucleotide and amino acid numbers used in this study are based on the Kresse strain (GenBank accession number U44978).

The clock-like behavior of the dataset was visualized using the regression of the root-to-tip genetic distance inferred from the Maximum Likelihood tree against the sampling time in the software Path-O-Gen v.1 (DRUMMOND et al. 2003). The population dynamic was estimated with a Bayesian Markov chain Monte Carlo (MCMC) method, using BEAST version 1.7.1 (DRUMMOND and RAMBAUT 2007). These analyses were run using the GTR DNA substitution model with partitions into codon positions, performing 500 million generations through the MCMC and subsampling each 10 000 generations. The population dynamic model used was the Bayesian skyline coalescent (stepwise) model and the molecular clock model used was a relaxed clock with an uncorrelated log-normal distribution of rates. Additionally, different models testing the population size change through time were estimated (constant, lineal and stepwise) using the GTR DNA substitution model with partitions into codon positions, performing 10 million generations through the MCMC and subsampling each 10 000 generations. The fitness of both models was compared using the marginal likelihood, adopting the method of NEWTON AND RAFTERY (1994), with modification (SUCHARD and

REDELINGS 2006). The strength of the statistical evidence was quantified using the Bayes factors (KASS and RAFTERY 1995).

The root-to-tip analysis was constructed to examine whether the samples exhibited adequate temporal structure. Since the obtained value of R^2 for the regression analysis was 0.9845, a strong temporal fitness was confirmed for the dataset. Finally, the Bayesian skyline coalescent model was used to estimate the epidemiological history and evolutionary dynamics of the PPV over time. The comparison of the null hypothesis (constant population size) with the hypothesis in which population change is allowed indicated that the data fit better with the hypothesis that allow variability (Table 4). The resulting demographic inference is shown in Figure 10. The effective population size of PPV underwent a period of relative steadiness until around 1982. After 1982, a moderate but continuous decrease is evident until the recent strains. The beginning of the population decrease matches with the period in which vaccination campaigns were broadly introduced.

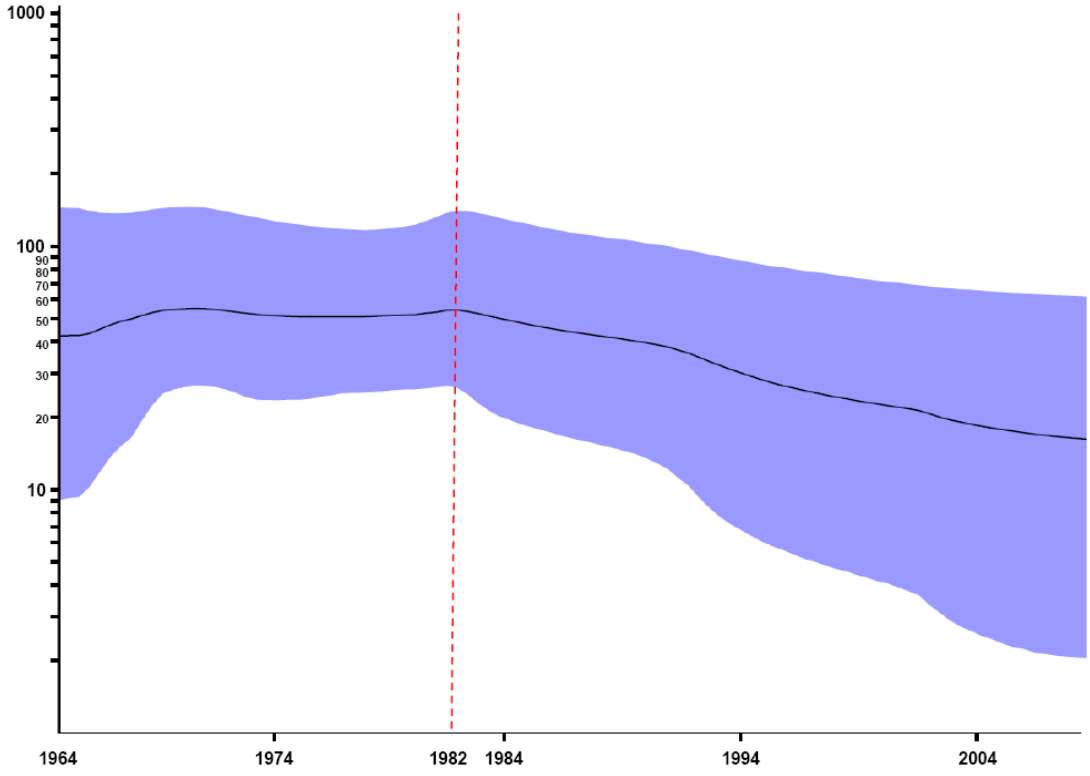


Figure 10. Skyline plot of the PPV population dynamics. The solid black line represents the mean value of the skyline plots. The blue area represents the limits of 95% higher posterior probability density. Time is shown in years across the X bar and the effective population size is shown in the Y bar.

Table 4. Comparison of the null-hypothesis (constant population size) with the hypothesis that allows population variability.

	Model 1	Model 2	Model 3
Tree prior	Constant	Lineal	Stepwise
Maximum likelihood	-1770.3356	-1772.9462	-1762.8145
95% higher posterior probability density difference	49.00	43.66	43.56
Log 10 Bayes' factor*	2.488	0.851	-

*Values between 0.0-0.5 provide no evidence; 1.0-1.5 provide weak evidence; >1 provide strong-to-decisive evidence [adapted from van BALLEGOOIJEN et al. (2009) and KASS and RAFTERY (1995)].

A main explanation for this phenomenon could be the broad use of certain PPV vaccines in the last 30 years. This can be stipulated, since a reduction in the genetic diversity is assumed to be strictly related to a more difficult or reduced virus transmission (HALLORAN and HOLMES 2009; van BALLEGOOIJEN et al. 2009), as expected in a vaccinated population. Another important factor that can influence the genetic diversity of the virus is the behavior of the host population (HALLORAN and HOLMES 2009). In the last decades, the demographics of the virus infection and its spreading potential with larger swine breeding/growth facilities possible changed. Additionally, new sanitary measures and disinfectants were adopted in the 1980s, probably affecting the viral population dynamics as well.

As several other factors could have influenced the PPV dynamic in the last decades, the *in vitro*-model was used to show that antibody pressure is able to cause a higher or lower genetic drift. In that model, we expected to observe a viral escape mechanism in viruses submitted to the antibody pressure. However, for both strains (NADL2 and Challenge), we observed less nucleotide and amino acids substitutions in the viruses when cultivated under antibody pressure in comparison with the same viruses cultivated without this pressure. This was particularly evident for the NADL2 strain submitted to the homologous antibody pressure. Regarding the heterologous Challenge strain, the emergence of non-synonymous and synonymous substitutions was quite similar for the virus cultivated with and without antibodies. As antibodies raised against the NADL2 virus do not efficiently neutralize the Challenge virus (ZEEUW et al. 2007; JÓŹWIK et al 2009), the pressure implemented by the anti-NADL2-antibodies should be milder compared to that raised against the homologous

strain NADL2. Under a strong pressure, most mutations may be deleterious, and the genetic diversity was lower for the NADL2 strain.

The selective forces that drive parvovirus evolution remain uncertain. Apparently, as in the *in vitro*-model a lower genetic diversity was observed in the presence of antibodies neutral selection seems to be more important for PPV than adaptive evolution.

In wild boar populations, phylogenetic analysis indicated that PPV is more diverse than in domestic pigs (CADAR et al. 2012). The authors of that study observed that the amino acid substitution rate per year was higher than that found in a study examining samples from domestic pigs (STRECK et al. 2011). As possible explanations for the wild boar viruses' higher variability, the dissemination of the same virus in herds by mixing animals of different sources and the extensive vaccination in domestic swine were hypothesized as factors that reduce PPV variability in these populations. Taken into consideration with the findings in the *in vitro* and *in silico* models, it can be assumed that vaccine failures and non-vaccinated populations (e.g. wild boars) rather than escape mutations due to vaccine-pressure may have played a major role in the emergence of new PPV-phenotypes.

In conclusion, the widely used vaccination programs had influenced the genetic diversity of porcine parvoviruses. To further minimize the risk of the emergence of new phenotypes the use of homologous vaccines along with a constant monitoring of the PPV appears advisable.

ACKNOWLEDGMENTS

This research was supported by the Deutscher Akademischer Austausch Dienst (DAAD, Germany) and Coordenação de Aperfeiçoamento Pessoal de Nível Superior (CAPES, Brazil). We thank Dietlinde Woll, Linda Oettel and Martin Pfeffer for critically reading the manuscript.

REFERENCES

Bergeron J, Hérbert B, Tijssen P. Genome organization of the Kresse strain of porcine parvovirus: identification of the allotropic determinant and comparison with those of NADL-2 and field isolates. J Virol. 1996; 70:2508-2515.

Cadar D, Dán Á, Tombácz K, Lőrincz M, Kiss T, Becskei Z, Spînu M, Tuboly T, Cságola A. Phylogeny and evolutionary genetics of porcine parvovirus in wild boars. Infect Genet Evol 2012; 12:1163-1171.

Drummond AJ, Pybus OG, Rambaut A. Inference of evolutionary rates from molecular sequences. Adv Parasitol. 2003; 54:331-358.

Drummond AJ, Rambaut A. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol. 2007; 7:214.

Fernandes S, Boisvert M, Tijssen P. Genetic elements in the VP region of porcine parvovirus are critical to replication efficiency in cell culture. J Virol. 2011; 85:3025-3029.

Halloran ME, Holmes EC. Invited commentary: Evaluating vaccination programs using genetic sequence data. Am J Epidemiol. 2009; 170:1464-1466.

Jóźwik A, Manteufel J, Selbitz HJ, Truyen U. Vaccination against porcine parvovirus protects against disease, but does not prevent infection and virus shedding after challenge infection with a heterologous virus strain. J Gen Virol. 2009; 90:2437-2441.

Kass RE, Raftery AE. 1995. Bayes factors. J Am Stat Assoc. 1995; 90(430):773-795.

Mengeling WL. Porcine parvovirus: frequency of naturally occurring transplacental infection and viral contamination of fetal porcine kidney cell cultures. Am J Vet Res. 1975; 36:41-44.

Newton MA, Raftery AE. 1994. Approximate Bayesian inference with the weighted likelihood bootstrap. J R Stat Soc (B). 1994; 56:3-48.

Shangjin C, Cortey M, Segalés J. Phylogeny and evolution of the NS1 and VP1/VP2 gene sequences from porcine parvovirus. Virus Res. 2009; 140:209-215.

Simpson AA, Hébert B, Sullivan GM, Parrish CR, Zádori Z, Tijssen P, Rossmann M. The structure of porcine parvovirus: comparison with related viruses. J Mol Biol. 2002; 315:1189-1198.

Soares RM, Cortez A, Heinemann MB, Sakamoto SM, Martins VG, Bacci M, De Campos FM, Richtzenhain LJ. Genetic variability of porcine parvovirus isolates

revealed by analysis of partial sequences of the structural coding gene VP2. J Gen Virol. 2003; 84:1505-1515.

Streck AF, Bonatto SL, Homeier T, Souza CK, Gonçalves KR, Gava D, Canal CW, Truyen U. High rate of viral evolution in the capsid protein of porcine parvovirus. J Gen Virol. 2011; 92:2628-2636.

Suchard MA, Redelings BD. BAli-Phy: simultaneous Bayesian inference of alignment and phylogeny. Bioinformatics. 2006; 22(16):2047-2048.

Truyen U, Streck AF. Porcine parvovirus. In: Zimmerman J, Karriker L, Ramirez A, Schwartz K, Stevenson G (ed). Diseases of Swine. 10th ed. Oxfort: John Wiley & Sons Inc; 2012. p. 447-455.

van Ballegooijen WM, van Houdt R, Bruisten SM, Boot HJ, Coutinho RA, Wallinga J. Molecular sequence data of hepatitis B virus and genetic diversity after vaccination. Am J Epidemiol. 2009; 170:1455-1463.

Zeeuw EJL, Leinecker N, Herwig V, Selbitz HJ, Truyen U. Study of the virulence and cross-neutralization capability of recent porcine parvovirus field isolates and vaccine viruses in experimentally infected pregnant gilts. J Gen Virol. 2007; 88:420-427.

Zimmermann P, Ritzmann M, Selbitz HJ, Heinritzi K, Truyen U. VP1 sequences of German porcine parvovirus isolates define two genetic lineages. J Gen Virol. 2006; 87:295-301.

CHAPTER 5 - ANALYSIS OF PORCINE PARVOVIRUSES IN TONSILS AND HEARTS FROM HEALTHY PIGS REVEALED HIGH PREVALENCE AND GENETIC DIVERSITY IN GERMANY

André Felipe Streck[1,2], Timo Homeier[1], Tessa Foerster[1], Stefan Fischer[3], Uwe Truyen[1]

Author´s affiliations:

1. Institute of Animal Hygiene and Veterinary Public Health, Faculty of Veterinary Medicine, University of Leipzig, An den Tierkliniken 1, 04103, Leipzig, Germany.

2. CAPES Foundation, Ministry of Education of Brazil, Setor Bancário Norte, Quadra 2, Bloco L, Lote 06, 70040-020, Brasília, Brazil.

3. Veterinary and Food Monitoring Office, am Schlachthof 8a, 06667, Weißenfels, Germany.

ABSTRACT

In the present study, tonsils and hearts from 100 pigs were collected in a German slaughterhouse in 2010 and tested for porcine parvoviruses (PPV, PPV2, PPV3 and PPV4). PPV was observed in 60/100 hearts and 61/100 tonsils and, PPV2 in 55/100 hearts and 78/100 tonsils. PPV3 and PPV4 were found in 20/100 and 7/100 of the tonsils tested, but not in the heart samples. Positive samples of PPV, PPV2 and PPV3 were analyzed by nucleotide sequencing and their phylogenetic analysis revealed at least two distinct lineages for each virus in the German samples. The high detection rate of the PPV, PPV2 and PPV3 in healthy animals and their genetic diversity highlights the importance of the continuous monitoring of these viruses and their zoonotic potential.

Archives of Virology, 2012, Accepted

INTRODUCTION

Parvoviruses are small DNA viruses infecting a variety of animal hosts, in which they may cause asymptomatic or sub-clinical infections as well as severe or even fatal disease. These viruses belong to the *Parvoviridae* family, which is sub-classified into two subfamilies: *Densovirinae* and *Parvovirinae* (TIJSSEN et al. 2001). The latter containing the most

important genera for the veterinary and human medicine in the whole family (*Amdovirus*, *Bocavirus*, *Dependovirus*, *Erythrovirus* and *Parvovirus*).

Porcine parvovirus (PPV) is considered to be one of the major causes of reproductive failure in swine. Clinical signs of PPV infections are characterized by the reoccurrence of estrus, abortion and the delivery of mummified and stillborn fetuses [reviewed by TRUYEN and STRECK (2012)] usually described with the acronym SMEDI (stillbirth, mummification, embryonic death and infertility). It was already demonstrated that PPV has a high substitution rate in the capsid gene, similar to that in other parvoviruses and in RNA viruses (SHACKELTON and HOLMES 2006; SHACKELTON et al. 2005; STRECK et al. 2011a). Therefore, the emergence of new PPV strains may interfere with the efficacy of the currently used vaccines, recommending the continuous monitoring of the swine population for PPV.

In recent years, by means of molecular techniques, the DNA sequences of several novel viruses have been identified. For parvoviruses affecting swine, the first of the newly identified viruses was the virus H-1 in sera from pigs in Myanmar (HIJIKATA et al. 2001), originally described as porcine parvovirus 2 (PPV2). In 2008, a virus closely related to the human parvovirus 4 was found in slaughtered pigs in Hong Kong (LAU et al. 2008) and initially termed as porcine hokovirus, here designated as porcine parvovirus 3 (PPV3). In 2010, another parvovirus was found in lungs from a diseased pig co-infected with porcine circovirus type 2 and designated porcine parvovirus 4 (PPV4) (CHEUNG et al. 2010). The impact of these viruses for the pig industry remains unknown.

In order to monitor the occurrence of new strains of PPV and to study the distribution of novel parvoviruses (PPV2, PPV3 and PPV4), hearts and tonsils of clinically healthy swine were collected at a slaughterhouse and examined for these viruses.

MATERIAL AND METHODS

SAMPLES

Tonsils and hearts (apex) from 100 pigs (around six months old) were collected in a German slaughterhouse in December 2010. The animals originated from thirteen farms (here termed A to M) located in seven German Federal States (Hessen, Lower Saxony, Mecklenburg-Vorpommern, Saxony, Saxony-Anhalt, Schleswig-Holstein and Thuringia). One gram of each tissue was macerated using mortar and pestle and resuspended as a 20% (v/v) suspension in phosphate-buffered saline solution (PBS, pH 7.2). Homogenates were cleared by centrifuging at 4000 *g* for 5 min and filtered (0.2 µm pore size). Clarified supernatants (200 µL) were used for total nucleic acid extraction using the QIAamp DNA Mini kit (Qiagen, Germany), according to the manufacturer´s instructions.

DNA AMPLIFICATION AND SEQUENCE ANALYSIS OF THE VP-GENE AND FULL-LENGTH GENOMES

Primers for the PPV2, PPV3 and PPV4 (Table 5) were designed using the Primer3 program (http://primer3.sourceforge.net/). The selected primers were locally aligned using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the GenBank content to avoid homology with other viral sequences. The PCRs specific for PPV, PPV2, PPV3 and PPV4 were carried out in single reactions using 10 nmol from each dNTP (Rapidozym, Germany), 10 pmol of the respective primer pair (Table 5), one unit of Taq polymerase (Rapidozym, Germany), enzyme buffer (according to the manufacturer's instruction) and 2 µL of DNA template in a total volume of 25 µL completed with bi-distilled water. The amplification was performed with an initial heating at 95℃ for 2 min utes, followed by 35 cycles consisting of 30 seconds at 95℃ for denaturation, 30 seconds at 55° C for annealing, 30 seconds at 72℃ for extension, and a final extension at 72℃ for 5 minu tes. Amplification products were submitted to electrophoreses in 1.5% agarose gels with 89 mM Tris-Base, 89 mM of boric acid, 2 mM EDTA Na_2 (TBE, pH 8.0) buffer and stained with 1,5 µL (per 100 mL of agarose gel) of Midori Green DNA Stain (Nippon Genetics Europe, Germany).

Table 5. Primers for the detection of the PPV, PPV2, PPV3 and PPV4.

Primer	Sequence	Location
PPV_S	GGGGGAGGGCTTGGTTAGAATCAC	2974-2997
PPV_AS	ACCACACTCCCCATGCGTTAGC	3149-3170
PPV2_F	AGATTCTTGCAGGCCGTAGA	909-928
PPV2_R	CCAAGGGTCAGCACCTTTTA	1096-1115
PPV3_F	GTGGCAGTGATATTGCATCG	992-1011
PPV3_R	TGGCAGTCATTGAATGGAAA	1219-1238
PPV4_F	ACAAGGTGGAGGAACGTTTG	3069-3088
PPV4_R	TTCCATGAGGGAGAGGATTG	3289-3308

In order to determine the sensitivity of each PCR, a plasmid harboring the target sequence of each PCR was generated. Briefly, PPV2, PPV3 and PPV4 positive samples were amplified as described previously. The amplicons were purified with the NucleoSpin purification kit (Macherey-Nagel, Germany) and cloned into the pDRIVE vector (Qiagen, Germany) using One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen, Germany). The transformed cells were grown on LB (Luria Bertani, Carl Roth GmbH, Germany) plates supplemented with 100 µg/ml ampicillin and incubated overnight at 37°C. Positive colonies were subcultured in LB broth (Carl Roth GmbH, Germany) with ampicillin and incubated at 37℃ for 8 hours. Plasmids were extracted using the PeqGOLD Plasmid Miniprep Kit I (Peqlab, Germany) and quantified by UV spectroscopy. For PPV, a plasmid containing the whole virus (Kresse strain) was used (FERNANDES et al. 2009). The copy number of each plasmid was calculated according the equation described in WHELAN et al. (2003). Finally, successive 10-fold dilutions of each plasmid were performed and tested for the respective PCR reaction.

Randomly selected positive samples of PPV, PPV2 and PPV3 were further amplified using the primer pairs listed in Supplementary Table 1. PCR was performed using the HotStarTaq Master Mix Kit (Qiagen, Germany) in a final volume of 25 µL. The PCR was performed under following conditions: one cycle at 94℃ for 15 min followed by 40 cycles of 95℃ for 1 min, 55℃ for 1 min and 72℃ for 1 min, with final elong ation at 72℃ for 5 min. PCR products were purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Germany), sequenced using a BigDye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems, Germany) and analyzed on an ABI3100 Genetic Analyzer (Applied Biosystems, Germany) at the Zentraler Funktionsbereich DNA-Sequenzierung (University of Leipzig, Germany). Assembling of the obtained sequences to a full-length sequence was performed using the SeqMan program of the Lasergene software (DNASTAR). The nucleotide sequences of the complete VP1 gene of the PPV and nearly full-length genomes of the PPV2 and PPV3 viruses were submitted to the NCBI GenBank, and are available under the accession numbers KC296743- KC296752. The samples were named according their sample number (1 to 100), followed by an H (sample from heart) or a T (sample from tonsil) and the respective virus (PPV, PPV2 or PPV3).

PHYLOGENETIC ANALYSIS

Sequences deposited in GenBank (up to June 2012) were retrieved from the NCBI homepage (http://www.ncbi.nlm.nih.gov/). In addition, the samples generated in this study were included. Altogether, the PPV dataset consisted of 47 sequences of the VP1 gene. In this dataset only sequences isolated after 2002 and standard sequences (143a, 225b, 27a, NADL2 and Kresse strains) were used. The PPV2 and PPV3 datasets consist of 7 and 45

sequences of the putative VP gene, respectively. The Open Reading Frames (ORFs) were found with Artemis software (RUTHERFORD et al. 2000) and the alignment was performed with the MUSCLE software (EDGAR. 2004).

The phylogeny was estimated with a Bayesian Markov Chain Monte Carlo (MCMC) method, using BEAST version 1.7.2 (DRUMMOND and RAMBAUT 2007). These analyses were run using the GTR substitution model with partitions into codon positions, performing 10 million generations through the MCMC and sub-sampling each 1000 generations. The resulting data were analyzed using Tracer software (http://tree.bio.ed.ac.uk/software/tracer/) after removing a 10% “burn in” for each data and compared with different priors. A maximum clade credibility tree for each dataset was generated by the software TreeAnnotator v.1.7.1 (BEAST package). Phylogenetic trees were visualized with the software FigTree v.1.3.1 (http://tree.bio.ed.ac.uk/).

All nucleotide numbers used in the present study for the PPV refer to the Kresse strain (GenBank accession number U44978). Amino acid numbers are according to the VP2 protein of the same strain. The nucleotide number for the novel parvoviruses refer to: PPV2 strain YH14 (GU938301), PPV3 strain HK1 (EU200671) and PP4 strain JS0918-5598 (GU978966).

CELL CULTURES

For virus isolation, the following cell lines were used: porcine kidney 15 (PK-15, CCL-33), swine embryonic kidney (SPEV) and *swine* testicular epitheloid (STE, CRL 1746). PK-15 and SPEV cells were propagated in Dulbecco's Modified Eagle Medium (DMEM, Sigma–Aldrich, Germany) supplemented with 5% (v/v) Fetal Bovine Serum (FBS), grown and maintained at 37℃ in 5% CO_2. STE cells were cultivated in DMEM supplemented with 10% (v/v) FBS and grown and maintained at 37℃. For primary cultures, testicles, lungs and kidney from piglets (negative tested for PPV, PPV2, PPV3 and PPV4) were washed in PBS (pH 7.2) and gingerly minced. The enzymatic disaggregation was performed with Trypsin 0.25% (Sigma–Aldrich, Germany) agitated using a magnetic stirrer at 37℃ for 45 min. The cells were then clarified (centrifugation at 500 *g* for 5 min), propagated in DMEM supplemented with 10% (v/v) FBS and grown and maintained at 37℃ in 5% CO_2. Finally, blood was obtained from 4 months old pigs and peripheral blood lymphocytes were separated from heparinized blood on Ficoll-Hypaque (Sigma–Aldrich, Germany). After one wash with PBS (pH 7.2) and clarification (600 *g* per 5 min) the cells were resuspended in Roswell Park Memorial Institute Medium (RPMI 1640, Sigma–Aldrich, Germany). Non-adherent cells were collected and resuspended in RPMI 1640 medium plus 20 µg/mL of concanavalin A (Lektin, Applichem, Germany).

All cell-lines and cell-cultures were inoculated with 100 µL of the inoculum (macerated tissues, Supplementary Table 3), after five days of incubation the cells were frozen and thawed twice and 500 µL of this mixture were used to inoculate new cells. The second cell passage was incubated for 5 days and frozen-thawed twice again. DNA of the cell extract and medium surface was extracted and submitted to the PCR.

STATISTICAL ANALYSIS

The data were statistically analyzed using the Chi-Square-test to evaluate differences in the PCR results. All analyses were carried out using the statistical package IBM-SPSS Version 20. Values of $P < 0.05$ were considered statistically significant.

RESULTS

PCR

The sensitivities of the PCRs was determined to be 10, 50, 100 and 50 plasmid copies detected per reaction for PPV, PPV2, PPV3 and PPV4, respectively. As shown in Table 6, amplification products of the PPV could be observed in 60/100 hearts and 61/100 tonsils. PPV2 could be identified in 55/100 hearts and 78/100 tonsils. None of the heart samples but 20/100 tonsil samples were positive for PPV3. Furthermore, none of the hearts was positive for PPV4 whereas in 7/100 a positive result was observed in tonsils. In total, 77 animals were positive tested for PPV, 81 for PPV2, 20 for PPV3 and 7 for PPV4.

Table 6. Positive samples for the PPV, PPV2, PPV3 and PPV4 viruses according their respective herd (A-M) and federal state (HE: Hessen, NI: Lower Saxony, MV: Mecklenburg-Vorpommern, SN: Saxony, ST: Saxony-Anhalt, SH: Schleswig-Holstein and TH: Thuringia). Results displayed as (positive heart samples//positive tonsil samples).

Herd	Fed. State	PPV	PPV2	PPV3	PPV4
A (n=10)	TH	7//4	3//4	0//0	0//2
B (n=10)	MV	8//10	8//9	0//0	0//0
C/D (n=10)	SN	10//9	7//10	0//5	0//1
E/F (n=10)	NI	8//6	5//8	0//4	0//0
G (n=10)	ST	2//0	5//10	0//0	0//1
H (n=10)	SH	6//9	5//5	0//2	0//0
I (n=10)	HE	8//7	3//5	0//5	0//0
J/K (n=10)	HE/NI	8//10	5//8	0//0	0//2
L (n=10)	NI	1//6	5//9	0//4	0//1
M (n=10)	SN	2//0	9//10	0//0	0//0
Total		60^{a}//61^{a}	55^{b}//78^{b}	0//20	0//7

Upper letters showed statistical association.

In the heart samples, PPV and PPV2 co-infection was observed in 32/100 samples. In the tonsils, the greatest numbers of viral co-infection was observed for the PPV together with PPV2 (46/100), PPV together with PPV3 (15/100) and PPV2 together with PPV3 (15/100) (Table 7). Co-infection with PPV, PPV2 and PPV3 was observed in 12/100 samples and 2/100 samples were positive tested for the four viruses. No contamination was observed in the negative controls.

Table 7. Number of positive samples for each virus per tissue

	Heart				Tonsil			
Virus	PPV	PPV2	PPV3	PPV4	PPV	PPV2	PPV3	PPV4
PPV	60				61			
PPV2	32	55			46	78		
PPV3	0	0	0		15	15	20	
PPV4	0	0	0	0	4	5	2	7

GENETIC CHARACTERIZATION

The four sequences of the PPV VP1 gene analyzed (6T-PPV, 37T-PPV, 55T-PPV and 80T-PPV) show an identity of 98.4% - 100% with the sequences retrieved from the GenBank (altogether 47 PPV sequences, nucleotide number 2287 - 2314 joined with 2388 - 4549). Among the four sequences of this study the nucleotide identity ranged from 98.4% to 99.9%. The synonymous substitution rate ranged from two to 21 (overall average of fourteen) and the non-synonymous substitution rate from zero to fourteen (overall average of nine). For PPV2, the three sequences (6T-PPV2, 68T-PPV2 and 98T-PPV2) showed an identity of 94.4% - 98.7% with the sequences available (altogether seven PPV2 sequences, nucleotide number 240 - 5358).The identity between the three sequences was 94.7% - 98.4%. For PPV3, the three sequences (62T-PPV3, 70T-PPV3 and 85T-PPV3) had an identity of 95.3% - 99.7% with the sequences available (altogether 34 PPV3 sequences, nucleotide number 109 - 4180). Among the three sequences, the identity was 97.4% - 99.2%.

At the protein level, the PPV sequences 6T-PPV and 55T-PPV displayed a higher number of amino acid differences than 37T-PPV and 80T-PPV compared with standard strains (NADL2 and Kresse). A similar amino acid profile can also be found in the PPV strains 15425 (GenBank accession number JN400520) and 21620005_1h (GQ884035). On the other hand, the sequences 37T-PPV and 80T-PPV were almost identical to the virulent 27a strain (differences only at the sites A414S and E419Q) (Table 8).

Table 8. Amino acid substitution within PPV VP2 sequences.

	VP sequence							
Amino acid VP2	NADL2	Kresse	27a	15425	6T	37T	55T	80T
20	T	-	-	A	A	-	A	-
45	T	S	S	S	S	S	S	S
82	R	-	-	K	K	-	K	-
215	I	T	T	T	T	T	T	T
226	Q	-	-	E	-	-	E	-
228	Q	-	E	-	-	E	-	E
233	S	-	-	-	T	-	T	-
304	P	-	-	T	T	-	T	-
320	I	-	-	T	T	-	T	-
366	D	-	-	N	N	-	N	-
378*	D	G	G	G	G	G	G	G
383*	H	Q	Q	Q	Q	Q	Q	Q
407	K	-	-	N	N	-	N	-
414	A	-	S	-	S	-	S	-
419	E	-	Q	-	Q	-	Q	-
436*	S	P	T	P	P	T	P	T
565	R	K	K	K	K	K	K	K

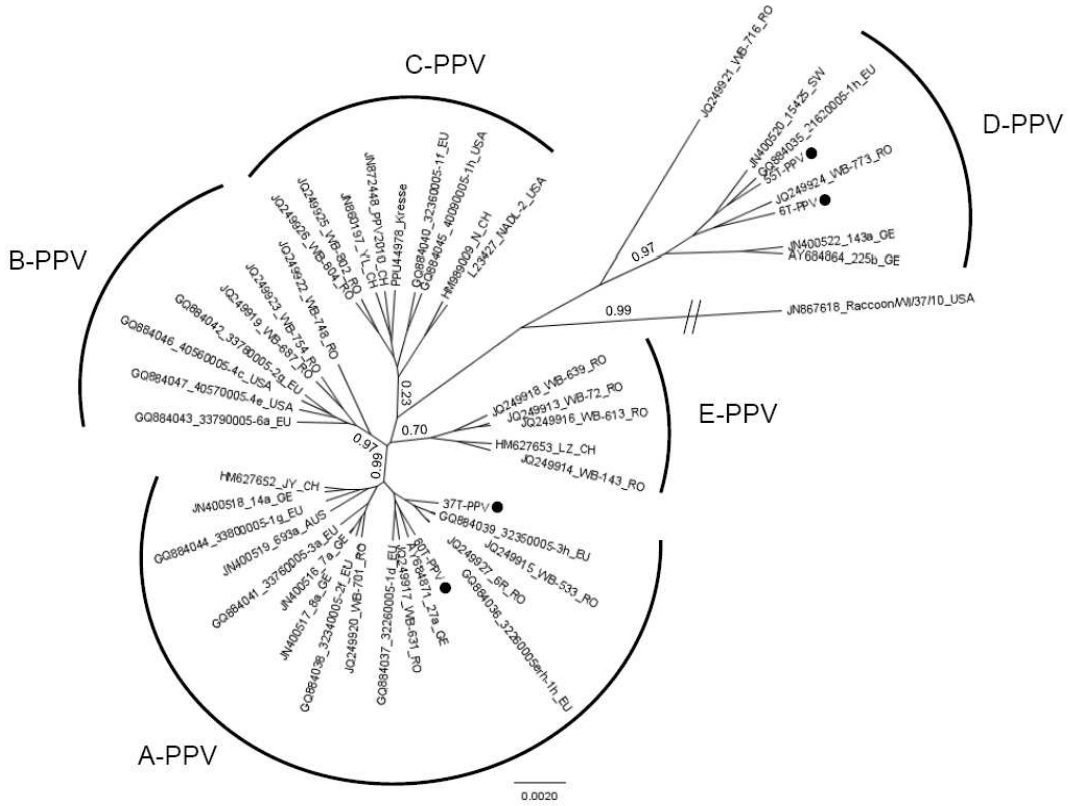
*Site considered responsible for the different tissue tropism (BERGERON et al. 1996).

PHYLOGENETIC ANALYSIS

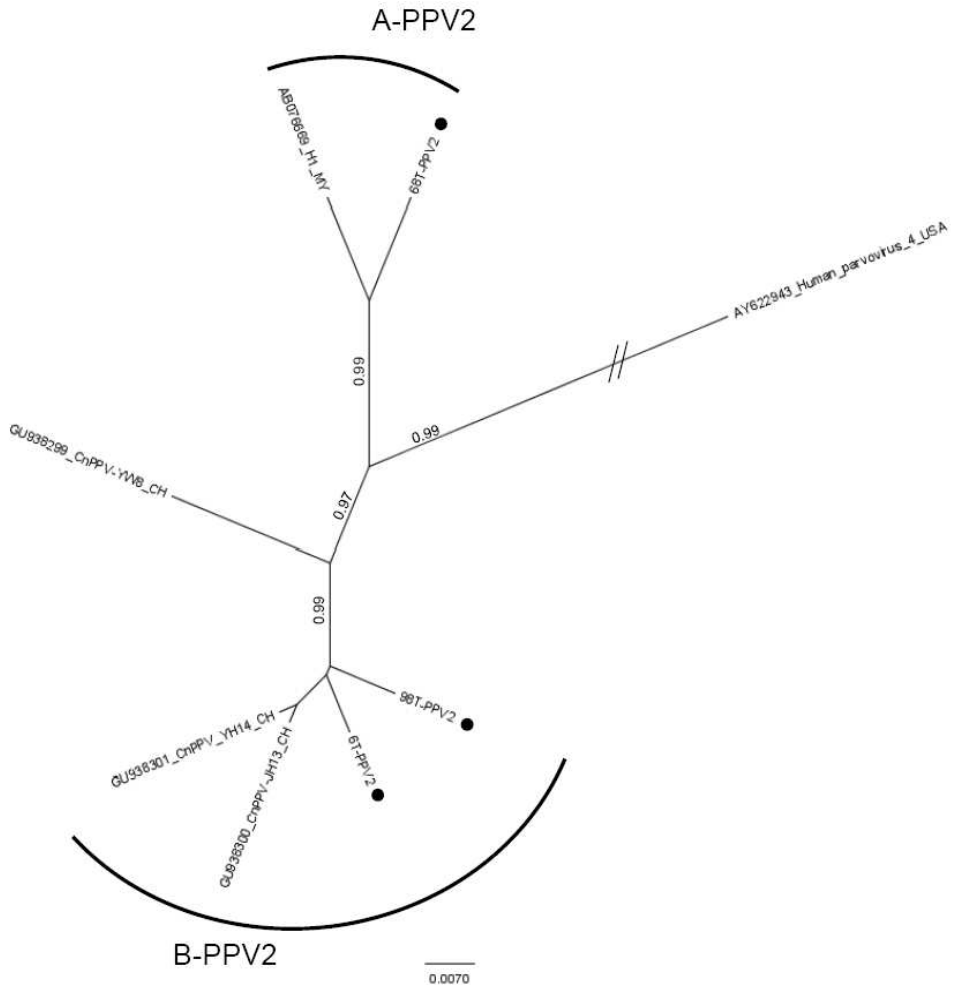
In the phylogeny, groups with posterior probability ≥ 0.90 were designated clusters and groups with low posterior probability (< 0.90) were designated clades. The Bayesian maximum clade credibility tree generated for the complete VP1 gene of the PPV revealed three clusters [here called A-PPV, B-PPV and D-PPV (posterior probability = 0.99, 0.97 and 0.97)] and two different clades [C-PPV and E-PPV (posterior probability < 0.90)]. The sequences 6T-PPV and 55T-PPV were located in the cluster D-PPV and the sequences 37T-PPV and 80T-PPV were located in the cluster A-PPV. From the sequences (isolated after 2002), the cluster A-PPV contains the higher proportion of new sequences in the DNA databanks (Figure 11).

The tree generated for the PPV2 putative VP gene displayed two clusters [A-PPV2 and B-PPV2 (posterior probability = 0.99 and 0.99]. The sequence 68T-PPV2 was located in the

cluster A-PPV2 and the sequences 6T-PPV2 and 98T-PPV2 were located in the cluster B-PPV2. Additionally, cluster A-PPV2 was formed with the sequence from Myanmar while cluster B-PPV2 by sequences from China. Finally, the tree generated for the PPV3 putative VP gene revealed two main clades [A-PPV3 and B-PPV3 (posterior probability < 0.90)]. The sequences 62T-PPV3 and 70T-PPV3 were located in the clade A-PPV3 and the sequence 85T-PPV3 was located in the clade B-PPV3. Clade A-PPV3 was formed mostly by sequences from China and clade B-PPV3 included sequences from Germany, Romania and the United Kingdom.



PPV2


A-PPV2
AB078689_H1_MY
68T-PPV2
AY622943_Human_parvovirus_4_USA
0.69
0.99
GU938299_ChPPV-YN9_CH
0.97
0.99
98T-PPV2
GU938301_ChPPV_YH14_CH
GU938300_ChPPV_JH13_CH
61T-PPV2
B-PPV2
0.0070

PPV3

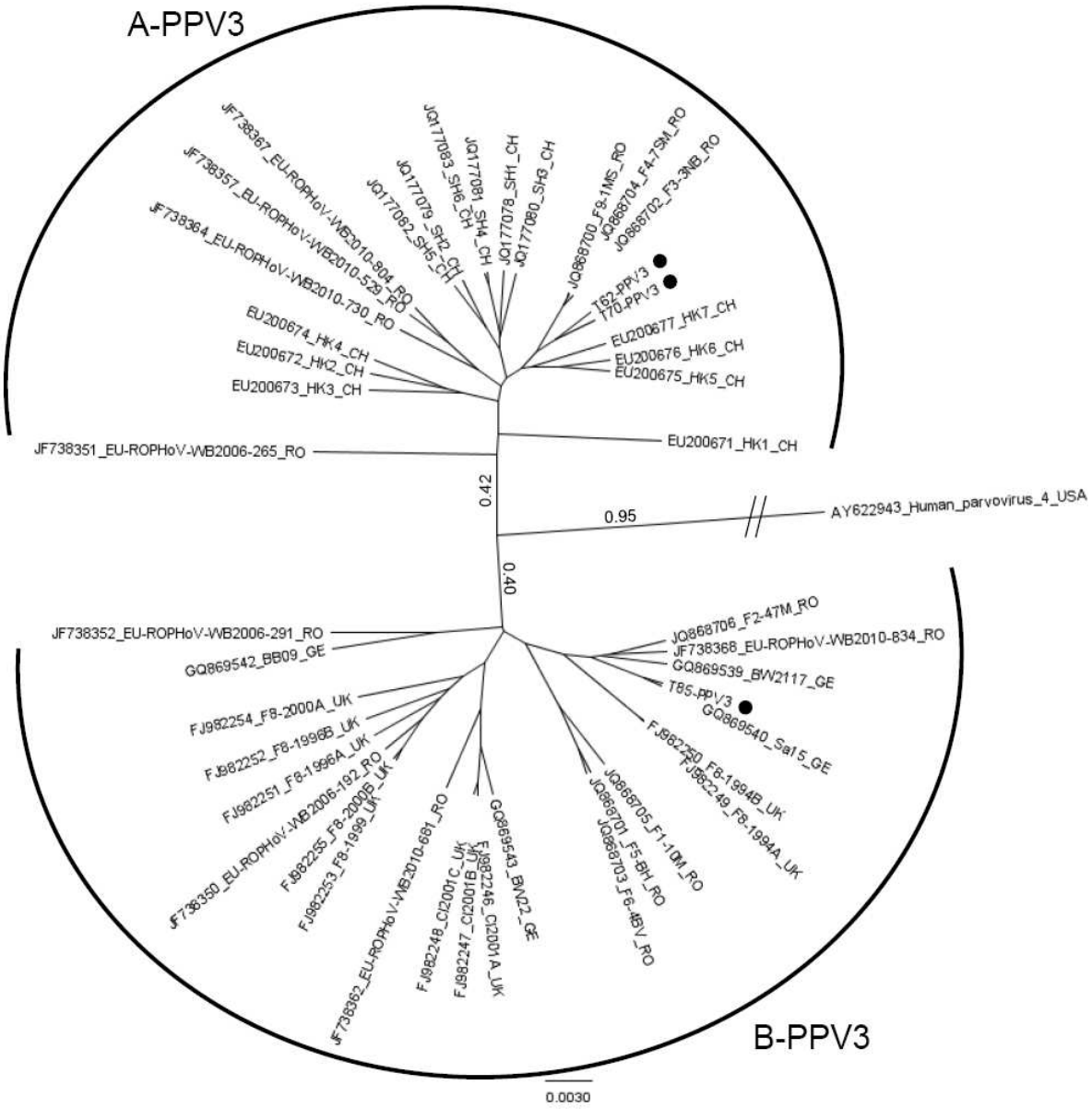


Figure 11. Phylogenetic tree for the PPV, PPV2 and PPV3 datasets. The scale axis indicates the substitutions per site. Posterior probabilities are indicated above the mainly branches. The GenBank accession number of each sequence is followed by the name (identification) and the location of isolation. The location is coded as: AUS (Austrian), CH (China), EU [Europe (no precise location available)], GE (Germany), MY (Myanmar), RO (Romania), SW (Switzerland), UK (United Kingdom) and USA (United States). The sequences from the present study are indicated with a black dot.

CELL CULTURE

After two passages in PK-15, SPEV and STE cell-lines and as well as in the primary culture (kidneys, lungs, testes and macrophages) no virus specific CPE was observed and no specific amplicons for PPV, PPV2, PPV3 or PPV4 could be generated in the PCR (supernatant and cell debris).

STATISTICAL ANALYSIS

Statistical associations among hearts and tonsils samples were observed for PPV and PPV2, individually. No statistical association was observed among different viruses (co-infection between PPV, PPV2, PPV3 or PPV4).

DISCUSSION

The high PPV DNA prevalence in hearts and tonsils observed in healthy fattening pigs was unexpected, since at that age these animals are usually separated from the sows (which can shed highly contaminated material from abortions). The animals were not vaccinated against PPV and, as in Germany no PPV live vaccines are licensed, the probability of vaccine DNA detection is minute.

Samples of tonsils were chosen due to their predominant role in a PPV infection. Tonsils are considered to be a primary replication site for PPV (TRUYEN and STRECK 2012). The high PPV prevalence (> 50%) may indicate a function of secondary replication for this tissue as well. As myocardium has been reported to support live-long presence of the human parvovirus B19 (KUETHE et al. 2009) and canine parvovirus (WALDVOGEL et al. 1991), heart samples were also included in the present study. In humans, prevalence of low DNA level of parvovirus B19 in the myocardium is a frequent finding in patients with heart disease and also in healthy patients (LOTZE et al. 2010). In pigs, PPV has already been demonstrated by nested-PCR in hearts of piglets presenting a non-suppurative myocarditis (BOLT et al. 1997), but its etiologic role is unknown.

The prevalence of low level of parvoviral DNA in humans is hypothesized to be caused by DNA of non replicating defective interfering particles or site specific integration in the chromosome (SÖDERLUND-VENERMO et al. 2002). In the swine host, the presence of the PPV DNA in serum with no correlation with the antibodies titers was already demonstrated (STRECK et al. 2011b), suggesting accordance with the hypothesis of DNA defective interfering particles, which are not capable of any immune system stimulation. Further studies should be performed in order to characterize the mechanism of persistence of PPV and parvovirus B19.

As the novel porcine parvoviruses are phylogenetically closely related to the novel human parvoviruses, those porcine viruses have recently been investigated. For PPV2, a high prevalence (20.7%) was already reported in pig lungs in USA in 2011 (XIAO et al. 2012). Here, even higher detection rates were obtained from hearts (55%) and tonsils (78%), strongly indicate that these tissues may be important for the viral infection. For PPV3, a high detection rate (18.7%) was observed in lungs from growing-finishing pigs in the USA [26]. In Germany, this virus was also detected in liver and serum (32.7%) from wild boars (ADLHOCH et al. 2010). For PPV4 only low detection rates were observed, confirming other reports (HUANG et al. 2010). Coincidently, in healthy humans a similar low to moderate DNA prevalence is observed for Human parvovirus 4 (high nucleotide identity to the PPV3) and Human bocavirus (with a similar genome structure to the PPV4) and a high prevalence is observed for parvovirus B19 as well (CORCIOLI et al 2010; KUETHE et al 2011).

To examine the properties of the infection in heart and tonsils, several cell-lines and primary cell cultures were inoculated with sixteen PCR-positive samples from PPV, PPV2, PPV3 and PPV4 (from tonsils and heart). PPV was firstly reported as a contaminant particle of primary cell culture from kidney and testicle and replication of PPV in these cells was essential for the further understanding of PPV biology {see SIEGL. (1976) for review}. Thus, it was expected that the novel parvoviruses have similar properties. The lack of virus replication in these cells may be due to the detection of non-infective particles of viral DNA or the primary cell cultures and the cell lines lack some property needed for the replication. Further studies are ongoing to define the tissue tropism of these viruses.

In the phylogenetic analysis, due to the high number of PPV sequences available from GenBank, the tree generated for the complete VP1 gene was calculated with sequences derived during the last ten years. In comparison with a previous phylogenetic study with PPV samples collected until September 2010 (STRECK et al. 2011a) a new clade (E-PPV) was formed with moderate posterior probability (0.70). To the standard strains 27a (sequence located in cluster A-PPV) and 225b (sequence located in cluster D-PPV) the *in vivo* pathogenicity was experimentally confirmed. Additionally, it was shown that antibodies raised against the vaccine strains have a low neutralization activity against these strains (ZEEUW et al. 2007), but no information about cross-neutralization and virulence of strains from the cluster B-PPV and clade E-PPV is available.

At the protein level, the sequences 37T-PPV and 80T-PPV were similar to the virulent strain 27a (AY684871). This strain was first detected in 2001 in Germany (ZIMMERMANN et al. 2006) and has since then been reported in several countries (STRECK et al. 2011a). The samples 6T-PPV and 55T-PPV had higher similarity with the strain 15425, a new strain (STRECK et al. 2011a), with unknown biological properties. A previous study with different

PPV-single-mutants has shown that the presence of amino acids 228 (E) and 436 (T) (as observed in the samples 37T-PPV and 80T-PPV) as well as the amino acids 414 (S) and 419 (Q) (as for samples 6T-PPV and 55T-PPV) increased the replication efficiency *in vitro* compared to the NADL2 original strain (data not shown). The amino acids 226 (E) in the sample 55T-PPV as well as the amino acids 233 (T), 304 (T), 320 (T), 366 (N) and 407 (N) for the samples 6T-PPV and 55T-PPV are located in the loops two, three and four of VP2 protein (Protein Data Bank accession 1K3V), it can be speculated that these substitutions may influence the immunogenicity or replication efficiency as well. For all the new samples, the amino acids substitutions (I-215-T, D-378-G, H-383-Q, S-436-P and R-565-K) that distinguish the non-virulent NADL2 strain (L23427) from the virulent Kresse strain (U44978) were similar to the Kresse sequence. The only exception was the site 436, at which the samples 37T-PPV and 80T-PPV have a serine (as in the strain 27a).

For the PPV2, there are few reports to enrich our phylogenetical analyses. It seems possible that the virus is divided in two main clusters; however, more sequences are needed to come to any meaningful conclusion. For PPV3, two distinct clades are evident. One is formed mostly by samples from China and the other includes samples from Canada, Germany and Romania. In Germany, five almost identical samples from PPV3 were already observed in wild boars (ADLHOCH et al. 2010) and were similar to the sequence 85T -PPV3 described here. The PPV2 and PPV3 sequences from this study were highly diverse, indicating that there is no geographic predominance of a single lineage of PPV2 or PPV3 in Germany.

In conclusion, DNA of PPV as well as of the novel PPV2, PPV3 and PPV4 was present in healthy animals (tonsils and hearts) and the phylogenetic analysis of the PPV, PPV2 and PPV3 demonstrated that these viruses have a high genetic diversity.

ACKNOWLEDGEMENTS

This research was supported by the Deutscher Akademischer Austausch Dienst (DAAD, Germany) and Coordenação de Aperfeiçoamento Pessoal de Nível Superior (CAPES, Brazil). We thank Dietlinde Woll and Martin Pfeffer for critically reading the manuscript.

REFERENCES

Adlhoch C, Kaiser M, Ellerbrok H, Pauli G. High prevalence of porcine Hokovirus in German wild boar populations. Virol J. 2010; 7:171.

Bergeron J, Hérbert B, Tijssen P. Genome organization of the Kresse strain of porcine parvovirus: identification of the allotropic determinant and comparison with those of NADL-2 and field isolates. J Virol. 1996; 70:2508-2515.

Bolt DM, Häni H, Müller E, Waldvogel AS. Non-suppurative myocarditis in piglets associated with porcine parvovirus infection. J Comp Pathol. 1997; 117:107-118.

Corcioli F, Zakrzewska K, Fanci R, De Giorgi V, Innocenti M, Rotellini M, Di Lollo S, Azzi A. Human parvovirus PARV4 DNA in tissues from adult individuals: a comparison with human parvovirus B19 (B19V). Virol J. 2010; 7:272.

Cheung AK, Wu G, Wang D, Bayles DO, Lager KM, Vincent AL. Identification and molecular cloning of a novel porcine parvovirus. Arch Virol. 2010; 155:801-806.

Drummond AJ, Rambaut A. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol. 2007; 7:214.

Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004; 32:1792-1797.

Fernandes S, Tijssen P. Seamless cloning and domain swapping of synthetic and complex DNA. Anal Biochem. 2009; 385:171-173.

Hijikata M, Abe K, Win KM, Shimizu YK, Keicho N, Yoshikura H. Identification of new parvovirus DNA sequence in swine sera from Myanmar. Jpn J Infect. 2001; 54:244-245.

Huang L, Zhai SL, Cheung AK, Zhang HB, Long JX, Yuan SS. Detection of a novel porcine parvovirus, PPV4, in Chinese swine herds. Virol J. 2010; 7:333.

Kuethe F, Lindner J, Matschke K, Wenzel JJ, Norja P, Ploetze K, Schaal S, Kamvissi V, Bornstein SR, Schwanebeck U, Modrow S. Prevalence of parvovirus B19 and human bocavirus DNA in the heart of patients with no evidence of dilated cardiomyopathy or myocarditis. Clin Infect Dis. 2009; 49:1660-1666.

Lau SK, Woo PC, Tse H, Fu CT, Au WK, Chen XC, Tsoi HW, Tsang TH, Chan JS, Tsang DN, Li KS, Tse CW, Ng TK, Tsang OT, Zheng BJ, Tam S, Chan KH, Zhou B, Yuen KY. Identification of novel porcine and bovine parvoviruses closely related to human parvovirus 4. J Gen Virol. 2008; 89:1840-1848.

Lotze U, Egerer R, Glück B, Zell R, Sigusch H, Erhardt C, Heim A, Kandolf R, Bock T, Wutzler P, Figulla HR. Low level myocardial parvovirus B19 persistence is a frequent finding in patients with heart disease but unrelated to ongoing myocardial injury. J Med Virol. 2010; 82:1449-1457.

Rutherford K, Parkhill J, Crook J, Horsnell T, Rice P, Rajandream MA, Barrell B. Artemis: sequence visualization and annotation. Bioinformatics. 2000; 16:944-945.

Shackelton LA, Holmes EC. Phylogenetic evidence for the rapid evolution of human B19 erythrovirus. J Virol. 2006; 80:3666-3669.

Shackelton LA, Parrish CR, Truyen U, Holmes EC. High rate of viral evolution associated with the emergence of carnivore parvovirus. Proc Natl Acad Sci USA. 2005; 102:379-384.

Siegl G. The Parvoviruses, in: Gard S, Hallauer C (eds) Virology Monographs, 15th ed. Wien, New York: Springer-Verlag; 1976. p 47-52.

Söderlund-Venermo M, Hokynar K, Nieminen J, Rautakorpi H, Hedman K Persistence of human parvovirus B19 in human tissues. Pathol Biol. 2002; 50:307-316.

Streck AF, Bonatto SL, Homeier T, Souza CK, Gonçalves KR, Gava D, Canal CW, Truyen U. High rate of viral evolution in the capsid protein of porcine parvovirus. J Gen Virol. 2011a; 92:2628-2636.

Streck AF, Gava D, Souza CK, Gonçalves KR, Bortolozzo FP, Wentz I, Canal CW. Presence of porcine parvovirus in sera from pigs is independent of antibody titers. Berl Munch Tierarztl Wochenschr. 2011b; 124:242-246.

Tijssen P, Agbandje-McKenna M, Almendral JM, Bergoin M, Flegel TW, Hedman K, Kleinschmidt JA, Li D, Pintel D, Tattersall P. Parvoviridae In: King MQ, Adams MJ, Carstens E, Lefkowitz EJ (eds) Virus Taxonomy: Classification and Nomenclature of Viruses: Ninth Report of the International Committee on Taxonomy of Viruses. San Diego: Elsevier; 2011.

Truyen U, Streck AF. Porcine parvovirus In: Zimmerman J, Karriker L, Ramirez A, Schwartz K, Stevenson G (eds) Diseases of Swine, 10th Ed Oxfort: John Wiley & Sons Inc; 2012. p 447-455.

Waldvogel AS, Hassam S, Weilenmann R, Tratschin JD, Siegl G, Hänichen T, Briner J, Pospischil A. Retrospective study of myocardial canine parvovirus infection by in situ hybridization. Zentralbl Veterinarmed B. 1991; 38:353-357.

Whelan JA, Russell NB, Whelan MA. A method for the absolute quantification of cDNA using real-time PCR. J Immunol Methods. 2003; 278(1-2):261-269.

Xiao CT, Gerber PF, Giménez-Lirola LG, Halbur PG, Opriessnig T.Characterization of porcine parvovirus type 2 (PPV2) which is highly prevalent in the USA. Vet Microbiol. 2012; Epub ahead of print

Xiao CT, Giménez-Lirola LG, Halbur PG, Opriessnig T. Increasing porcine PARV4 prevalence with pig age in the US pig population. Vet Microbiol. 2012; Epub ahead of print

Zeeuw EJL, Leinecker N, Herwig V, Selbitz HJ, Truyen U. Study of the virulence and cross-neutralization capability recent porcine parvovirus field isolates and vaccine viruses experimentally infected pregnant gilts. J Gen Virol. 2007; 88:420-427.

Zimmermann P, Ritzmann M, Selbitz HJ, Heinritzi K, Truyen U. VP1 sequences of German porcine parvovirus isolates define two genetic lineages. J Gen Virol. 2006; 87:295-301.

CHAPTER 6 - DISCUSSION

Porcine parvovirus is endemic in most areas of the world and one the most important causes of reproductive failure in pigs worldwide. Since there is a protective humoral immunity after infection, regular vaccination of breeding sows has become a common practice. The commercial vaccines represent inactivated virus and were used worldwide after 1980s with results considered satisfactory (reviewed by TRUYEN and STRECK 2012).

The described conservative character of the PPV genome have probably contributed to the few monitoring reports of PPV in the 1980s and 1990s. As eukaryotic cells have a cellular polymerase complex, with high efficiency in the polymerization of new DNA copies (due to their efficient repair unit) and consequent low substitution rate (around 10^{-9} till 10^{-10} substitutions per nucleotide per year), the parvoviruses, that use the host polymerase to replication, have their genome considerered to be low mutational, with a substitution rate close to the host (DUFFY et al. 2008). On the contrary, RNA viruses, that have their own RNA polymerase (without repair unit), have a substitution rate around 10^{-3} till 10^{-5} (DRAKE. 1993).

More recently, several studies about mutation dynamic showed that additional factors (e.g. genomic size, oxidation, deamination and methylation) may drastically affect the viral mutation rates (XIA and YUEN 2005; DUFFY et al. 2008). Some DNA viruses (mainly *ss* DNA) evolve swiftly whereas some RNA viruses do not (DUFFY et al. 2008). The first estimation of a high substitution rate in *ss* DNA viruses came from a study reporting the emergence of the canine parvovirus 2 (CPV-2) from the feline panleukopenia virus (FPLV). For both viruses, a substitution rate of 10^{-4} was estimated, and, in the main branch where CPV-2 emerges, a substitution rate of 7.1×10^{-3} was calculated in the period of time between 1968 and 1978 (SHACKELTON et al. 2005). After that, high evolution rates were observed for other *ss* DNA as well, including the human parvovirus B19 (SHACKELTON et al. 2006), the circovirus sen-V (UMEMURA et al. 2002) and the tomate yellow left curl virus (DUFFY and HOLMES 2008). For PPV, in a study amplifying and analyzing no more than 800 base pairs, the virus was described as homogeneous, with only few amino acid changes in the capsid (SOARES et al. 2003). As PPV, CPV-2 and human parvovirus have a close related genomes, a conservative genome only in the PPV seemed to be incongruent.

This question was addressed in the first manuscript titled "**High rate of viral evolution in the capsid protein of porcine parvovirus"** (Chapter 3). In this study, samples from Austria, Brazil, Germany and Switzerland had their genome sequenced (NS1 and VP genes). Additionally, the NS1 gene of several older samples (including 143a, 27a, 225b, Challenge,

KBSH and others) was sequenced as well. Since there were a lower number of PPV NS1 sequences available in the DNA databases in comparison to the VP sequences, this approach allowed us to analyze both genes with a fair number of sequences.

As results, a high evolutionary rate (around 10^{-4} nucleotides substitution per year) was observed for PPV capsid genes (complete and partial VP datasets) and a more moderate evolution rate was observed for PPV non-structural genes (around 10^{-5}). The capsid gene had a lower and higher *highest posterior density* (HPD) quite similar to the mean rate providing strong support to the data, and the root-to-tip analysis indicated that both capsid protein datasets have also strong temporal structure. Additionally, the complete and partial VP dataset led to similar results, indicating that the partial dataset (shorter sequences, but a higher number available at GenBank) is a plausible alternative for phylogenetic studies. Despite that, a higher variation in the HPD range and a median temporal regression score were observed for the non-structural dataset. Probably, the non-structural proteins of the PPV could be more evolutionary stable, resulting in few arguments for the algorithm, and consequently, uncertainty in the analysis.

The differences of the VP and NS1 genes datasets for PPV were higher in comparison to similar studies about canine parvovirus and human B19 erythrovirus (SHACKELTON et al. 2005; SHACKELTON and HOLMES 2006). That a structural gene evolves swiftly compared to the non-structural genes suggests that immune selection and tropism shifts could be important for the PPV evolution. Moreover, the high substitution rate observed in the VP datasets is similar to the substitution rate range observed for *ss* RNA viruses (DUFFY et al. 2008), indicating that the PPV is virus capable of faster adaptive evolution.

The wild phenotypes found in this study displayed the amino acids substitutions mainly located in the protein loops. These amino acid sites are further displaced from the center of the PPV capsid in comparison with other capsid sites. Differently, the amino acids substitutions in the old non-virulent strain NADL2 (isolated in 1976) and the old virulent Kresse (isolated in 1985) were mainly located in β-strands, more close to the center of the capsid (SIMPSON et al. 2002). The amino acids substitutions on the surface observed in the new phenotypes indicate viral changes for the cellular binding and antigenic binding activities, indicating that the virus is currently under an adaptation process.

The phylogenetic trees estimated for the VP1 complete and partial genome contained four and seven clades/clusters, respectively. Furthermore, with a molecular clock associated to the phylogenetic tree analysis, it could be shown that the main divergences between the isolates were introduced in the last 10-30 years for both VPs datasets. As there are few samples before 1980, to know if the high substitution rate is a recent event or if it is continuous through time was not possible. Recently, it was hypothesized that vaccination

could introduce a selective pressure, resulting in new vaccine escape phenotypes. That was suggested since antibodies raised with vaccine strains had reduced neutralizing activity against new phenotypes (ZEEUW et al. 2007).

To address this question, population dynamics, an evolutionary branch that studies the changes in population size, together with the biological and environmental factors that can influence these changes, was used. For viral populations, the accuracy of a vaccine program evaluation using population dynamics requires some conditions. First, the dataset should be large and embrace enough samples to provide epidemiologic-scale resolution to the analysis. Second, the data need to have a temporal structure. Third, all the processes that can potentially influence genetic diversity and insert uncertainties in demographic inferences should be considered (HALLORAN and HOLMES 2009). For PPV, the sequences available in GenBank/EMBL/DDBJ already provide a sufficient temporal fine-grained dataset (according the root-to-tip regression in the first and second manuscript). However, the size and mainly the geographic distribution of the present dataset are still poor. Currently, the publication of PPV DNA sequences is mostly performed by research groups distributed in Brazil, China and Germany. Moreover, the continuous development of handling techniques and disinfectants could interfere in the analysis as well.

As alternative, in the second manuscript titled **"Population dynamics and *in vitro* antibody pressure of porcine parvovirus indicates decrease of variability"** (Chapter 4) an additional *in vitro* approach was performed in cell lines infected with PPV and continually submitted to antibody pressure. Cell lines infected with PPV and non-submitted to antibody pressure were used as controls. As results, a lower genetic diversity was observed in the viruses submitted to the antibody pressure. That was more evident in the strain NADL2 (antibody homologous and non-virulent strain) submitted to the antibody pressure. The difference of this strain cultivated without pressure compared to the strain under pressure was of four distinct amino acids. In the Challenge strain (antibody heterologous and virulent strain), the strain cultivated without pressure had only one amino acid substitution more. Probably, as the antibodies were raised using the NADL2 strain, the consequent antibody pressure against the NADL2 was higher. Moreover, the NADL2 strain had higher cellular infection and replication capacity in compare to PPV viruses with distinct genetic combinations (FERNANDES et al. 2011). Thus, it can be expected that NADL2 strain had more generations per passage. It can be suggested that these both factors together contributed for the difference observed in the NADL2 strains with antibody pressure and without pressure.

In the *in silico* model a moderate and continuous reduction in genetic diversity was observed after 1985. A main explanation for this phenomenon could be the worldwide use of PPV

vaccines in the last 30 years. As a reduction in the genetic diversity is assumed to be strictly related to a more difficult or reduced virus transmission (van BALLEGOOIJEN et al. 2009). In that, the reduction of genetic diversity caused by vaccination seems to be plausible. However, the population dynamic algorithm can be strongly influenced by other non-evolutionary processes, like sanitary measures, disinfectants and animals separation in categories. Additionally, a small number of samples and biased sampling could also interfere with the result (HALLORAN and HOLMES 2009). In our study, the sampling size could be considered appropriate compared with other viral datasets (FIRTH et al. 2010), but a randomly sampled location cannot be reached.

It was expected to observe a viral escape in those viruses submitted to the antibody pressure as well in the sequences retrieved from GenBank. However, it was observed that viruses cultivated under antibody pressure were more evolutionary stable compared to the same viruses cultivated without this pressure; and for the PPV sequences, a decrease in the genetic diversity also was observed matching with the beginning of vaccination. As hypothesis, under a selective pressure, most mutations may be deleterious, reducing the genetic diversity. It also indicates that, neutral evolution seemed to be more important for the emergence of new phenotypes than adaptive evolution.

For the wild boar populations, a recent analysis indicated that PPV in these animals had higher diversity compared with the virus in domestic pigs (CADAR et al. 2012). In a previous report (CADAR et al. 2012), the obtained nucleotide substitution rate per year was higher than the rate found in the report containing only domestic pigs' samples (first manuscript of this dissertation). As possible explanations there are i. higher viral variability present in wild boars; ii. the dissemination of the same virus in herds by mixing animals of different sources; iii. the extensive vaccination in domestic swine as factors reducing the PPV variability in these populations.

The amino acids changes in the new phenotypes are mainly located close to the capsid surface (first manuscript) with possible importance for the cell-virus interaction or immune response. It has already been demonstrated that these phenotypes could have advantages compared to some vaccine strains, since antibodies raised against the vaccine strains (strain NADL2 and IDT) have a lower neutralization activity against field strain 27a and others (ZEEUW et al. 2007; JÓŹWIK et al. 2009). In this context, the dominance of some phenotypes in the domestic swine viral population and the consequent decrease of variability could be the result of an adaptation.

The third manuscript of this dissertation described the detection of novel parvoviruses. Recently, several asymptomatic or covert virus infections were firstly described (SIJUN et al. 2011), mainly with the development of the next generation sequencing technologies and

numerous bioinformatics methods, as the BLAST program (Basic Local Alignment Search Tool) (ALTSCHUL et al. 1990). For parvoviruses, the discovery of the high nucleotide substitution rate suggests that, in special occasions, these viruses could undergo rapid evolution and generate new genotypes or species. This was observed in the CPV-2, where an evolutionary pattern in the FPLV originating the CPV-2 in a short range of years was estimated (SHACKELTON et al. 2005). Thereafter, novel parvoviruses were found in several species, with a higher number of discover viruses in humans (YOO et al. 1999; NGUYEN et al. 2002; FRYER et al. 2006; LAU et al. 2008; LINDNER and MODROW 2008). In pigs, after the first description of the virus H-1 (called porcine parvovirus 2) in sera from pigs in Myanmar (HIJISAKA et al. 2001), several novel parvoviruses were further identified. For these parvoviruses, their biologic properties, epidemiology and veterinary-importance are still unknown.

In the third manuscript, titled **"Analysis of porcine parvoviruses in tonsils and hearts from healthy pigs revealed high prevalence and genetic diversity in Germany"** (Chapter 5), tonsils and hearts from 100 pigs were collected in a German slaughterhouse in 2010 and tested for PPV and the novel parvoviruses [porcine parvovirus 2 (PPV2), porcine parvovirus 3 (PPV3, also known as porcine hokovirus) and porcine parvovirus 4 (PPV4)]. As result, a high DNA prevalence was observed for PPV and PPV2 in hearts and tonsils, as well for PPV3 only in tonsils.

For PPV, tonsils are considered to be a primary replication site for PPV (TRUYEN and STRECK 2012). Since a very high detection rate was observed in this tissue, a secondary replication function can be suggested. In hearts, PPV has already been demonstrated in piglets presenting a non-suppurative myocarditis (BOLT et al. 1997). For humans, prevalence of low DNA level of parvovirus B19 in myocardium is a frequent finding in patients with heart disease and also in healthy patients (KUETHE et al. 2009; LOTZE et al. 2010). The maintaince of a persistent infection in this tissue with parvovirus B19 is speculated to be caused by DNA defective interfering particles or site specific integration in the chromosome. Another possible explanation may be that this particular tissue provides a good protective place from the immune system (SODERLUND-VENERMO et al. 2002). As this subject is speculative, further studies should be performed in order to characterize the persistence properties of parvoviruses.

The tropism for a specific tissue is still unknown for the PPV2, PPV3 and PPV4. However, virus DNA presence in hearts and tonsils suggests that both tissues may play a role in the viral replication or persistence. Since the heart muscle of swine is designated to human and animal food industry, a zoonotic potential of these viruses can be highlighted. For PPV, a viral contamination in human products was already observed in the Hyate C porcine factor

VIII concentrate designated for hemophilic patients. In this particular case report, no clinical implications were found (SOUCIE et al. 2000). However, as PPV can replicate in primary cell culture from humans (HALLAUER et al. 1971) a transmission between these hosts appears plausible.

In the PPV amino acid analysis, the sequences 37T-PPV and 80T-PPV were almost identical to the virulent 27a strain. This phenotype was first detected in 2001 in Germany (ZIMMERMANN et al. 2006) and spread successfully across several countries always related to severe diseases (as reported in the first manuscript). In this study, the 27a similar strains were detected in non-vaccinated and clinically healthy animals as well. The evidence of this phenotype in healthy animals may indicate that apart from the escape potential from the current vaccines (ZEEUW et al. 2007), this phenotype could possess higher infection or replication efficiency abilities in comparison with concurrent phenotypes.

The phylogenetic analysis of PPV was calculated with sequences obtained during the last ten years (from the DNA databases) together with sequences considered standard (143a, 225b, 27a, NADL2 and Kresse strains). The sequences 6T-PPV and 55T-PPV were located in the cluster D-PPV. The sequences 37T-PPV and 80T-PPV were located in the cluster A-PPV and were nearly identical to the German 27a strain. In comparison with the phylogenetic analysis from the first manuscript (samples retrieved up to September 2010), a new clade (E-PPV) emerged with moderate posterior probability (0.70) in a period not longer than two years. Unfortunately, for the samples grouped in this clade, as well in cluster B, no information about their cross-neutralization and virulence properties was available. Further studies on these groups can be important to understand veterinary importance of these samples. For the PPV2, the phylogenetical analysis reveals that the virus is divided in two main clusters. However, more sequences and reports are necessary to come to any meaningful conclusion. Finally, for PPV3, two distinct clades are evident as well. Geographic relation could be observed, since one clade is formed mostly by samples from China and the other clade includes samples from Canada, Germany and Romania. In Germany, five (almost) homologous samples from PPV3 have already been observed in wild boars (ADLHOCH et al. 2010) and were nearly identical to the sequence T85-PPV3 described in this study. For the PPV, PPV2 and PPV3 the phylogeny demonstrated that these viruses have a high genetic diversity and no geographic dominance of one group could be observed in Germany.

CHAPTER 7 - CONCLUSIONS

In the last years, new PPV phenotypes emerged in Austria, Brazil, Germany and Switzerland. These viruses could be observed in abortions and in clinical healthy pigs as well.

The PPV DNA was present in hearts and tonsils of fattening pigs with a high detection rate. The novel PPV2 was highly prevalent in both tissues and the PPV3 only in tonsils.

The amino acids substitutions of the new PPV phenotypes were located mainly at the capsid surface and the outgrowth of a distinct surface profile from the vaccine strains (mainly based on the NADL2 strain) can be observed.

The phylogenetic analysis of the PPV, PPV2 and PPV3 demonstrated that these viruses have a high genetic diversity and indicates that there is no dominance of a single phenotype of PPV, PPV2 or PPV3 in Germany.

The phylogenetic tree associated with a molecular clock for PPV revealed that the new clades/cluster emerged for both structural datasets in the last 10-30 years.

A decrease in genetic diversity of PPV was observed in the presence of antibodies in an *in vitro* model or after vaccination in the GenBank content. Since antibodies may have reduced neutral selection, vaccine failures and infections in non-vaccinated populations were most likely responsible for the emergence of new PPV phenotypes.

CHAPTER 8- SUMMARY

André Felipe Streck

Studies on genetic properties of porcine parvoviruses

Institute of Animal Hygiene and Veterinary Public Health

Faculty of Veterinary Medicine, Leipzig University

Submitted in December 2012

75 Pages, 11 Figures, 8 Tables, 116 References, 3 Appendices

Keywords: Porcine parvovirus, substitution rate, population dynamic, novel parvoviruses.

Porcine parvovirus (PPV) is considered to be one of the most important causes of reproductive failure in swine. Fetal death, mummification, stillbirths and delayed return to estrus are some of the clinical signs commonly associated with PPV infection in a herd. The virus genome is considered to be conservative, with substitution rates near to that of their host. However, it has been shown that some parvoviruses exhibit a substitution rate close to that commonly determined for RNA viruses. In this scenario, new PPV phenotypes may reduce the effectiveness of the currently used vaccines, recommending the continuous monitoring of the currently prevalent PPV strains. In addition, a number of novel porcine parvoviruses have been described during the last decade, but the importance and characteristics of these viruses remain unknown. In the present dissertation, three studies were performed to address the PPV genetic variability, to monitor the emergence of new PPV strains and the prevalence of novel parvoviruses.

In the first study, recent PPV field isolates from Austria, Brazil, Germany and Switzerland were sequenced and analyzed. These samples, together with sequences retrieved from GenBank, were included in three datasets (viral protein complete gene, viral protein partial gene and non-structural protein complete gene). For each dataset, the nucleotide substitution rate was determined and a molecular clock estimated. The analysis revealed that for the new strains, the amino acids substitutions were located mainly in the viral capsid loops. Only the capsid protein datasets present the higher suitability for phylogenetic analysis. In them, a higher divergence was found, with three well defined clusters. By inferring the evolutionary dynamics of the PPV sequences, a nucleotide substitution rate of approximately 10^{-4} substitutions per site per year was found for these datasets. An association of the phylogenetic tree with the molecular clock revealed that the main divergence of the PPV strains for the viral protein occurred in the last 30 years.

In the second study, the population dynamic of PPV isolates from swine herds was analyzed using PPV complete protein gene and partial sequences deposited in GenBank. The population dynamic of the virus was calculated using a Bayesian approach with a Bayesian skyline coalescent model. Additionally, an *in vitro* model was performed by twenty-one consecutives passages of the Challenge strain (a virulent field strain) and NADL2 strain (a vaccine strain) in PK15 cell-line supplemented with polyclonal antibodies raised against the vaccine strain (negative control was not supplemented). The Bayesian analysis indicated a decrease in the population diversity over the years and the predominance of some PPV strains. In agreement, the *in vitro* study revealed that a lower number of mutations appeared for both viruses in the presence of anti-PPV antibodies in comparison with the control passages without antibodies.

In the third study, tonsils and hearts from 100 pigs were collected in a German slaughterhouse in 2010 and tested for PPV, porcine parvovirus 2 (PPV2), porcine parvovirus 3 (PPV3) and porcine parvovirus 4 (PPV4). Positive samples of PPV, PPV2 and PPV3 were sequenced. PPV was observed in 60/100 hearts and 61/100 tonsils and PPV2 in 55/100 hearts and 78/100 tonsils. PPV3 and PPV4 could not be detected in the heart samples but 20/100 and 7/100, respectively, of the tonsils were tested positive. The phylogenetic analysis of the PPV, PPV2 and PPV3 sequences revealed that the German samples could be divided in at least two clusters or clades for each virus.

Altogether, it can be concluded that PPV is continuously evolving. Apparently, PPV vaccines largely used in the last 30 years probably have reduced the genetic diversity of the virus and induced the predominance of strains with distinct capsid profile from the original vaccine-based strain. Moreover, the high prevalence of the PPV, PPV2 and PPV3 and their genetic diversity highlight the importance of the continuous monitoring of these viruses.

CHAPTER 9 - ZUSAMMENFASSUNG

André Felipe Streck

Studien über die genetischen Varianten der Porcinen Parvoviren

Institut für Tierhygiene und Öffentliches Veterinärwesen

der Veterinärmedizinischen Fakultät, Universität Leipzig

Eingereicht im Dezember 2012

75 Seiten, 11 Abbildungen, 8 Tabellen, 116 Literaturangaben, 3 Anhangen

Schlüsselwörter: porcinen Parvoviren, Substitutionsrate, Populationsdynamik und neue Parvoviren

Die Infektion mit porcinen Parvoviren (PPV) zählt zu den wichtigsten Ursachen von Fruchtbarkeitsstörungen beim Schwein. Fetaler Tod, mumifizierte Feten, Totgeburten und verzögerter Östrusbeginn sind klinische Symptome, die mit einer PPV-Infektion in einer Herde assoziiert sind. Die Nukleotid-Substitutionsraten von PPV und des eukaryontischen Wirtes liegen in der gleichen Größenordnung; das PPV-Genom ist entsprechend stark konserviert. Kürzlich konnte jedoch für einige Parvoviren eine deutlich höhere Nukleotid-Substitutionsrate, wie sie bei RNA-Viren anzutreffen ist, gezeigt werden. In diesem Zusammenhang könnten neue PPV-Typen auftreten, die die Effektivität der derzeit verwendeten Impfstoffe reduzieren könnten. Eine kontinuierliche Überwachung der zirkulierenden PPV ist daher geboten. Des Weiteren wurde in den letzten zehn Jahren eine Reihe neuartiger, porciner Parvoviren beschrieben, wobei deren klinische Bedeutung unbekannt ist. In der vorliegenden Arbeit wurden drei Studien durchgeführt, die die genetischen Variabilität von PPV, die Entstehung neuer PPV-Typen und die Prävalenz der neuartigen Parvoviren beschreiben.

In der ersten Studie wurden PPV-Feldisolate aus Brasilien, Deutschland, Österreich und der Schweiz sequenziert und analysiert. Diese Proben wurden, zusammen mit Sequenzen aus öffentlichen Datenbaken (GenBank), zu drei Datensätzen zusammengefügt [Virusprotein (vollständiges Gen), Virusprotein (partielles Gen) und das vollständige Gen des Nicht-Strukturproteins]. Für jeden Datensatz wurde die Nukleotid-Substitutionsrate bestimmt und eine molekulare Uhr berechnet. Die Analysen zeigten, dass die Aminosäuresubstitutionen bei den neuartigen PPV-Typen hauptsächlich in den *loops* des Viruskapsids lokalisiert waren. Eingehende phylogenetische Analysen wurden mit den beiden Virusprotein-Datensätzen (vollständiges und partielles Gen) durchgeführt. In beiden Datensätzen wurde im Vergleich mit der Analyse des Nicht-Strukturproteins eine höhere Divergenz mit drei gut

definierten Clustern gefunden. Unter Einbeziehung der evolutionären Dynamik der PPV-Sequenzen, wurde eine Nukleotid-Substitutionsrate von annähernd 10^{-4} Substitutionen pro Position und Jahr in beiden Datensätzen detektiert. Die Verknüpfung der phylogenetischen Analyse mit der molekularen Uhr zeigte, dass in den letzten 30 Jahren die Hauptunterschiede der PPV-Stämme im Kapsidprotein lokalisiert waren.

In der zweiten Studie wurde die Populationsdynamik der PPV-Isolate von Schweinen, unter Einbeziehung des vollständigen Proteingens und Teilsequenzen aus den öffentlichen Datenbaken (GenBank), analysiert. Die Populationsdynamik des Virus wurde durch eine Bayesian-Methode mit einem *Bayesian skyline coalescent* Model berechnet. Zusätzlich wurde eine *in vitro* Untersuchung von 21 aufeinanderfolgenden Passagen durchgeführt. Hierzu wurde ein virulenter Feldstamm (Stamm Challenge) sowie ein Impfstamm (Stamm NADL2) in einer PK-15 Zelllinie vermehrt. Das Zellkulturmedium war mit polyklonalen Antikörpern gegen den Impfstamm supplementiert (Negativkontrolle ohne Antikörper-Zusatz). Die Bayesian-Analyse zeigte eine Abnahme der Populationsdiversität mit der Zeit sowie die Dominanz einiger PPV-Stämme. Die *in vitro* Studie ergab, dass bei beidem Viren die Anwesenheit von anti-PPV Antikörpern, im Vergleich zu der Kontrollpassage ohne Antikörper, eine geringeren Anzahl von Mutationen bewirkt.

In der dritten Studie wurden im Jahr 2010 Tonsillen und Herzen von 100 Schweinen in einem deutschen Schlachthof gesammelt. Die Organe wurden auf PPV, porcines Parvovirus 2 (PPV2), porcines Parvovirus 3 (PPV3) und porcines Parvovirus 4 (PPV4) untersucht. Positive Proben von PPV, PPV2 und PPV3 wurden im Anschluss sequenziert. PPV wurde in 60 Herzen sowie 61 Tonsillen, PPV2 in 55 Herzen und 78 Tonsillen nachgewiesen. PPV3 und PPV4 konnten in keiner Herzprobe detektiert werden, aber in 20 (PPV3) bzw. sieben (PPV4) Tonsillen. Die phylogenetischen Analysen der PPV, PPV2 und PPV3 Sequenzen verdeutlichten, dass die Isolate für jedes Virus mindestens zwei Cluster oder Clades bilden.

Insgesamt konnte eine kontinuierliche Evolution von PPV festgestellt werden. In den letzten 30 Jahren führte möglicherweise die starke Nutzung von PPV-Impfstoffen zu einer Abnahme der genetischen Diversität des Virus. Die neuen Typen weisen innerhalb der PPV-Population eine deutliche Ausbreitungstendenz auf. Darüber hinaus unterstreicht die hohe Prävalenz von PPV, PPV2 und PPV3 und ihre genetische Diversität die Bedeutung der kontinuierlichen Überwachung dieser Viren.

CHAPTER 10 - REFERENCES

Adlhoch C, Kaiser M, Ellerbrok H, Pauli G. High prevalence of porcine Hokovirus in German wild boar populations. Virol J. 2010; 7:171.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990; 215:403-10.

Andrews CH. Generic names of viruses of vertebrates. Virology. 1970; 40:1070-1.

Bachmann PA. Vorkommen und Verbreitung von Picodna (Parvo) - virus beim Schwein. Zentralbl Veterinarmed B. 1969; 16:341.

Bergeron J, Hérbert B, Tijssen P. Genome organization of the Kresse strain of porcine parvovirus: identification of the allotropic determinant and comparison with those of NADL-2 and field isolates. J Virol. 1996; 70:2508-15.

Bernhard W, Kasten FH, Chang C. Étude cytochimique et ultrastructurale de cellules infecteé par le virus K du rat et le virus H-I. Compte rendu hebdornadaire des séances de l'Académie des sciences. 1963; 2:1566-9.

Berns KI, Hauswirth WW. Adeno-associated virus DNA structure and replication. In: Berns KI. The Parvoviruses. New York: Plenum Press; 1983. p. 1-31.

Blomström AL, Belák S, Fossum C, McKillen J, Allan G, Wallgren P, Berg M. Detection of a novel porcine boca-like virus in the background of porcine circovirus type 2 induced postweaning multisystemic wasting syndrome. Virus Res. 2009; 146:125-29.

Boisvert M, Fernandes S, Tijssen P. Multiple pathways involved in porcine parvovirus cellular entry and trafficking toward the nucleus. J Virol. 2010; 84:7782-92.

Bolt DM, Häni H, Müller E, Waldvogel AS. Non-suppurative myocarditis in piglets associated with porcine parvovirus infection. J Comp Path. 1997; 117:107-18.

Burger D, Gorham JR, Ott RL. Protection of cats against feline panleukopenia following mink virus enteritis vaccination. Small Anim Clin. 1963; 3:611-4.

Cadar D, Cságola A, Lorincz M, Tombácz K, Spînu M, Tuboly T. Distribution and genetic diversity of porcine hokovirus in wild boars. Arch Virol. 2011; 156:2233-39.

Cadar D, Dán Á, Tombácz K, Lőrincz M, Kiss T, Becskei Z, Spînu M, Tuboly T, Cságola A. Phylogeny and evolutionary genetics of porcine parvovirus in wild boars. Infect Genet Evol. 2012; 12:1163-71.

Cartwright SF, Huck RA. Viruses isolated in association with herd infertility, abortions and stillbirths in pigs. Vet Rec. 1967; 81:196-7.

Cartwright, SF, Lucas M, Huck RA. A small heamagglutinating porcine DNA virus. I. Isolation and properties. J Comp Path. 1969; 79:371-7.

Cartwright SF, Lucas M, Huck RA. A small haemagglutinating porcine DNA virus. II. Biological and serological studies. J Comp Path. 1971; 81:145-55.

Chapman MS, Rossmann MG. Structure, sequence and function correlations among parvoviruses. Virology. 1993; 194:491-508.

Chen HY, Li XK, Cui BA, Wei ZY, Li XS, Wang YB, Zhao L, Wang ZY. A TaqMan-based real-time polymerase chain reaction for the detection of porcine parvovirus. J Virol Methods. 2009; 156:84-8.

Cheung AK, Wu G, Wang D, Bayles DO, Lager KM, Vincent AL. Identification and molecular cloning of a novel porcine parvovirus. Arch Virol. 2010; 155:801-6.

Dea S, Elazhary MASY, Martineau GP, Vaillancourt J. Parvovirus-like particles associated with diarrhea in unweaned piglets. Can J Comp Med. 1985; 49:343-5.

Dias AS, Gerber PF, Araújo AS, Auler PA, Gallinari GC, Lobato ZIP. Lack of antibody protection against Porcine circovirus 2 and Porcine parvovirus in naturally infected dams and their offspring. Res Vet Scie. 2012. Epub ahead of print.

Drake JW. Rates of spontaneous mutations among RNA viruses. Prod Nat Acad Sci USA. 1993; 90:4171–5.

Duffy S, Holmes EC. Phylogenetic evidence for rapid rates of molecular evolution in the single-stranded DNA begomovirus tomato yellow leaf curl virus (TYLCV). J Virol. 2008; 82:957–65.

Duffy S, Shackelton L, Holmes EC. Rates of evolutionary change in viruses: patterns and determinants. Nat Genet. 2008; 9:267-76.

Farr GA, Zhang L, Tattersall P. Parvoviral virions deploy a capsid-tethered lipolytic enzyme to breach the endosomal membrane during cell entry. Prod Nat Acad Sci USA. 2005; 102:17148–53.

Fernandes S, Boisvert M, Tijssen P. Genetic elements in the VP region of porcine parvovirus are critical to replication efficiency in cell culture. J Virol. 2011; 85:3025-9.

Firth C, Kitchen A, Shapiro B, Suchard MA, Holmes EC, Rambaut A. Using time-structurated data to estimate evolutionary rates of double-stranded DNA viruses. Mol Biol Evol. 2010; 27:2038-51.

Fryer JF, Kapoor A, Minor PD, Delwart E, Baylis SA. Novel parvovirus and related variant in human plasma. Emerg Infect Dis. 2006; 12:151-4.

Girod A, Wobus CE, Zádori Z, Ried M, Leike K, Tijssen P, Kleinschmidt JA, Hallek M. The VP1 capsid protein of adeno-associated virus type 2 is carrying a phospholipase A2 domain required for virus infectivity. J Gen Virol. 2002; 83:973-8.

Hallauer C, Kronauer G. Nachweis von Gelbfiebervirus-Haemagglutinin in menschlichen Explantaten. Arch Gesamte Virusforsch. 1960; 10:267-86.

Hallauer C, Kronauer G, Siegl G. Parvoiruses as contaminants of permanent human cell lines. I. Virus isolation from 1960-1970. Arch Gesamte Virusforsch. 1971; 35:80-90.

Halloran ME, Holmes EC. Invited commentary: Evaluating vaccination programs using genetic sequence data. Am J Epidemiol. 2009; 170:1464-6.

Hampton EG. Viral antigen in rat embryo in culture infected with the H-I virus isolated from transplantable human tumours: cytochemical studies. Cancer Res. 1964; 24:1534-43.

Hao X, Lu Z, Sun P, Fu Y, Cao Y, Li P, Bai X, Bao H, Xie B, Chen Y, Li D, Liu Z. Phylogenetic analysis of porcine parvoviruses from swine samples in China. Virol J. 2011; 8:320.

Harbinson CE, Chiorini JA, Parrish C. The parvovirus capsid odyssey: from the cell surface to the nucleus. Trends in Microbiol. 2008; 16:208-14.

Hijisaka M, Abe K, Win KM, Shimizu YK, Keicho N, Yoshikura H. Identification of new parvovirus DNA sequence in swine sera from Myanmar. Jpn J Infect Dis. 2001; 54: 244-5.

Hohdatsu T, Baba K, Ide S, Tsuchimoto M, Nagano H, Yamagami T, Yamagishi H, Fujisaki Y, Matumoto M. Detection of antibodies against porcine parvovirus in swine sera by enzyme-linked immunosorbent assay. Vet Microbiol, 1988; 17:11-9.

Hoggan MD. Small DNA viruses. In: Maramorosch K, Kurstak E. Comparative Virology. New York: Academic Press; 1971. p. 43-79.

Huang L, Zhai SL, Cheung AK, Zhang HB, Long JX, Yuan SS. Detection of a novel porcine parvovirus, PPV4, in Chinese swine herds. Virol J. 2010; 7:333.

Johnson RH. Isolation of swine parvovirus in Queensland. Aust Vet J. 1973; 49:157-9.

Johnson RH, Collings DF. Experimental infection of piglets and pregnant gilts with a parvovirus. Vet Rec. 1969; 85:446.

Johnson RH, Donaldson-Wood CR, Allender U. Observations on the epidemiology of porcine parvovirus. Aust Vet J. 1976; 52:80-4.

Joo HS, Donaldson-Wood CR, Johnson RH. A standartized heamagglutination inhibition test for porcine parvovirus antibody. Aust Vet J. 1976a; 52:422-4.

Joo HS, Donaldson-Wood CR, Johnson RH. Observations on the pathogenesis of porcine parvovirus infection. Arch Virol. 1976b; 51:123-9.

Joo HS, Johnson RH. Observations on rapid diagnosis of porcine parvovirus in mammified foetuses. Aust Vet J. 1977; 53(2):106-7.

Józwik A, Manteufel J, Selbitz HJ, Truyen U. Vaccination against porcine parvovirus protects against disease but does not prevent infection and virus shedding of a heterologous challenge virus. J GenVirol. 2009; 90:2437-41.

Karasaki S. Size and ultrastructure of the H-viruses as determined with the specific antibodies. J Ultra Mol Struct R. 1966; 16:109-22.

Kilham L, Olivier LJ. A latent virus of rats isolated in tissue culture. Virology. 1959; 7:428-37.

Kresse JI, Taylor WD, Stewart WW, Emisse KA. Parvovirus infection in pigs with necrotic and vesicle-like lesions. Vet Microbiol. 1985; 10:525-31.

Kuethe F, Lindner J, Matschke K, Wenzel JJ, Norja P, Ploetze K, Schaal S, Kamvissi V, Bornstein SR, Schwanebeck U, Modrow S. Prevalence of parvovirus B19 and human

bocavirus DNA in the heart of patients with no evidence of dilated cardiomyopathy or myocarditis. Clin Infect Dis. 2009; 49:1660-6.

Ladekjær-Mikkelsen AS, Nielsen J. A longitudinal study of cell-mediated immunity in pigs infected with porcine parvovirus. Viral Immunol. 2002; 15:373-84.

Lau SK, Woo PC, Tse H, Fu CT, Au WK, Chen XC, Tsoi HW, Tsang TH, Chan JS, Tsang DN, Li KS, Tse CW, Ng TK, Tsang OT, Zheng BJ, Tam S, Chan KH, Zhou B, Yuen KY. Identification of novel porcine and bovine parvoviruses closely related to human parvovirus 4. J Gen Virol. 2008; 89:1840-8.

Lawson JR. Infectious infertility of swine. Reports of the Meeting of the Expert Panel of Livestock Infertility; Rome, Italy; 1961. p. 67.

Lenghaus C, Forman AJ, Hale CJ. Experimental infection of 35, 50 and 60 day old pig foetuses with porcine parvovirus. Aust Vet J. 1978; 54(9):418-22.

Lindner J, Modrow S. Human bocavirus - a novel parvovirus to infect humans. Intervirology. 2008; 51:116-22.

Liu S, Vijayendran D, Bonning BC. Next Generation Sequencing Technologies for Insect Virus Discovery. Viruses. 2011; 3:1849-69.

Lotze U, Egerer R, Glück B, Zell R, Sigusch H, Erhardt C, Heim A, Kandolf R, Bock T, Wutzler P, Figulla HR. Low level myocardial parvovirus B19 persistence is a frequent finding in patients with heart disease but unrelated to ongoing myocardial injury. J Med Virol. 2010; 82:1449-57.

Lucas MH, Cartwright SF, Wrathall AE. Genital infection of pigs with porcine parvovirus. J Path. 1974; 84:347-50.

Madsen ES, Madsen KG, Nielsen J, Jensen MH, Lei JC, Have P. Detection of antibodies against porcine parvovirus nonstructural protein NS 1 may distinguish between vaccinated and infected pigs. Vet Microbiol. 1997; 54:1-16.

Mahnel H. Virus-like particles from hog cholera infected tissue cultures and demonstrated in the electron microscope. Proceedings of the FAO/OIE meeting on hog cholera and African swine fever; Rome, Italy; 1965.

Mayor HD, Melnick JL. Small deoxyribonucleic-acid-containing viruses (Picodnavirus group). Nature. 1966; 210:331-2.

Mayr A, Mahnel H. Cultivation of hog cholera virus in pig kidney cultures with cytopathogenic effect. Zentralbl Bakteriol Orig. 1964; 195:157-66.

Mengeling WL. Porcine parvovirus: frequency of naturally occurring transplacental infection and viral contamination of fetal porcine kidney cell cultures. Am J Vet Res. 1975; 36:41-4.

Mengeling WL. Porcine parvovirus: properties and prevalence of a strain isolated in the United States. Am J Vet Res. 1972; 33:2239-48.

Mengeling WL, Brown TT, Paul PS, Gutekunst DE. Efficacy of an inactivated virus vaccine for prevention of porcine parvovirus-induced reproductive failure. Am J Vet Res. 1979; 40(2):204-7.

Mengeling WL, Cutlip RC. Reproductive disease experimentally induced by exposing pregnant gilts to porcine parvovirus. Am J Vet Res. 1976; 37:1393-400.

Mengeling WL, Cutlip RC, Barnett D. Porcine parvovirus: pathogenesis, prevalence, and prophylaxis. Proceeding of the International Pig Veterinary Society Congress; Zagreb, Croatia; 1978. p. 15.

Mengeling WL, Lager KM, Vorwald AC. The effect of porcine parvovirus and porcine reproductive and respiratory syndrome virus on porcine reproductive performance. Anim Reprod Scie. 2000; 60-61:199-210.

Miao LF, Zhang CF, Chen CM, Cui SJ. Real-time PCR to detect and analyze virulent PPV loads in artificially challenged sows and their fetuses. Vet Microbiol. 2009;138:145-9.

Morimoto T, Kurigi H, Miura Y, Sugimori T, Fujisaki Y. Isolation of Japanese encephalitis virus and hemagglutinating DNA virus from the brain of still born piglets. Nat I Anim Health Q. 1972; 12:127-36.

Nguyen QT, Wong S, Heegaard ED, Brown KE. Identification and characterization of a second novel human erythrovirus variant, A6. Virology. 2002; 301:374-80.

Nuesch JP, Cotmore SF, Tattersall P. Sequence motifs in the replicator protein of parvovirus MVM essential for nicking and covalent attachment to the viral origin: identification of the linking tyrosine. Virology. 1995; 209:122-35.

Oravainen J, Heinonen M, Tast A, Virolainen JV, Peltoniemi OAT. High Porcine parvovirus antibodies in sow herds: prevalence and associated factors. Reprod Domest Anim. 2005; 40:57-61.

Pan Y, Zeng Q, Zhu C, Hua X, Wang M, Pan K, Cui L. Frequency and characterization of porcine hokovirus (PHoV) in domestic pigs in eastern China. Arch Virol. 2012; 157:1785-8.

Payne JE, Heals TF, Preston RE. Morphology of a small DNA virus. Virology. 1964; 23:109-13.

Paul PS, Mengeling WL. Oronasal and intramuscular vaccination of swine with a modified live porcine parvovirus vaccine: multiplication and transmission of the vaccine virus. Am J Vet Res. 1984; 45:2481-5.

Paul PS, Mengeling WL, Brown TT. Replication of porcine parvovirus in peripheral blood lymphocytes, monocytes, and peritoneal macrophages. Infect Immun. 1979; 25:1003-7.

Paul PS, Mengeling WL, Pirtle EC. Duration and biological half-life of passively-acquired colostral antibodies to porcine parvovirus. Am J Vet Res. 1982. 43:1376-9.

Qinq L, Lv J, Li H, Tan Y, Hao H, Chen Z, Zhao J, Chen H. The recombinant nonstructural polyprotein NS1 of porcine parvovirus (PPV) as diagnostic antigen in ELISA to differentiated from vaccinated pigs. Vet Res Commun. 2006; 30:175-90.

Rasbech NO. A review of the causes of reproductive failure in swines. Brit Vet J. 1969; 125:599-616.

Rose JA, Berns KI, Hoggan MD, Koczot FJ. Evidence for a single-stranded adenovirus associatedvirus genome: formation of a DNA density hybrid on release of viral DNA. Proc Nat Acad Sci USA. 1969; 64:863-9.

Ruckerbauer GM, Dulac GC, Boulanger P. Demonstration of parvovirus in Canadian swine and antigenic relationships with isolates from other countries. Can J Comp Med. 1978; 42(3):278-85.

Rudek Z, Kwiatkowska L. The Possibility of detecting fetal lymphocytes in the maternal blood of the domestic pig, Sus scrofa. Cytogenet Cell Genet. 1983; 36:580-3.

Shackelton LA, Holmes EC. Phylogenetic evidence for the rapid evolution of human B19 erythrovirus. J Virol. 2006; 80:3666-9.

Shackelton LA, Parrish CR, Truyen U, Holmes EC. High rate of viral evolution associated with the emergence of carnivore parvovirus. Prod Nat Acad Sci USA. 2005; 102:379-84.

Shangjin C, Cortey M, Segalés J. Phylogeny and evolution of the NS1 and VP1/VP2 gene sequences from porcine parvovirus. Virus Res. 2009; 140:209-15.

Siegl G. The Parvoviruses. In: Gard S, and Hallauer C, Eds. Virology Monographs. 15. Edn. Wien, New York: Springer-Verlag; 1976. p. 47-52.

Simpson AA, Hébert B, Sullivan GM, Parrish CR, Zádori Z, Tijssen P, Rossmann M. The structure of porcine parvovirus: comparison with related viruses. J Mol Biol. 2002; 315:1189-98.

Soares RM, Cortez A, Heinemann MB, Sakamoto SM, Martins VG, Bacci M, De Campos FM, Richtzenhain LJ. Genetic variability of porcine parvovirus isolates revealed by analysis of partial sequences of the structural coding gene VP2. J Gen Virol. 2003; 84:1505-15.

Soares RM, Durigon EL, Bersano JG, Richtzenhain LJ. Detection of porcine parvovirus DNA by the polymerase chain reaction assay using primers to the highly conserved nonstructural protein gene, NS-1. J Virol Methods. 1999; 78(1-2):191-8.

Söderlund-Venermo M, Hokynar K, Nieminen J, Rautakorpi H, Hedman K. Persistence of human parvovirus B19 in human tissues. Pathol Biol. 2002; 50:307-16.

Soucie JM, Erdman DD, Evatt BL, Anderson LJ, Török TJ, El-Jamil M, Barnhart E, Tepper M, Burrill HN, Pickett AM, Mengeling WL. Investigation of porcine parvovirus among persons with hemophilia receiving Hyate:C porcine factor VIII concentrate. Transfusion. 2000; 40:708-11.

Steitz TA. Structural studies of protein-nucleic acid interaction: the sources of sequence-specific binding. Q Rev Biophys. 1990; 23:205-80.

Streck AF, Gava D, Souza CK, Gonçalves KR, Bortolozzo FP, Wentz I, Canal CW. Presence of porcine parvovirus in sera from pigs is independent of antibody titers. Berl Munch Tierarztl Wochenschr. 2011; 124:242-6.

Szelei J, Zadori Z, Tijssen P. Porcine parvovirus. In: Kerr JR, Cotmore SF, Bloom ME, Linden RM, Parrish CR, Eds. Parvoviruses. 1. edn. London: Hodder Arnold Publication; 2006. p. 435-446.

Thacker BJ, Joo HS, Winkelman NL, Leman AD, Barnes DM. Clinical, virologic, and histopathologic observations of induced parvovirus infection in boars. Am J Vet Res. 1987; 48:763-6.

Thomson GR, Prozesky L. Porcine parvovirus infection. In: Coetzer JAW, Thomson GR, Tustin RC, Eds. Infectious Diseases of Livestock. South Africa: Oxford University Press; 1994. p. 888-94.

Tijssen P, Agbandje-McKenna M, Almendral JM, Bergoin M, Flegel TW, Hedman K, Kleinschmidt JA, Li D, Pintel D, Tattersall P. Parvoviridae. In: King MQ, Adams MJ, Carstens E, Lefkowitz EJ, Eds. Virus Taxonomy: Classification and Nomenclature of Viruses: Ninth Report of the International Committee on Taxonomy of Viruses. San Diego: Elsevier; 2011.

Tinsley TW, Longwoth JF. Parvoviruses. J Gen Virol. 1976; 20:71-6.

Truyen U, Streck AF. Porcine parvovirus. In: Zimmerman J, Karriker L, Ramirez A, Schwartz K, Stevenson G, Eds. Diseases of Swine. 10. Edn. Oxfort: John Wiley & Sons Inc; 2012. p. 447-55.

Tsao J, Chapman MS, Agbandje M, Keller W, Smith K, Wu H, Lau M, Smith TJ, Rossmann MG, Compans RW, Parrish CR. The three-dimensional structure of canine parvovirus and its functional implications. Science. 1991; 251:1456-64.

Umemura T, Tanaka Y, Kiyosawa K, Alter HJ, Shih JWK. Observation of positive selection within hypervariable regions of a newly identified DNA virus (SEN virus). FEBS Lett. 2002; 510:171-4.

van Ballegooijen WM, van Houdt R, Bruisten SM, Boot HJ, Coutinho RA, Wallinga J. Molecular sequence data of hepatitis B virus and genetic diversity after vaccination. Am J Epidemiol. 2009; 170:1455-63.

van Leengoed LA, Vos J, Gruys E, Rondhuis P, Brand A. Porcine Parvovirus infection: review and diagnosis in a sow herd with reproductive failure. Vet Q. 1983; 5:131-41.

Vendeville A, Ravallec M, Jousset FX, Devise M, Mutuel D, López-Ferber M, Fournier P, Dupressoir T, Ogliastro M. Densovirus infectious pathway requires clathrin-mediated endocytosis followed by trafficking to the nucleus. J Virol. 2009; 83:4678-89.

Vihinen-Ranta M, Yuan W, Parrish CR. Cytoplasmic trafficking of the canine parvovirus capsid and its role in infection and nuclear transport. J Virol. 2000; 74:4853-9.

Westenbrink F, Veldhuis MA, Brinkhof JMA. An enzyme-linked immunosorbent assay for detection of antibodies to porcine parvovirus. J Virol Methods. 1989; 23:169-78.

Wilhelm S, Zeeuw EJL, Selbitz HJ, Truyen U. Tissue distribution of two field isolates and two vaccine strains of porcine parvovirus in foetal organs after experimental infection of pregnant sows as determined by real-time PCR. J Vet Med B. 2005; 52:323–6.

Xiao CT, Giménez-Lirola LG, Halbur PG, Opriessnig T Increasing porcine PARV4 prevalence with pig age in the U.S. pig population. Vet Microbiol. 2012 Epub ahead of print.

Xia X, Yuen KY. Differential selection and mutation between dsDNA and ssDNA phages shape the evolution of their genomic AT percentage. BMC Genet. 2005; 6:20.

Xie Q, Chapman MS. Canine parvovirus capsid structure, analyzed at 2.9 Å resolution. J Mol Biol. 1996; 264:497-520.

Yasuhara H, Matsui O, Hirahara T, Ohgtani T, Tanaka ML, Kodama K, Nakai M, Sasaki N. Characterization of parvovirus isolated from diarrheic feces of a pig. Jpn J Vet Sci. 1989; 51:337-44.

Yoo BC, Lee DH, Park SM, Park JW, Kim CY, Lee HS, Seo JS, Park KJ, Ryu WS. A novel parvovirus isolated from *Manchurian chipmunks*. Virology. 1999; 253:250-8.

Zádori Z, Szelei J, Tijssen P.SAT: a late NS protein of porcine parvovirus. J Virol. 2005; 79(20):13129-38.

Zeeuw EJL, Leinecker N, Herwig V, Selbitz HJ, Truyen U. Study of the virulence and cross-neutralization capability of recent porcine parvovirus field isolates and vaccine viruses in experimentally infected pregnant gilts. J Gen Virol. 2007; 88:420-7.

Zimmermann P, Ritzmann M, Selbitz HJ, Heinritzi K, Truyen U. VP1 sequences of German porcine parvovirus isolates define two genetic lineages. J Gen Virol. 2006; 87:295-301.

CURRICULUM VITAE

Personal details

André Felipe Streck

Tarostrasse 8

04103 Leipzig

afstreck@hotmail.com

Place of birth: São Leopoldo, Brazil

Births date: 09.01.1983

Nationality: German and Brazilian

Marital status: Single

Education

School

1988-2000 – Colégio Sinodal, São Leopoldo, Brazil

University

2001-2006 – Veterinary Medicine study, Federal University of Rio Grande do Sul

Post-graduation

2007-2009 – Post-graduate master's degree in Scientiae Veterinariae, Federal University of Rio Grande do Sul

2010 – Admission in the post-graduate doctor's degree in Medicinae Veterinariae in the Institute of Animal Hygiene and Veterinary Public Health, Faculty of Veterinary Medicine from the University of Leipzig

Leipzig, 19.12.2012

SUPPLEMENTARY TABLE 1

SUPPLEMENTARY TABLE: Primers used in the PPV genome amplification

Primer pairs	Sequence (5´ - 3´)	Location	Target
P1-NS1	TGC TTC AGA CTG CAC TTC G TCY CTT AAT TTT ATT CTT TCA ACT GG	232-250 793-818	NS1
P2-NS1	AAA TGG TTC AGA AAA CAA TTA AAC AA GTC ATA TGC TGT TTT TGT TCT TGC	721-746 1291-1314	NS1
P3-NS1	GCT CAA ACC GGA GGA GAA AA GTT TAA CAT TCT GTC TCT TAT TGG TTG	1231-1250 1798-1824	NS1
P4-NS1	GGA AGC AAA CAA ATT GAA CCA CAC CAC CCC CTT AAA ACT ACC	1702-1722 2320-2340	NS1
PPV I	ATG CAT CAT TGG GGA AAT GT TTC GTC GTG TTC TTT TGC TG	1913-1932 2443-2462	VP1
PPV II	CAG CAA AAG AAC ACG ACG AA CCC CAT GCG TTA GCA TCT AT	2443-2462 3117-3136	VP1/2
PPV III	GGG AGG GCT TGG TTA GAA TC TCC TAC CTG AGC TGG CCT AA	2950-2969 3772-3791	VP1/2
PPV IV	TTA GGC CAG CTC AGG TAG GA TTT CTG CTG TTG TTG TGT GTT G	3772-3791 4417-4437	VP1/2
PPV V	TCA AAC TTT TGG TGG AAA GGA GGG CGA CCA ACT AAC TCA AA	4341-4361 4899-4918	VP1/2
D5	GGC CAC TAA TAT GTG CTT GGT T TTG GAG CAG GTC TTT TTC CTG G	1854-1875 2697-2718	VP1/2
D7	CCA TGG TTA CCT ACA AAA CCA ACT C ACA GGT GCA GTA TTG TTT AGT GCT GTT	3378-3401 4135-4159	VP1/2

SUPPLEMENTARY TABLE 2

SUPPLEMENTARY TABLE: Primers used in the PPV VP1 gene and in the PPV2, PPV3 genome amplification

Primer pairs	Sequence (5´ - 3´)	Location
PPV I	ATGCATCATTGGGGAAATGT	1913-1932
	TTCGTCGTGTTCTTTTGCTG	2443-2462
PPV II	CAGCAAAAGAACACGACGAA	2443-2462
	CCCCATGCGTTAGCATCTAT	3117-3136
PPV III	GGGAGGGCTTGGTTAGAATC	2950-2969
	TCCTACCTGAGCTGGCCTAA	3772-3791
PPV IV	TTAGGCCAGCTCAGGTAGGA	3772-3791
	TTTCTGCTGTTGTTGTGTGTTG	4417-4437
PPV V	TCAAACTTTTGGTGGAAAGGA	4341-4361
	GGGCGACCAACTAACTCAAA	4899-4918
PPV D5	GGCCACTAATATGTGCTTGGTT	1854-1875
	TTGGAGCAGGTCTTTTTCCTGG	2697-2718
PPV D7	CCATGGTTACCTACAAAACCAACTC	3378-3401
	ACAGGTGCAGTATTGTTTAGTGCTGTT	4135-4159
PPV2 P1	CGGACCGGAAGTCGCCGAATG	16-36
	TGATCGCCTTCCCACCAGCG	675-694
PPV2 P2	CGGCACAAAGGCCCGAGGG	555-573
	AGCGGGACCGAACAGCCAGA	1234-1253
PPV2 P2-new[a]	GCGGCTCTTGAGAGGCCCGG	1558-1577
PPV2 P3	CGCAGGGAACTCGTAGCAGGGA	1053-1074
	TGGAGCTACCGGTGCTACGGC	1725-1745
PPV2 P4	GCTCCGAGACCTCCCCAGCT	1643-1662
	CTCTCCGCCCGCCAGAGTTG	2355-2374
PPV2 P5	GGTAAGCGGCCATGAGCGCT	2249-2268
	TCTCTTCAGGTGCCGGCCGA	2925-2944
PPV2 P6	CTGGGGTACCTGGGCAGAGCTT	2787-2808
	AATGCGCACAATCCCACCCCC	3589-3609
PPV2 P6-new[a]	GGTGGTTTCTGGGATAAAATTAAGG	2467-2491
	GATGAGCTCGTCGTACCGCTCATCG	3183-3207
PPV2 P7	CCGCCCGCCCAAAAACCAAGA	3499-3519
	AAAGAACTCCGTGTCCGCGCT	4210-4230
PPV2 P8	GGTACAACCCTCCCCAATACGCCT	4118-4141
	CAGAGGACCGAGCAAAGGCCAT	4675-4696
PPV2 P8-new[a]	CCGAGAGCATCCGGGCATTGGC	3676-3697
	AAACCCCTGAGGAAATATGTCC	4443-4464
PPV2 P9	TCGCCAGGGGGAGGTAGAGAGAA	4629-4651

	GGGTCGCCGGACTCCGTGTAA	5238-5258
PPV2 P10	CGAGCGCCCCCTCTGTTACG	4941-4960
	AGGTCGGACCGGAAGTCGCC	5570-5589
PPV3 P1	GTTCCGGTTGCGTATTTCC	2-20[b]
	TGTTAGTCCAAGCATACCAGACA	627-649
PPV3 P2	TGTATCAGTGCGACCTGAC	566-584
	TGGCAGTCATTGAATGGAAA	1219-1238
PPV3 P3	TCTTATGGCTGCGTGAATTG	1186-1205
	AGACACGTCCGTGGACTCAT	1748-1767
PPV3 P4	GGCAGCCGATGAGTCCAC	1740-1757
	CACATGGGAGAAGGCTTGTT	2342-2361
PPV3 P5	CGAGCCATCATCATGGAGTA	2271-2290
	ATCGTACCGTTCATCGTGGT	2933-2952
PPV3 P6	GCTAAGGGACCAGTGGATGA	2902-2921
	ATGGAGTCCGCATACCCATA	3483-3502
PPV3 P7	GGGGCACTCATTTCTCTGAT	3359-3378
	CTGGCCTTTTCCACTTAGGA	4053-4072
PPV3 P8	GCGATCATGACAGGGGTAGA	4021-4040
	AGCCACCCAGTAGGGGATAC	4626-4645
PPV3 P9	CCTATTCCCTGCTGCTGTGT	4530-4549
	TTGTTGCATCAGCAATGAATC	5006-5026[b]

[a]Additional primers needed for some sequences
[b]Number according the PPV3 strain HK3 (EU200673).

SUPPLEMENTARY TABLE 3

SUPPLEMENTARY TABLE: Samples of heart (H) and tonsil (T) submitted to cell inoculation and their respective PCR results for PPV, PPV2, PPV3, PPV4 and PBoV.

Samples	PPV	PPV2	PPV3	PPV4	PBoV
8H	-	+	-	-	-
14H	+	+	-	-	-
26H	+	-	-	-	-
41H	-	+	-	-	-
43H	+	+	-	-	-
44H	-	+	-	-	-
68H	+	+	-	-	-
92H	-	+	-	-	-
9T	-	+	-	-	-
37T	+	+	+	-	-
40T	+	+	+	-	-
43T	-	+	-	-	-
62T	-	+	+	-	-
65T	-	-	+	-	-
86T	+	+	+	+	-
90T	-	+	+	-	-

FIGURE CAPTION

TABLE CAPTION

ACKNOWLEDGEMENT

My studies and this dissertation would not have been possible without the guidance, help and friendship of several persons who in one way or another contributed and assisted me in this important academic and personal experience at the University of Leipzig:

First, my utmost gratitude to Prof. Dr. Uwe Truyen, my supervisor in this period. Prof. Truyen accepted me as an international DAAD student without knowing me in person, and thereafter, always kindly and patiently supported me for fulfillment of the academic requirements. I learned much about microbiology, virology and parvovirology with Prof. Truyen, and for sure, have much more to learn with him.

Prof. Martin Pfeffer for the valuable insights and for his patience with the manuscripts' corrections.

Dr. Timo Homeier for the friendship and unfailing support as adviser. I will always kindly remember our discussions about soccer and microbiology evolution.

Dr. Carolin Karnath, Tessa Foerster, Dana Rüster and Monika Schneider for the friendship and encouragement to complete this study. The promises to visit me in Brazil will be seriously remembered.

Nadja Leinicker for the excellent support and for teaching me the labor basics of a parvovirologist.

All colleagues from the department and laboratory who directly or indirectly helped me to conclude this study.

My Brazilian friends and colleagues (including Dr. Danielle Gava, Luciane Pinto, Carine Kunzler and Prof. Cláudio Canal) for always encouraging and supporting me.

The University of Leipzig for accepting me as a son in its 600th year.

The Brazilian and German governments, that provided financial support for this dissertation. For sure, I will always remember and do my best to honor this investment.

Last but not least, my family, that even in distance, always gave me support and stood by my side in every moment. This dissertation is dedicated to them.