UNIVERSITÁ DEGLI STUDI DI MILANO
Facoltà di Medicina Veterinaria
Dipartimento di Scienze veterinarie e Sanità pubblica

PhD COURSE OF
VETERINARY HYGIENE AND ANIMAL PATHOLOGY
XXVIII cycle

Title

Detection and molecular analysis of Pseudorabies virus strains isolated in Italy

PhD Student
Dott. Enrica Sozzi
Matr. R10132

PhD Supervisor
Dott. Guido Grilli

PhD Coordinator
Prof. Giuseppe Sironi

Academic Year
2014-2015

List of Abbreviations

- Akaike's information criterion (AIC)
- Amino-acid (aa)
- Aujeszky's disease (AD)
- Bayesian information criterion (BIC)
- cellular herpesvirus entry mediator (HVEM)
- Central nervous system (CNS)
- cytopathic effect (CPE)
- European (EU)
- internal one sequence (IRS)
- maximum likelihood (ML)
- Monoclonal antibody (Mab)
- National Center for the Biotechnology Information (NCBI)
- polymerase chain reaction (PCR)
- Porcine kidney 15 (PK 15)
- Pseudorabies virus (PRV)
- Real time PCR (rt PCR)
- Suid herpesvirus 1 (SHV-1)
- terminal one sequence (TRS)
- unique long (UL)
- unique short (US)
- Virus neutralization (VN)

Abstract

Study n. 1: Aujeszky Disease: serological and virological surveillance in Italy during 2012-2014

Although wild boar can act as a persistent Aujeszky's disease (AD) reservoir, limited data are available on long-term epidemiology in free-ranging wild boar living in areas where industrial swine herds are limited. Hence, this study provides crucial information, which fills this knowledge gap, on the natural dynamics of AD infection. From 3260 sera sampled during eight hunting seasons, 162 (4.97%) were tested positive. Factors, including the animal's age class, and the sampling year, had significant effects on the probability of the wild boar being seropositive, while wild boar mean abundance per area, yearly abundance and the total number of pig farms, as well as interactions among age, year and sex, were not significant. In particular, a positive trend of seroprevalence was observed over the years, with values ranging from 2.1 to 10.8%. This long-term surveillance showed an increase in seroprevalence with a higher probability of being seropositive in older individuals and the independence of wild boar seropositivity from the likelihood of contact with pigs in the area.

Study n. 2: Genomic characterization of pseudorabies virus strains isolated from swine in Italy

In this study, we undertook the genomic characterization of 44 pseudorabies virus (PRV) strains originated on pig farms isolated in Italy during 1984–2010. The characterization was based on partial sequencing of the UL44 (gC) and US8 (gE) genes. Thirty six porcine PRV strains, which were closely related to those isolated in Europe and America in the last 20 years belong to cluster B in both phylogenetic trees. Six porcine strains that do not belong to cluster B are related in both gE and gC phylogenetic trees to the 'old' porcine PRV strains isolated in the 1970s and 1980s. In the last two decades, the presence of these strains in domestic pig populations has been reduced drastically, whereas they are prevalent in wild boar. The two remaining strains have an interesting

genomic profile, characterized by the gC gene being closely related to the old porcine PRV strains, and the gE gene being similar to that of recently isolated strains. These results provide interesting insight into the genomic characterization of PRV strains and reveal a clear differentiation between the strains isolated from wild boar and those originating from domestic pigs.

Study n. 3: Detection and molecular analysis of pseudorabies virus strains isolated from dogs and wild boar in Italy

Aujeszky's disease (AD) is one of the most economically important diseases of farmed pigs. Wild boars can act as reservoirs and might represent a potential threat for domestic animals, including dogs. The aim of this study was to report the results of an AD survey based on the Pseudorabies virus (PRV) genome detection in samples of dogs clinically suspected of AD and of wild boars collected during four consecutive hunting seasons in the period 2010–2014. Genomic characterization was based on the partial gC sequence of the Italian strains and the comparison with those from domestic pigs and European PRV strains circulating in wild boars. The Italian PRV strains were mainly distributed into three different clusters and revealed two interesting findings. First, there was a clear distinction between the viral strains that were isolated from dogs used for hunting and subsequently traced back to wild boars and the strains that were isolated from working dogs and subsequently found to be closely related to domestic pigs. Second, the Italian epidemiological situation was found to be different from those of European countries in that the Italian situation was characterized by the presence of both the typical Italian clades 1 and 2 and supported by new patterns of aa deletions/insertions. Italian clade 1 included strains from hunting dogs and two Italian wild boars, and Italian clade 2 grouped with recent strains from dogs that were unable to hunt and domestic pigs that were related to one old reference strain (S66) and not

included elsewhere. Molecular and phylogenetic analyses of PRV strains are therefore necessary to improve the understanding of the distribution of the PRV clusters and their evolution.

The results of the present Thesis have been published or submitted for publication in international scientific peer-reviewed journal:

- Sozzi, E., Moreno, A., Lelli, D., Cinotti, S., Alborali, L., Nigrelli, A., Luppi, A., Bresaola, M., Catella, A., Cordioli, P. 2014. Genomic characterization of pseudorabies virus strains isolated from Italy. Transboundary Emerging Dis. 61(4), 334-340

- Moreno A., E. Sozzi, D. Lelli, M. Chiari, P. Prati, G. L. Alborali, M. B. Boniotti, P. Cordioli, A. Lavazza (2014). "Genomic characterization of Pseudorabies virus strains isolated from wild boar and dogs in Italy", 8th Annual EPIZONE meeting 23-25 September 2014, Copenhagen, Denmark. Manuscript in preparation.

- Moreno A., E. Sozzi, G. Grilli, L. R. Gibelli, D. Gelmetti, D. Lelli, M. Chiari, P. Prati, G. L. Alborali, M. B. Boniotti, A. Lavazza, P. Cordioli. "Detection and molecular analysis of Pseudorabies virus strains isolated from dogs and a wild boar in Italy", Vet Microbiol. 2015 Jun 12;177(3-4):359-65

Index of contents

General introduction	9
Historical Background	10
Aetiology	12
Classification and nomenclature	12
Virus-cell interaction	16
Virus-host interaction	20
In the absence of specific immunity	20
Role of envelope glycoprotein gC	21
Role of envelope glycoprotein gD	21
Role of envelope glycoprotein gE	21
Role of envelope glycoprotein gI	22
Role of other viral proteins	23
In the presence of specific immunity	23
Immunogenicity of PRV glycoproteins	25
Virus-respiratory tract interactions	26
Vaccination and control	30
Epidemiology	**31**
Clinical signs and pathogenesis	**32**
Acute PRV infection of swine	**32**
PRV latency in swine	**34**
Lethality of pseudorabies infection in non-native hosts	**35**
Lesions	**35**

Diagnosis **36**

Identification of the agent 37

Virus isolation 37

Identification of virus by the polymerase chain reaction 38

Serological tests 40

Hypothesis and objectives **42**

Study n. 1: Aujeszky Disease: serological and virological surveillance in Italy during 2012-2014

Introduction **46**

Materials and methods **47**

Study area and Sampling 47

Laboratory analysis 47

Results **48**

Discussion **52**

Study n. 2: Genomic characterization of pseudorabies virus strains isolated from swine in Italy

Introduction 54

Materials and methods **54**

Results **55**

Phylogenetic analysis based on gE 55

Phylogenetic analysis based on gC 56
Discussion **57**
Figures and tables **60**

Study n. 3: Detection and molecular analysis of pseudorabies virus strains isolated from dogs and wild boar in Italy

Introduction **66**
Materials and methods **67**
Animals 67
Laboratory investigations 67
Phylogenetic and molecular analysis 68
Results **69**
Laboratory investigations 69
Phylogenetic and molecular analysis 69
Discussion **71**
Figures and tables **74**

General conclusions **79**
References **85**

General Introduction

Historical Background

Aujeszky’s disease (AD) or pseudorabies is a disease with a long history. In the nineteenth century, the disease was linked with central nervous disorders in cattle, dogs and cats, characterized by itching, rubbing, exhaustion and paralysis. At that time, pigs did not come into the picture. In the USA, the oldest descriptions of a disease that closely resembles Aujeszky’s disease was called “mad-itch” in cattle and was referred to in the agricultural magazines “Cultivator” (1839) and “New England Farmer” (1844) (Hanson, 1954). Similar reports were made later in Europe. In 1889, Strebel (1889) published his findings on four cows with itching in Switzerland (“Juckkrankheit”) and mentioned that he had seen five similar cases in the past in his region. The Hungarian veterinary surgeon Aladar Aujeszky was the first to demonstrate the infectious origin of the disease and to forward the idea that the disease was distinct from rabies (Aujeszky , 1902).

Figure n. 1: Aujeszky Aladar

He could induce nervous symptoms and death in rabbits within a period of 48 h with tissue suspensions from an ox with the following clinical picture: excitation and nasal pruritus, followed by convulsions and death within half a day. He was successful in repeating his experiment with

brain material from a cat and a dog which died quickly after showing similar symptoms. Aujeszky was convinced that he dealt with a virus that was different from rabies virus based on some specific observations, such as short time of incubation, quick course of disease and infectivity of blood. Afterwards, Schmiedhofer (1910) , Shope (1931 and 1934) and Elford and Galloway (1936) brought the necessary proofs to demonstrate that a virus with a size between 100–150 nm was the cause of the disease based on ultrafiltration experiments. Reagan *et al*. were the first to visualize the virus (1952). Based on immunological studies, virus morphology, intranuclear inclusions and ether sensitivity, Sabin (1934) and Kaplan and Vatter (1959) classified Aujeszky's disease virus (ADV)/pseudorabies virus (PRV) in the herpesvirus group. PRV was linked with sporadic problems in pigs world-wide and pigs were identified as a reservoir between 1920 and 1940 (Kohler and Kohler , 2003). During the intensification of pig breeding in the nineteen-fifties, nineteen-sixties and nineteen-seventies, increasing incidences of severe clinical outbreaks were reported in swine. Several pig specialists are convinced that an increase in virulence occurred during this period, however a cell biological and molecular explanation is lacking.

Aetiology

Classification and nomenclature

PrV belongs to the family of the *Herpesviridae*, subfamily *Alphaherpesvirinae,* genus *Varicellovirus*. The general structure of a pseudorabies virion is given in Figure n.2.

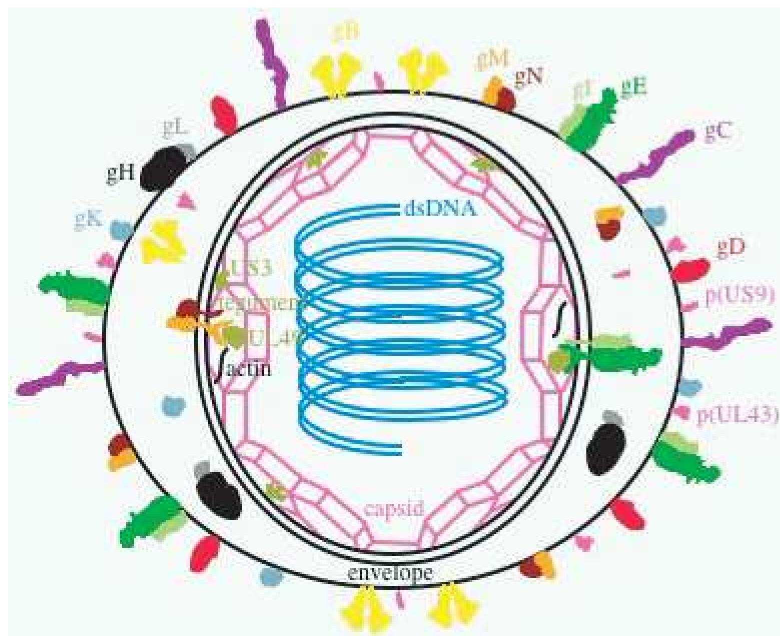


Figure n. 2: Structure of a pseudorabies virion

The virion consists of a double stranded DNA genome of approximately 150 kbp, surrounded by a capsid, tegument and envelope. The genome of PRV belongs to the class D genomes of the herpesviruses. It consists of two unique regions, a long one (unique long, UL) and a short one (unique short, US) flanked by two repeat sequences, an internal one (IRS) and a terminal one (TRS) [4]. The whole genome has been sequenced and published by Klupp *et al.* (2004). The different genes within the genome of the alphaherpesviruses are designated by a two letter code UL or US depending on its position in the unique long (UL) or unique short (US), followed by a number. The number gives the place of the gene within each specific region. The capsid of alphaherpesviruses encloses and protects the large genome. It consists of 162 capsomers, 150 hexons (one hexon

consists of 6 molecules VP5 (protein expressed by UL19 (pUL19)) and 6 molecules VP26 (p(UL35)) and 12 pentons (11 pentons consist of 5 molecules of VP5 (p(UL19)); 1 penton consists of 12 molecules of p(UL6) and forms the cylindric entry pore for newly produced dsDNA), both linked by triplexes (one molecule of VP19C (p(UL38)) and two molecules of VP23 (p(UL18)), all nicely arranged in an icosahedral lattice (Newcomb *et al.*, 1999) (Figure n.3).

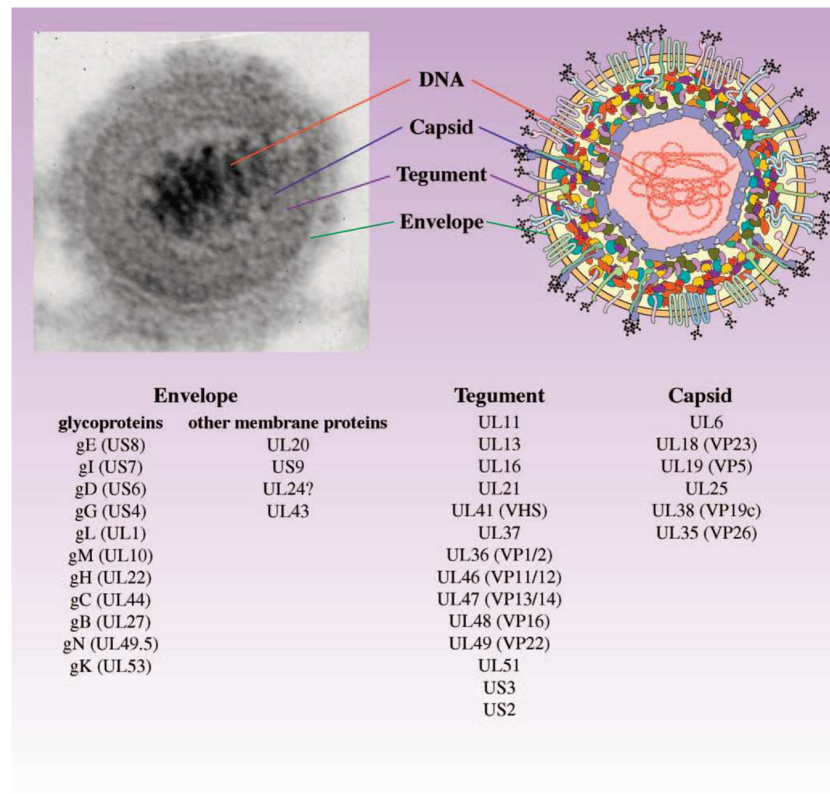


Figure n. 3: Structure of the PRV virion. PRV virions are composed of four structural elements. The double-stranded DNA genome is housed in an icosahedral capsid. The tegument is a collection of approximately 12 proteins organized into at least two layers, one which interacts with envelope proteins and one that is closely associated with the capsid. The envelope is a lipid bilayer infused with transmembrane proteins, many of which are modified by glycosylation. Listed are proteins thought to be components of the virion; however, not all proteins are represented in the cartoon.

The space between the capsid and the envelope is filled with tegument proteins, which comprise besides viral proteins cellular actin. Tegument proteins are important during entry, priming the cell for virus replication, primary envelopment at the inner nuclear membrane and secondary envelopment at *trans*-Golgi vesicles (Mettenleiter., 2006). The envelope is a bilayered phospholipid membrane which is pinched off from the cell membrane during assembly at *trans*-Golgi vesicles. It contains 10 glycoproteins (gB, gC, gD, gE, gH, gI, gK, gL, gM, gN) with gB forming homodimers, gE/gI, gH/gL and gM/gN forming hetero oligomers (Mettenleiter., 2000) and at least 2 nonglycosylated proteins (p(UL43), p(US9)) (Brideau *et al*., 1998; Klupp *et al*., 2005). The envelope proteins play important roles in binding, internalization, envelopment, egress, cell-associated spread, induction of protective immunity and immune evasion. Different domains in both the extra- and intra-envelope regions are important for these functions (Figure n.4).

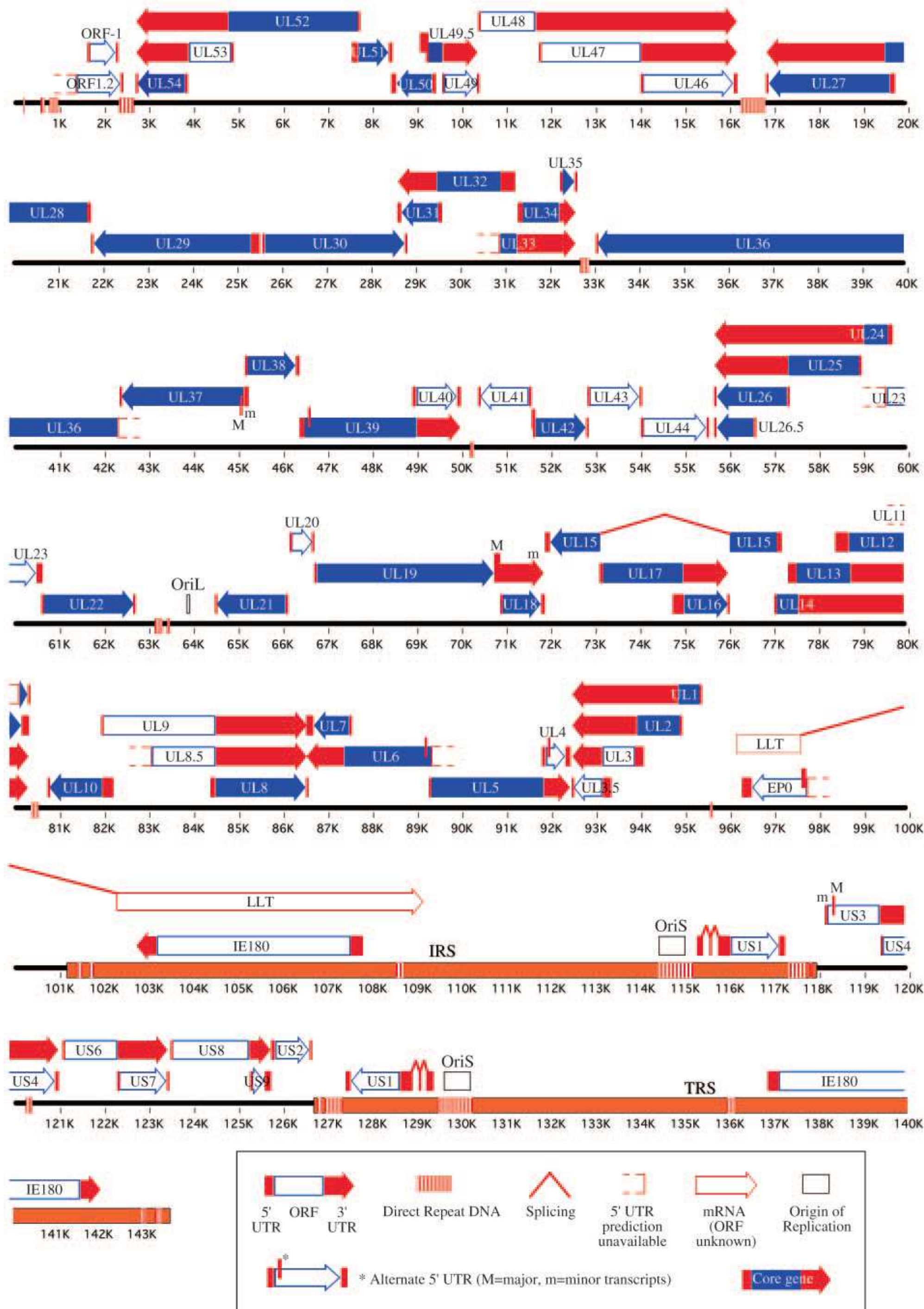


Figure n. 4: Linear map of the PRV genome: predicted gene and transcript organization. The PRV genome consists of a long and a short unique segment, named UL and US, respectively. The US region is flanked by the inverted repeats IRS and TRS. The predicted locations of core and accessory genes, transcripts, DNA repeats, splice sites, and the origin of replication are indicated.

Virus-cell interactions

An overview of the PRV replication is given in Figure n.5.

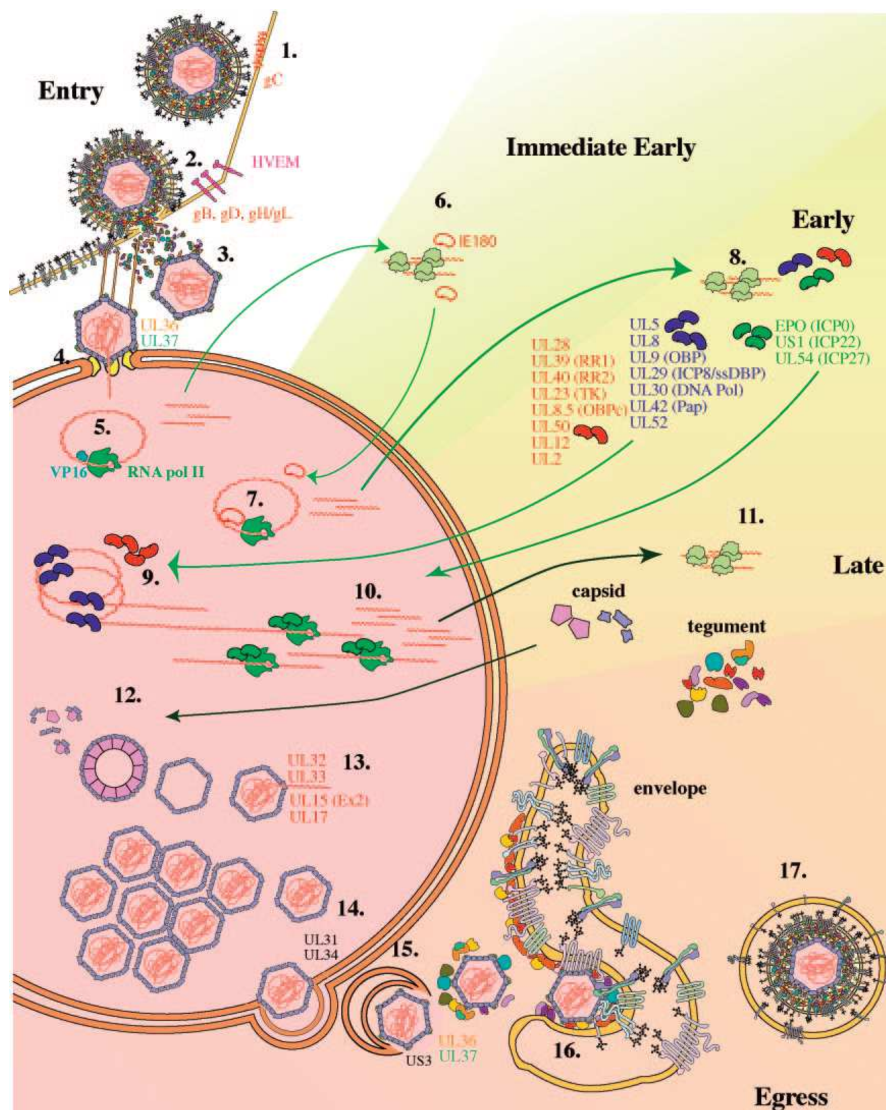


Figure n. 5: Replication cycle of PRV. 1. Entry begins with attachment or binding of the virus particle to the cell surface. In PRV, this initial binding step is an interaction between gC in the virion envelope and heparan sulfate on the surface of the cell. 2. The next steps of entry require gD, gB, gH, and gL. In PRV, although gD is not essential for membrane fusion or cell-cell spread, gD interacts with the cellular herpesvirus entry mediator (HVEM) and is required for entry of extracellular virus (penetration). 3. After fusion of the virion envelope with the cell membrane, the

capsid and tegument proteins are released into the cell. The viral tegument proteins begin takeover of the host cell protein synthesis machinery immediately after entering the cell. 4. The capsid and tightly bound inner tegument proteins are transported along microtubules to the cell nucleus. 5. The VP16 tegument protein localizes to the nucleus independent of the capsid and transactivates cellular RNA polymerase II transcription of the only immediate-early protein of PRV, the HSV ICP4 homolog IE180. 6. IE180 protein expressed in the cytoplasm is transported back to the nucleus. 7. There, it transactivates RNA polymerase II transcription of the early genes. 8. Early proteins fall into two main categories. The first category comprises 15 proteins involved in viral DNA synthesis. 9. Seven of these proteins (UL5, UL8, UL9/OBP, UL29/ssDNABP, UL30/DNA Pol, UL42/Pap, and UL52) (shown in blue) are essential for replication of the viral DNA. DNA replication occurs by a rolling-circle mechanism. 10. The second category comprises three proteins thought to act as transactivators of transcription (EP0, US1, and UL54). 11. Onset of DNA synthesis signals the start of the late stage of the PRV replication cycle and synthesis of true late proteins. 12. The capsid proteins are transported to the nucleus, where they assemble around a scaffold composed of the product of the UL26 and UL26.5 genes. 13. The mature capsid is composed of five proteins (UL19/VP5, UL18/VP23, UL25, UL38, and UL35). The product of the UL6 gene acts as a portal for insertion of the genomic DNA into the capsid. UL32, UL33, UL15 (Ex2), and UL17 are all involved in cleavage and packaging of the viral DNA. 14. During primary envelopment, the fully assembled nucleocapsid buds out of the nucleus, temporarily entering the perinuclear space. This process involves the products of the UL31 and UL34 genes along with the US3 kinase. 15 and 16. The nucleocapsid (15) loses its primary envelope and (16) gains its final envelope by associating with tegument and envelope proteins and budding into the trans-Golgi apparatus. 17. The mature virus is brought to the cell surface within a sorting compartment/vesicle derived from the envelopment compartment.

PRV has developed an ingenious complex system to enter a host cell. It consists of a cascade of interactions between viral and cellular components. The attachment is initiated by an unstable binding mediated by the viral envelope glycoprotein gC and cellular heparin sulfate proteoglycans exposed at the plasma membrane (Mettenleiter *et al*., 1990). Next, PRV is firmly bound to the cell by glycoprotein gD which is interacting with at least one of three cellular receptors: nectin 1, nectin 2 and CD55 (Mettenleiter., 2002; Spear *et al*., 2000). The presence of different receptors for gD may explain the extreme pantropic character of PRV and the possibility to infect non-porcine mammals. Binding is followed by fusion coordinated by gB, gD and gH/gL which all probably find their own not yet identified cellular counterparts (Klupp *et al*., 1997; Rauh *et al.,* 1991). Next, capsids of alphaherpesviruses are transported to the nucleus via microtubules (Smith *et al*., 2002). Upon arrival in the nucleus, the PRV genome is transcribed in a cascade-like fashion (Ben-Porat *et al*., 1985). First, immediate-early (IE) genes are transcribed during the first 2 h after nucleus entry. For this transcription the host nuclear machinery is used. IE180 is the only PRV IE protein. It is activating not only promoters of PRV genes (US4(gG), UL12 (deoxyribonuclease), UL22 (gH), UL23 (thymidine kinase) and UL41 (viral host shut off)) but also promoters of cellular genes and genes of other viruses (cross-activation) (Chang *et al*., 2004; Ou *et al.,* 2002; Taharaguchi *et al*., 1994; Wong *et al*., 1997; Wu *et al*., 1988). Early (E) genes are the next group of genes that are active. Like IE180, several of them (EP0, UL54 and UL48) are regulating the expression of genes of PRV and the cell (Fuchs *et al*., 2002a; Ono *et al*., 1998; Schwartz *et al*., 2006). Other E genes are producing proteins which are important for nucleotide synthesis (UL23, UL39/UL40, UL50) and DNA replication (UL5, UL8, UL9, UL29, UL30, UL42, UL52). The UL23-encoded thymidine kinase is phosphorylating deoxythymidine into deoxythymidine-triphosphate, one of the four building stones of DNA. This non-essential enzyme in cell cultures is important for the replication of PRV in differentiated cells such as neurons in vivo (Kit *et al*., 1987). UL39 and UL40 products are forming a

viral ribonucleotide reductase, which reduces ribonucleotides into deoxyribonucleotides (Kaliman *et al*., 1994) . The absence of this enzyme strongly attenuates the virus for replication in pigs (De Wind *et al*., 1993). The UL50 encodes a dUTPase which is cleaving dUTP into dUMP and pyrophosphate. dUMP may be enzymatically changed into dTMP and subsequently into dTTP. Deletion of UL50 results in a reduced virus replication in pigs (Jons *et al*., 1997). In analogy with herpes simplex virus, the proteins encoded by UL5, UL8 and UL52 are predicted to form a heterotrimeric primase-helicase complex, which together with p (UL9), is believed to recognize the site of initiation of DNA synthesis and unwinds the supercoiled DNA (Lehman *et al*., 1999). Products of UL30 and UL42 form the DNA-dependent DNA polymerase which uses the rolling-circle mechanism to produce a long head-to-tail concatemeric DNA strand (Berthomme et al., 1995). The precise mechanism is still not completely understood, and still controversially discussed. Finally late (L) genes become expressed. They mainly encode structural proteins, such as capsid and tegument proteins and envelope (glyco)proteins. All capsid proteins enter the nucleus for the formation of the capsids. Scaffolding proteins encoded by UL26 and UL26.5 aid in the construction of the capsids. The concatemeric DNA is cleaved into monomeric forms and simultaneously pulled into newly formed capsids through the cylindric entry pore encoded by UL6 (special penton of the capsid). The nucleocapsid is then ready for the primary envelopment at the inner nuclear membrane and subsequent de envelopment at the outer nuclear membrane. At least three viral proteins encoded by US3, UL34 and UL31 have been put forward to be crucial players in this process (Mettenleiter ., 2006). The following steps are proposed: p(UL31) is a tegument protein which takes care of the first positioning of the nucleocapsids at the inner membrane where p(UL34), a transmembrane protein, is anchored. By budding, primary enveloped particles are entering the lumen and by fusion the nucleocapsids are released in the cytoplasm (Fuchs *et al*., 2002b). Next, the nucleocapsids are transported to the *trans*-Golgi vesicles, the site

for the secondary envelopment process. The cytoskeletal structures and motor proteins necessary for the transport to this site are not characterized yet. It is very well possible that tegument proteins encoded by US3, UL36 and UL37 which are found on the outside of cytoplasmic nucleocapsids, are involved in this migration with p(UL36) physically interacting with p(UL37) (Fuchs *et al*., 2004; Klupp *et al*., 2002). The envelope glycoproteins are anchored in the membranes of TGN vesicles, presenting their cytoplasmic tails in the cytosol. On these tails, tegument proteins are assembled. P(UL11) and p(UL49) have already been localized at this site. The latter tegument protein interacts with the tails of gE and gM (Fuchs *et al*., 2002c). Finally, the virus is released by exocytosis.

Virus-host interactions

In the absence of specific immunity

The virulence of PRV is determined by its capacity to replicate and to invade in the pig. The primary site of replication is situated in the nasal cavity, tonsils, pharynx and lungs (Sabo *et al*., 1968; Sabo *et al*., 1969; Wittmann *et al*., 1980; Miry and Pensaert, 1989; Pol, 1990; Kritas *et al*., 1994a; Kritas *et al*., 1994b). The extent of replication at the different localisations depends on the amount of virus in the inoculum and on the route of inoculation. After replication in the epithelium the virus spreads to the underlying connective tissue in a very short time. At 24 h PI, viral antigens are already found in the nasal cavity in groups of epithelial cells, fibrocytes and nerve cells. At 48 h PI, viral antigens are detected in larger plaques which are extended over epithelium and underlying connective tissue. including nerves and endothelial walls. These plaques of local virus replication undergo extensive necrosis. A similar ‘plaque-wise’ spread is also found in the tonsils and lungs. Virus can spread from the primary sites to distant secondary replication sites by lymph, blood and nerves. Both cell-free and cell-associated viremia are possible

(Nauwynck and Pensaert, 1995a). Virus replication at secondary sites such as draining lymph nodes, olfactory bulb. medulla, spleen, kidneys, ovaries and uterus starts at 48 h PI. It is less extensive than at the primary sites and is clearly reduced with the onset of the immune response starting from 7 days PI. Despite the presence of a humoral and cellular immunity, virus can be isolated from tonsils up to 18 days PI (Sabo *et al*., 1969) and from nasal swabs up to 13 days PI (Pensaert *et al*., 1990; Vannier *et al*., 1990). The role which different viral proteins play in this pathogenesis picture has been studied by different research groups by means of deletion mutants.

Role of envelope glycoprotein gC

Somewhat different data exist on the effect of the deletion of gC on virus replication and virulence in pigs. In neonatal pigs no effect was found while in 3-week old pigs virulence was reduced (Mettenleiter *et al*., 1989; Kritas *et al*., 1994a; Kritas *et al*., 1994b). In what way age influences the virulence is not clear at present.

Role of envelope glycoprotein gD

By the use of a phenotypically complemented gD mutant the role of gD in neuro-invasion of PRV became clear (Mulder *et al*., 1995). The loss of gD did not inhibit but only slowed down viral spread over the neuronal pathways up to the central nervous system. This proved that the trans-synaptic spread of PRV in pigs may occur in a direct cell-associated way.

Role of enuelope glycoprotein gE

The viral glycoprotein gE forms a non-covalently linked complex with g1, which becomes expressed in the cell membrane of the infected cell and is present in the viral envelope. The absence of the entire gE glycoprotein or deletion of two amino acids (val-125 and cys-126) results in a reduction of virulence (Jacobs *et al*., 1993; Kritas *et al*., 1994a; Kritas *et al*., 1994b). After an

intranasal inoculation central nervous disorders, respiratory problems, anorexia and fever are absent and pigs do not succumb. This reduction in virulence reflects well the impaired capacity to invade, which can be found at different neuronal levels. Although an infection with the gE^- mutant gives a similar number of foci of infection in the epithelium of the nasal mucosa early after infection (48 h PI) in comparison with its parental strain. the plaque-wise spread into the underlying connective tissue was clearly affected. The reason for this can be found in the less efficient release of the virus and the modulating role that gE plays in the direct cell-to-cell spread. The Ka gE^- mutant also demonstrates a reduced invasion and spread into the central nervous system. This has been demonstrated for both the olfactory and trigeminal pathway. A much lower number of infected neurons was detected on the first neuronal level and almost no infection was found in more distant levels in the central nervous system. The lower capacity to invade into the nervous tissues is probably an accumulation of different effects such as (i) the smaller plaques formed in the nasal mucosa causing a lower number of neuronal extensions which are reached and a lower number of neurons which become infected, (ii) the deficient virus release, (iii) the affected cell-to-cell spread at the site of the synapses and (iv) an impaired anterograde transport, as demonstrated in the trigeminal pathway (Kritas *et al*., 1995). Furthermore, it has been shown that virus titers in internal organs such as liver, kidneys, adrenal glands and spleen of gE-negative PRV infected pigs are much lower than those obtained with the parental strain. In conclusion, we can state that gE forms an important invasion and virulence determinant.

Role qf envelope glycoprotein gI

PRV without gI is able to replicate in the nasal mucosa to the same extent as the parental strain does (Kritas *et al*., 1994a,b). Although this mutant can invade in the central nervous system along neurons, the total number of infected neurons is clearly reduced in both the trigeminal and

olfactory pathways. Parallel with its replication pattern, the g1 mutant has an intermediate position concerning its virulence in pigs between the parental strain and the gE mutant. This information indicates that although gE/gI forms a complex, this conformation is not essential for all functions. The viral glycoprotein gE is able to perform some of the functions in the absence of g1.

Role of other viral proteins

Deletion of the gene coding for gG or 28 K, two proteins of which the function has not been identified so far, does not affect the virulence indicating the minor roles these proteins play in the replication in pigs (Kimman *et al*., 1992). The US3 mutant which does not express the protein kinase PK (US31 demonstrated a reduced virulence in pigs (Kimman, 1994a; Kimman, 1994b). PK (US3) has been shown to have an in vitro phosphorylation capacity of a major virion phosphoprotein of 112 kDa (Zhang *et al*., 1990). Although the function of the formed phosphoprotein is unknown, it is necessary for an efficient replication in vitro. The reduced capacity to replicate may explain its reduced virulence. No data are available at present, on the effect of a deletion of the gene encoding another protein kinase PK (UL13). The role of two viral enzymes involved in nucleic acid metabolism, thymidine kinase (TK) and ribonucleotide reductase (RR) has already been clarified. Ribonucleotide reductase reduces the ribonucleotidediphosphates to desoxy-products which can be used for DNA synthesis. Thymidine kinase is necessary for the phosphorylation of deoxythymidine to its monophosphate product. As both enzymes are present in dividing cells but not in non-dividing cells such as neurons it is easy to understand that TK and RR mutants have a reduced virulence to pigs (Kit *et al*., 1985: de Wind *et al*., 1993).

In the presence of specific immunity

The degree with which replication of PRV may be restricted in pigs depends on the immunisation approach. Although a total inhibition is found in the nasal cavity and pharynx in pigs which are reinfected 1 and 2 months after the primary infection, viral antigens can already be detected in a small number of free alveolar cells and alveoli in the lungs (Miry and Pensaert, 1989). From these findings it was hypothesized that alveolar macrophages first become infected which transmit the virus afterwards to alveolar epithelium and underlying connective tissue via a direct cell-to-cell contact. In pigs immune after vaccination the protection is less efficient as viral antigens were not only present in the deeper airways but also in the pharynx, tonsils and nasal cavity (Miry and Pensaert, 1989; Wittmann *et al*., 1980). The viral antigen positive cells were located in clusters mainly in the epithelium. Despite the presence of a vaccination immunity a cell-associated viremia can still be found (Wittmann *et al*., 1980). This allows PRV to reach inner organs. When an infected leucocyte sticks in the microcirculation, PRV may spread to the contacting cells in a plaque-wise manner. The formation of a single plaque will not result in signs except in the uterine circulation. Once PRV reaches the fetal tissues the infection will spread over the uterus contents till abortion occurs (Nauwynck and Pensaert, 1992). In recent experiments of the author, the role of glycoproteins which become expressed outside the carrier blood mononuclear cell in adhesion to endothelial cells and further spread through the maternal placenta has been elucidated. The adhesion of the infected blood mononuclear cells to the capillary wall was mediated by a physiological process and not by the expressed viral glycoproteins. The latter seems logical as antibodies directed against them completely inhibit cell adhesion (Hanssens *et al*.. 1993). After adhesion PRV is able to spread through the maternal placenta which consists of endothelial cells, fibrocytes and uterine epithelial cells in a direct cell-associated way as shown by the use of a gD^- deletion mutant. After injection of gD^- PRV infected mononuclear cells in the uterine artery gD^- PRV spreads through the maternal placenta in order to infect fetal tissue. gD^- PRV was

demonstrated in the fetuses 2 weeks after inoculation. From the different pathogenetic studies it can be concluded that the cell-associated spread of PRV enables the virus to evade immunity in vaccinated pigs.

Immunogenicity of PRV glycoproteins

In the course of the present thesis, it became clear that the different envelope glycoproteins are responsible for important interactions of PRV with host cells. Thus, it is not surprising that the immunity induced against these specific viral proteins is very effective in the protection of the pig, not only clinically but also virologically. The specific immunity against the envelope glycoproteins consists of two components: antibodies and cytotoxic T-lymphocytes. Antibodies can act by themselves (direct way) or mediate some antiviral activities with the help of complement and leucocytes (indirect way). Direct effects are obtained by blocking the glycoprotein site which is active during virus entry, cell adhesion and cell fusion. So has been found that some monoclonal and polyclonal antibodies directed against gB, gC and gD neutralize PRV in the absence of complement (Wathen *et al*., 1985; Ben-Porat *et al*., 1986; Eloit *et al*., 1990; Marchioli *et al*., 1988; Coe and Mengeling, 1990) that monospecific antisera against gB and gC inhibit the adhesion of infected cells in suspension (Hanssens *et al*., 1993) and that monoclonal antibodies against gB, gD and gE reduce the plaque size in infected monolayers (Nauwynck and Pensaert, 1995b). Indirect effects are possible not only with the previously mentioned antibodies but also with antibodies bound to functionally inactive regions of the envelope glycoproteins. The bound antibodies may activate complement, causing damage of the membrane in which the envelope glycoproteins are anchored. This may lead to cell death and virus inactivation. This is the reason why non-neutralizing monoclonal antibodies against gB, gC, gD and gE may cause inactivation of the virus in the presence of complement (Wathen *et al*., 1985; Fuchs *et al.,* 1990; Nakamura *et al*., 1990).

Antibodies attached to the expressed viral glycoproteins may also cause cell death after interaction with Fc receptors on phagocytes (ADCC by monocyte/macrophages and neutrophils). Porcine polyclonal antisera against gC show a high ADCC-activity in the presence of polymorphonuclear cells, whereas antisera against gB and gD had minimal ADCC-activities (Iglesias *et al*., 1990). Although antibodies against other viral proteins such as gG, the IEP 180 and nucleocapsid proteins do exist, they are immunologically of no importance. The reason for this is that these proteins are active only inside the infected cell where they cannot be reached by antibodies. Cytotoxic T-lymphocytes directed against gC in combination with MHCI are effective in eliminating infected cells (Zuckerman *et al*., 1990).

Virus-respiratory tract interactions

The general pathogenesis picture of PRV infections in pigs can be summarized as follows. The virus primarily replicates in the respiratory tract, especially the upper respiratory tract, spreads along cranial nerves to the brains and via lymph and blood to internal organs, with the reproductive organs being important targets. Replication in the respiratory tract, central nervous system and reproductive organs is responsible for pathological changes causing respiratory, nervous and reproductive disorders. Clear changes in virus-host interactions have been reported over time which point to differences in virulence of the virus. The original reports of Aujeskzy's disease were only describing nervous disorders, mainly in cattle and dogs and rarely in pigs. The involvement of the respiratory tract in the pathogenesis was not at all clear at that moment. Shope (1934) was the first one to demonstrate that PRV was present in nasal secretions of pigs for several days after intranasal infection and Mc Ferran and Dow (1964) showed that PRV was shed from 1 to 9 days after inoculation in the nasal secretions and up to 17 days intermittently. Researchers of the Veterinary Research Laboratories (Stormont, Belfast, Northern Ireland) discovered clear

pathogenetic changes between strains which were isolated in their region over time. The Northern Ireland PRV strain 1 (NIA-1) originally isolated in the nineteen-sixties was found to replicate in the nasal and pharyngeal mucosa starting from 24 h post inoculation. During the replication of at least one week, no respiratory disease and gross pathological lesions were found in the respiratory tract. After 48 h, virus was detected in the central nervous system where it was spreading over time. The dissemination via lymph and blood and replication in internal organs was very restricted. The pathogenesis of infections with NIA-2, which was isolated one decade later, behaved somewhat similar. The more pronounced respiratory lesions (rhinitis and pneumonia) were attributed to the way of inoculation (Baskerville, 1971). The virus was administered via aerosol, which allows the virus to reach deeper parts of the respiratory tract than when the virus is instilled intranasally. NIA-3 was isolated in the early nineteen-seventies during a severe outbreak, with 100% mortality in piglets during their first weeks of age. Several sows aborted. The extremely high mortality in piglets and abortions were new findings at that time. This strain was highly virulent for the respiratory tract. As soon as 30 h after intranasal inoculation, pyrexia was measured and 48 h post inoculation, severe depression, tremors of the extremities and sneezing with a mucopurulent nasal discharge were noticed (Pol *et al*., 1989). Pathologically, the nasal and pharyngeal mucosae were hyperemic at 24 h post inoculation and necrotic starting from 48 h post inoculation. This extremely aggressive form of Aujeszky's disease was not reported before. Virologically, the virus had spread within 24 h through the basement membrane and was already replicating in several fibrocytes in the lamina propria. At 48 h post inoculation, the epithelial cells were sloughed away and large parts of the underlying connective tissue were necrotic. In the nineteen-sixties-seventies, an increase in the invasive character of PRV at the level of the respiratory tract allowing the virus to find access to more nerves and to increase the viremia and to replicate to high levels in internal organs, including reproductive organs, was also reported in other countries. In Belgium,

a virulence switch has been recognized in the beginning of the nineteen-seventies (Pensaert *et al*., 1987). A strain isolated in 1971 (NS374) from a few piglets with central nervous disorders but without a high mortality, respiratory problems and reproductive problems on the farm of origin, was inoculated in piglets of 7 weeks of age. A restricted replication was found in the respiratory tract. A viremia was not detected and in the central nervous system the virus could only be demonstrated in the brainstem. Besides fever, other clinical signs were not observed. In contrast, the 75V19 strain, which was isolated during a severe PRV outbreak with respiratory, nervous and reproductive problems in 1975, was replicating to higher titers in the respiratory tract compared to the NS374, gave a clear viremia and was replicating in different regions of the central nervous system. Similar observations of increased virulence were done in other West European countries (Akkermans *et al*., 1980; Pittler, 1982; Toma *et al*., 1985). The basis for the increased virulence is not known. Interesting are the observations made by Bitsch (1980) in Denmark. A link was made between the characteristics in vitro and the behavior in the field. The more invasive strains demonstrated a better cell-associated spread (larger syncytia). A similar finding was done with Belgian strains (Nauwynck, 1993). Over time, the cell-associated spread of PRV isolates seems to have improved. In one way or another there seems to be an advantage in evolution for PRV to spread in a cell-associated way. The replication kinetics and characteristics of PRV in the respiratory tract are influenced by different factors, such as virus strain as illustrated above, inoculation route, virus titer in the inoculum, animal age and genetics and immune status. In naïve pigs, PRV replicates first in the respiratory tract, mainly in the upper part, consisting of the nasal cavity, tonsils and the oropharynx upon intranasal/peroral inoculation. It infects primarily the epithelial cells and within 24 h it crosses the basement membrane in order to infect all cell types in the underlying tissues in a plaque-wise fashion (fibrocytes, endothelial cells, mononuclear cells) (Kritas *et al*., 1994a; Kritas *et al*., 1994b; Miry and Pensaert, 1989; Sabo *et al*., 1968; Sabo *et al*.,

1969; Wittmann *et al*., 1980). How the virus penetrates easily through the mucus layer barrier on top of the epithelial cells and crosses the basement membrane barrier is not known and deserves more in depth studies. Replication in lower parts of the respiratory tract is restricted, except when virus is directly deposited in these locations, such as by intratracheal or aerosol inoculation (Baskerville, 1971; Miry and Pensaert, 1989). In the lungs, the virus replicates in all epithelial cell types and spreads plaque-wise without restrictions (Miry and Pensaert, 1989). Lung macrophages have also been identified as target cells (Iglesias *et al*., 1989). Between 2 and 5 days post inoculation, virus spreads in a cell-associated way over the whole mucosa and deep into the submucosa. Virus replication induces an enormous influx of phagocytes which as a first line defense start to attack the infected regions. The resulting massive destruction causes respiratory signs, such as sneezing, coughing, nasal discharge and dyspnea. During this invasion period, virus becomes transported over neurons, in blood and lymphatics and reaches important target organs such as the brain, lymphoid organs and pregnant uterus. Interferon-alpha is present starting from 2 till 7 days post inoculation and the concentration is inversely proportional to the intranasal virus replication (Nauwynck and Pensaert, 1995a). The latter shows the importance of interferon-alpha in the control of virus replication in the nose and pharynx. Using nasal mucosal explants, Pol *et al*. (1991) demonstrated that interferon-alpha is diminishing the spread of PRV in the connective tissue but not in the epithelial cells. The reason why epithelial cells are not protected is not understood and should be further analyzed. With the appearance of a general and local humoral and cellular immunity starting from 6–7 days post inoculation, virus becomes neutralized and inactivated and infected cells are lysed and cleared by phagocytes (Kimman, 1994a). With the onset of the specific immunity, the recovery phase starts. Despite the presence of these different arms of the immune system, virus is completely eliminated only after 13 days post inoculation in the nasal cavity and 18 days post inoculation from the tonsils (Pensaert *et al*., 1990; Vannier *et al*.,

1991). The ability to replicate at a low level in the presence of a specific immunity is indicative for the fact that PRV is able to overcome some antiviral actions of the immunity. This also forms the basis for replication of PRV in the respiratory tract of immune animals after previous infection or vaccination or after uptake of colostrum with anti-PRV antibodies. A reduced number of small foci of virus replication are still present in the respiratory tract, virus may still reach the trigeminal ganglion and virus may still be spread via infected monocytes in the blood. During the latter cell-associated viremia virus may still cross the placenta and infect fetuses (Nauwynck and Pensaert, 1992). The stronger the immune response and the more components of the immunity are activated, the better the protection. By using mutants, it is possible to study the role of certain non-essential viral envelope glycoproteins and viral enzymes in the replication and invasion capacities. PRV gC and gI negative mutants behaved like the wild type virus with respect to the number and size of the foci of infected cells in the mucosa/submucosa. In contrast, the absence of gE and gD resulted in a reduced number of infected cells and gave foci with smaller dimensions (Kritas *et al*., 1994a; Kritas *et al*., 1994b; Mulder *et al*., 1996). Concerning the enzymes, it was demonstrated that the absence of viral ribonucleotide reductase, dUTPase and US3-encoded protein kinase was reducing virus excretion in pigs, indicating that the replication was clearly affected (De Wind *et al*., 1993; Jons *et al*., 1997; Kimman *et al*., 1992). At what level these proteins are involved in the invasion of PRV through the different layers of the respiratory mucosa and submucosa is not known and merits further examination.

Vaccination and control

In order to guarantee a free trade of pigs in Europe, efforts have been made to eradicate PRV (Pensaert *et al*., 2004). In the past, PRV hindered free trade between countries such as Great Britain and several Scandinavian countries which were already free for decades by mainly a

stamping out policy and countries which were not free and wanted to export pigs to PRV free countries. Therefore, several exporting countries started programs to reach a PRV free status. For reaching this high sanitary status, huge efforts were necessary, especially in densely populated pig regions where the seroprevalence was high. In most of these regions a combination of vaccination during several years, which reduced virus circulation and seroprevalence, and culling at a moment of low seroprevalence was used, generally with success. This whole strategy was only possible with marker vaccines and a discriminating ELISA which is able to identify infected animals in a group of vaccinated animals (Van Oirschot *et al.*, 1996). For PRV, the choice fell on a gE deletion vaccine, based on several molecular features. Deleting gE is attenuating PRV by reducing the cell-associated transmission and neurological spread but does not hinder mass production in cell cultures and does not show a reduced induction of a protective immunity. Because latent virus may be reactivated and cause a new explosion of transmissions, it was generally feared that eradication programs would have met a lot of problems to be successful. This fear seemed to be unfounded in the field. At present, several countries are officially PRV free. Since PRV is still present in wild boars and feral swine in most of these countries, authorities should follow up the situation within this population and estimate the risk from this reservoir in the wild.

Epidemiology

Pseudorabies outbreaks occur in swine populations worldwide, resulting in substantial economic losses for affected countries. Many countries, such as Germany and the Netherlands, mounted successful agricultural campaigns to eradicate PRV from their swine populations. In 2001, Germany was listed as Aujeszky's disease-free. Eradication efforts included selective culling of PRV-positive herds, widespread vaccination programs with marker viruses such as gE-null vaccine strains, restricted importation of swine, and improved herd management practices to isolate

swine from potential reservoirs of infection such as wild boar (Muller *et al*., 2003; Stegeman *et al*., 1997; van Oirschot *et al*., 1986). In 2003, countries with documented cases of PRV (in alphabetical order) included Belarus, Brazil, Cuba, France, Hungary, Italy, Mexico, Panama, Poland, Portugal, Romania, Russia, Slovakia, Slovenia, Taiwan, and Ukraine. Pseudorabies outbreaks in Mexico are particularly troubling to U.S. state and federal agricultural officials, given Mexico's border with the United States. This underscores the importance of surveillance and monitoring of movement of animals across U.S. borders. Information regarding global pseudorabies outbreaks was provided by the World Organization for Animal Health/Office International des Epizooties (web site address: www.oie.int).

Clinical signs and pathogenesis

Acute PRV infection of swine

Outbreaks of PRV increased substantially coinciding with a dramatic increase in the intensity of swine production and close-quarter confinement of large numbers of pigs in swine barns. The increased stresses imposed on the swine coupled with closer contact provided ideal conditions for spread of PRV (Kluge *et al*., 1999). Primary viral replication occurs in the nasal and oropharyngeal mucosa (Masic *et al*., 1965). PRV is tropic for both respiratory and nervous system tissue of swine and viral particles enter sensory nerve endings innervating the infected mucosal epithelium. Morbidity and mortality associated with PRV infection varies with the age of the pig, overall health status of the animal, viral strain, and infectious dose. Younger swine are the most severely affected by PRV infection and typically exhibit symptoms of central nervous infection whereas older swine exhibit symptoms of respiratory disease (Kluge *et al*., 1999). For suckling piglets, the incubation period of PRV is typically 2 to 4 days. Initially, piglets are listless, febrile, and uninterested in nursing. Within 24 h of exhibiting these symptoms, the piglets will progressively

develop signs of central nervous system infection including trembling, excessive salivation, incoordination, ataxia, and seizures. Infected piglets may sit on their haunches in a “dog-like” position because of hind limb paralysis, lay recumbent and paddle, or walk in circles. Once piglets develop central nervous system abnormalities, they die within 24 to 36 h. Mortality of suckling pigs with pseudorabies is extremely high, approaching 100%. The cause of death in piglets has historically been attributed to viral encephalitis. However, recent work suggests that a peripheral host immune response to viral infection may be a significant determining factor in the death of infected animals (Brittle *et al*., 2004). Weaned pigs, ages 3 to 9 weeks of age, tend to develop symptoms highly reminiscent of those described for suckling pigs. However, the mortality rate is much lower. Typically, 50% of infected 3- to 4-week-old animals die. Pigs 5 to 10 weeks of age become listless and anorectic, and exhibit temperatures of 41 to 42°C within 3 to 6 days of infection. Animals often develop respiratory signs such as sneezing, nasal discharge, a severe cough, and difficulty breathing. Pigs with respiratory illness often lose significant body weight, a condition that translates directly into economic loss for swine producers. Signs of infection typically resolve after 5 to 10 days, with most pigs making a rapid recovery upon resolution of the fever and anorexia. If carefully nursed through their illness and treated for secondary bacterial infections when necessary, mortality rarely exceeds 10% (Kluge *et al*., 1999). In adult swine, respiratory signs are the hallmark of PRV infection although, sporadically, adult animals may exhibit central nervous system abnormalities varying from mild muscle tremors to violent convulsions. Although morbidity is quite high (approaching 100% of infected animals), mortality is relatively low (1 to 2% of infected animals). Typically, clinical signs appear in 3 to 6 days. Symptoms include a febrile response (41 to 42°C), listless behavior, lack of interest in eating, and mild to severe respiratory signs. These animals will typically exhibit rhinitis evidenced by sneezing and nasal discharge. The respiratory illness may progress to pneumonia with a harsh cough and

labored breathing. Sick animals will become emaciated and lose considerable body weight, resulting in financial losses for swine producers. The duration of clinical illness is 6 to 10 days, and infected animals typically recover rapidly (Kluge *et al*., 1999). Pregnant animals infected with PRV in the first trimester of pregnancy will usually reabsorb the fetuses in utero. If infection occurs within the second and third trimester of pregnancy, infection typically results in abortion, stillbirths, or weak piglets that die within 48 h of birth. PRV-induced infertility results from the virus crossing the placenta and infecting animals in utero. In fact, in a given litter, some pigs may be born normal, while others are weak and some are stillborn due to transplacental transmission of virus. Reproductive failure usually has a low incidence on an infected farm, occurring in 20% or less of pregnant animals (Kluge *et al*., 1999).

PRV latency in swine

During an acute infection, viral particles replicate in the oropharyngeal mucosa then enter sensory nerve endings innervating the site of infection. Retrograde transport of virus particles occurs in the maxillary branch of the trigeminal nerve (cranial nerve V), the glossopharyngeal nerve (cranial nerve IX), and the olfactory nerve (cranial nerve I) (Maes *et al*., 1997). The term latent infection of swine is used to describe a long-term infection in which the PRV genome is quiescent and PRV virions cannot be recovered. The predominant sites of PRV latency are the trigeminal ganglia (originally described as gasserian ganglia) and the sacral ganglia in feral swine. Similarly, the predominant sites of latent neuronal infection of the human alphaherpesviruses HSV-1 and HSV-2 are the trigeminal ganglia and the sacral ganglia (Whitley, 2001). Latent PRV genomes can be detected in the other neural tissues such as the olfactory bulb and brain stem (Rziha *et al*., 1986; Sabin, 1938; Sabo and Rajcani, 1976; Wheeler *et al*., 1991). Each latently infected cell body of the trigeminal ganglia harbors approximately 30 copies of the viral genome (Rziha *et al*., 1986). In

addition, tonsillar lymph nodes are reported sites of latency (Brittle *et al*., 2004; Brockmeier *et al*., 1993; Cheung *et al*., 1994; Sabo and Rajcani, 1976). However, the rate of PRV DNA detection in tonsils is often lower than that seen in trigeminal ganglia, and questions remain as to whether tonsils constitute a true site of latency rather than the site of low but persistent infection (Maes *et al*., 1997). The cell type harboring PRV within the tonsillar tissue remains unknown. Reactivation and shedding of virus in latently infected animals frequently occurs after stressful experiences. Stressors include concomitant disease conditions, vehicular transport, poor animal husbandry, farrowing (i.e., giving birth to piglets), and treatment with immunosuppressive agents, e.g., corticosteroids (Davies and Beran, 1980; Rziha *et al*., 1989, Rziha *et al*., 1986, Thawley *et al*., 1984; van Oirschot and Gielkens, 1984; Wittmann *et al*., 1983).

Lethality of pseudorabies infection in nonnative hosts

In addition to infection of its natural swine host, PRV infects a broad range of vertebrates. These include cattle, sheep, dogs, cats, goats, chickens, raccoons, possums, skunks, rodents, rabbits, guinea pigs, and, rarely, horses (Field and Hill, 1974; Gustafson, 1986; Kimman *et al*., 1991; McCracken *et al*., 1973; Pensaert and Kluge, 1989; Wittmann *et al*., 1980). Infection of carnivores, such as bears and wild felines, has also been reported to occur after consumption of raw PRV-infected meat (Capua *et al*., 1997; Glass *et al*., 1994; Zanin *et al*., 1997). Infection of nonnative hosts with a wild-type PRV strain is uniformly lethal. While PRV does not infect humans, experimental studies in nonhuman primates indicate that rhesus monkeys and marmosets are susceptible to infection. Other higher-order primates, such as chimpanzees, are not susceptible to infection (Enquist, 1999; Kluge *et al*., 1999).

Lesions

Focal necrotic and encephalomyelitis lesions occur in the cerebrum, cerebellum, adrenals and other viscera such as lungs (Figure n. 6), liver or spleen. In fetuses or very young piglets, white spots on liver are pathognomonic of their infection by the virus (Figure n. 7). Intranuclear lesions are frequently found in several tissues.

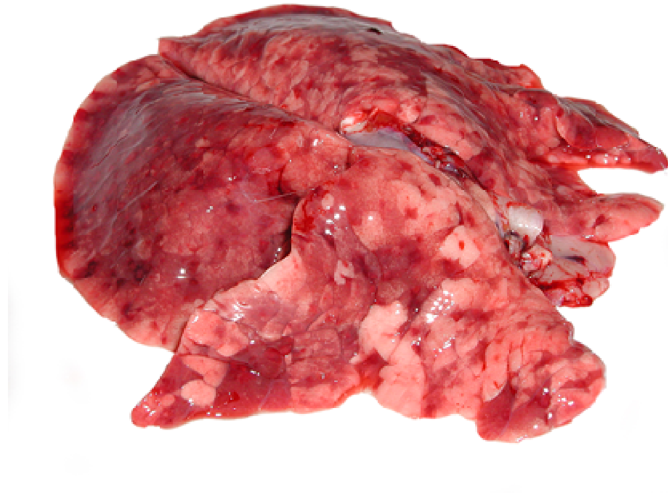


Figure n. 6: Focal necrotic lesions occur in the lungs

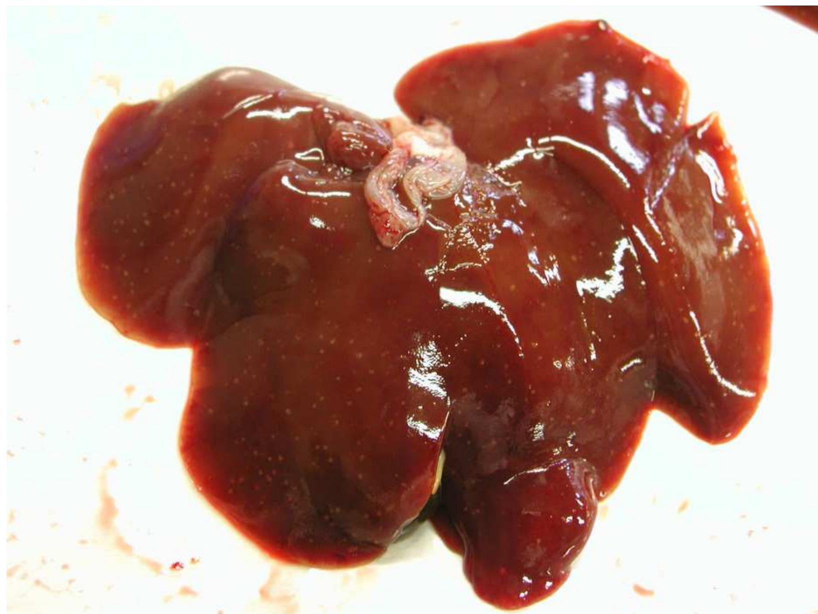


Figure n. 7: White spots on liver are pathognomonic in fetuses or very young piglets

Diagnosis

Identification of the agent

Virus isolation

The diagnosis of Aujeszky's disease can be confirmed by isolating the virus from the oro-pharyngeal fluid, nasal fluid (swabs) or tonsil swabs from living pigs, or from samples from dead pigs or following the presentation of clinical signs such as encephalitis in herbivores or carnivores. For post-mortem isolation of SHV-1, samples of brain, tonsil, and lung are the preferred specimens. In cattle, infection is usually characterized by a pruritus, in which case a sample of the corresponding section of the spinal cord may be required in order to isolate the virus. In latently infected pigs, the trigeminal ganglia is the most consistent site for virus isolation, although latent virus is usually non-infective unless reactivated, making it difficult to recover in culture. The samples are homogenized in normal saline or cell culture medium with antibiotics and the resulting suspension is clarified by low speed centrifugation at 900 *g* for 10 minutes. The supernatant fluid is used to inoculate any sensitive cell culture system. Numerous types of cell line or primary cell cultures are sensitive to SHV-1, but a porcine kidney cell line (PK-15) is generally employed. The overlay medium for the cultures should contain antibiotics (such as: 200 IU/ml penicillin; 100 µg/ml streptomycin; 100 µg/ml polymyxin; and 3 µg/ml fungizone). SHV-1 induces a cytopathic effect (CPE) (Figure n. 8) that usually appears within 24–72 hours, but cell cultures may be incubated for 5–6 days. The monolayer develops accumulations of birefringent cells, followed by complete detachment of the cell sheet. Syncytia also develop, the appearance and size of which are variable. In the absence of any obvious CPE, it is advisable to make one blind passage into further cultures. Additional evidence may be obtained by staining infected cover-slip cultures with haematoxylin and eosin to demonstrate the characteristic herpesviral acidophilic intranuclear inclusions with margination of the chromatin. The virus identity should be confirmed by

immunofluorescence, immunoperoxidase (Figure n. 9), or neutralisation using specific antiserum. The isolation of SHV-1 makes it possible to confirm Aujeszky's disease, but failure to isolate does not guarantee freedom from infection.

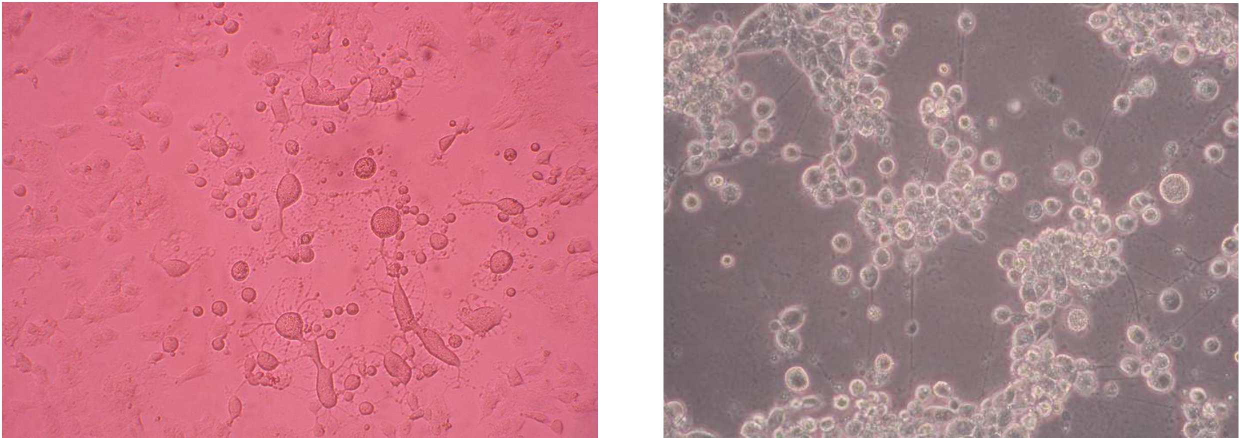


Figure n. 8: PRV: cytopathic effect on swine kidney cells

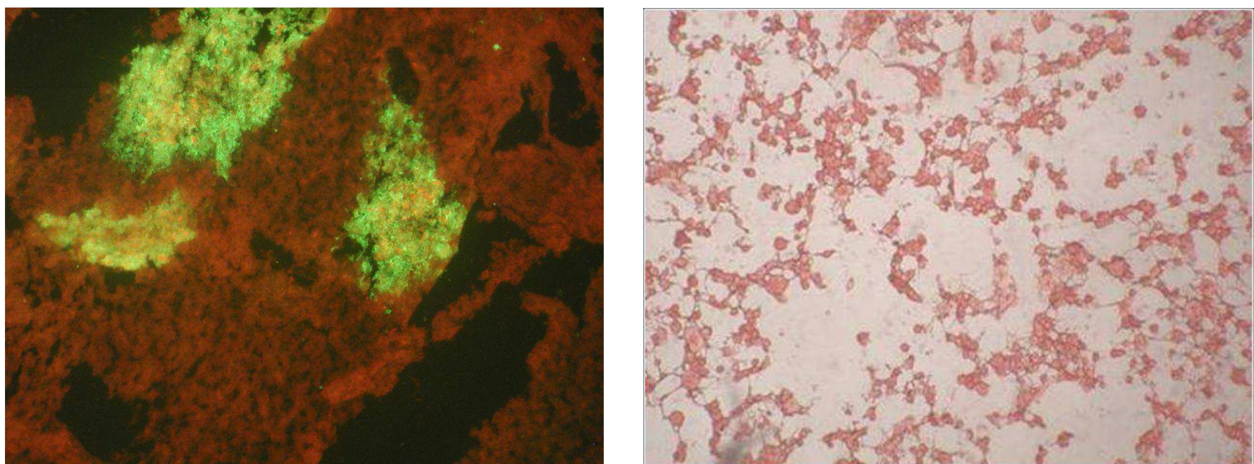


Figure n. 9: PRV: Immunofluorescence and immunoperoxidase

Identification of virus by the polymerase chain reaction

The polymerase chain reaction (PCR) can be used to identify SHV-1 genomes in secretions or organ samples. Many individual laboratories have established effective protocols, but there is as yet no internationally agreed standardized approach. The PCR is based on the selective amplification of a

specific part of the genome using two primers located at each end of the selected sequence. In a first step, the complete DNA may be isolated using standard procedures (e.g. proteinase K digestion and phenol–chloroform extraction) or commercially available DNA extraction kits. Using cycles of DNA denaturation to give single-stranded DNA templates, hybridisation of the primers, and synthesis of complementary sequences using a thermostable DNA polymerase, the target sequence can be amplified up to 106-fold. The primers must be designed to amplify a sequence conserved among SHV-1 strains, for example parts of the gB or gD genes, which code for essential glycoproteins, have been used (Mengeling *et al*., 1992; Van Rijn *et al*., 2004; Yoon *et al*., 2006). A real-time PCR has been developed that can differentiate gE-deleted vaccine viruses from wildtype virus based on the specific detection of gB and gE genes (Ma *et al*., 2008). However, the gE specific real-time PCR has a lower sensitivity than the gB-specific real-time PCR. The amplified product may be identified from its molecular weight as determined by migration in agarose gel, with further confirmation where possible by Southern hybridisation using a complementary probe. Recent techniques involve liquid hybridisation using enzyme-labelled probes, which give a color reaction after incubation with the appropriate substrate. More recent techniques include the use of fluorescent probes linked to an exonuclease action and real-time monitoring of the evolution of product, enabling simultaneous amplification and confirmation of the template DNA thus increasing the rapidity and specificity of the PCR assays. In all cases, the main advantage of PCR, when compared with conventional virus isolation techniques, is its rapidity; with the most modern equipment, the entire process of identification and confirmation can be completed within one day. However, because of the nature of the test, many precautions need to be taken to avoid contamination of samples with extraneous DNA from previous tests or from general environmental contamination in the laboratory. This may limit the value of the test for many laboratories unless care is taken to avoid DNA carry-over contamination. The use of an internal

control is necessary to avoid false negative results by ensuring adequate efficiency of DNA extraction and confirming the absence of PCR inhibitors in each sample. In practice, different systems can be used for detection of endogenous or exogenous gene (Hoffman *et al*., 2009).

Serological tests

Virus neutralisation (VN) has been recognised as the reference method for serology (Moennig *et al*., 1982), but for general diagnostic purposes it has been widely replaced by the enzyme-linked immunosorbent assay (ELISA) because of its suitability for large-scale testing (Moennig *et al*., 1982). The tests can be performed on a variety of matrices (e.g. serum, whole blood, milk, muscular exudates, and filter paper) but the preferred matrix is serum. A latex agglutination test has also been developed and can be used for screening for antibodies. It can differentiate between the immune response of naturally infected pigs and those that have been vaccinated with gE deleted vaccines (Yong *et al*., 2005). Kits for the test are commercially available (Schoenbaum *et al*., 1990). Serological tests are carried out only for pigs, as other animals (herbivores and carnivores) die too quickly to produce antibodies. In free areas where pigs are not vaccinated, an active epidemiological survey can be carried out using ELISA gB kits. As antibodies can be detected between 7 and 10 days post-infection, this serological tool can also be used in case of an outbreak suspicion, to confirm the infection of pigs. In area where pigs are vaccinated with gE deleted vaccines, the ELISA gE kits permit the differentiation between infected and vaccinated pigs (DIVA), but to assess the level of immunity induced by vaccination, gB ELISA kits or viral neutralisation should be used. Any serological technique used should be sufficiently sensitive to give a positive result with the OIE International Standard Reference Serum. This serum can be obtained from the OIE Reference Laboratory for Aujeszky's Disease in France. For international trade purposes, the test should be sensitive enough to detect the standard serum diluted 1/2. To

authorize pig movement from an area where deleted gE vaccines are used to a free area, serological assays should be able to detect at least the dilution of 1/8 for ELISA gE of the OIE reference standard serum as prescribed by the European Commission (2008).

Hypothesis and objectives

Hypothesis

Suid Herpesvirus (SHV-1) is the agent of Aujeszky's disease (AD), which is spread worldwide and causes great economic losses in the pig industry. Pigs are the primary host of the virus but a large number of other species can be naturally infected. SHV-1 belongs to *Herpesviridae* Family, *Alphaherpesvirinae* subfamily and has a double-stranded, linear DNA genome belonging to the D class. The genome is characterized by two regions i.e. UL and US, with the US region flanked by the internal repeat sequences (IRS and TRS respectively). Out of the high number of viral proteins encoded by the genome, 11 are glycosylated membrane proteins involved in virus entry, egress, cell to cell spread and modulation of immune response. The control and eradication of AD have been taken into account by many countries and considerable efforts have been focussed on the elucidation of the epizootiology of the infection. The characterization of SHV-1 strains by means of identification restriction fragment length polymorphism (RFLP) analysis has greatly contributed to the implementation of these programs. Genomic characterization of SHV-1 has been routinely performed using RFLP but, recently, some studies based on the sequencing of two genes (US8 and UL44), encoding gE and gC proteins respectively, have been reported. In Italy, an AD national monitoring program has been applied since 1997. This was able to achieve a substantial reduction of serum prevalence in the first five years but SHV-1 is still present in the Italian swine population causing economic losses due to a reduction in reproductive performances and body weight. In many other countries of Europe AD has been eradicated in the last decade in domestic pigs but not in wild swine population. Despite the progress that has been made in controlling and eliminating SHV-1 in domestic pigs, there is mounting evidence that SHV-1 infections are more widespread in wild swine across the world than originally thought. The understanding of the extent of SHV-1 infections in these wild populations and of the threat to domestic swine is still fragmentary.

Objectives

Study n.1

To report the results of a Aujeszky Disease monitoring programme performed in Northern Italy during the period 2012-2014. This was based on control of all respiratory, nervous and reproductive forms, genome detection and virus isolation, serological investigations. To analyse the distribution of the Aujeszky Disease and evaluate the epidemiological situation in our area.

Study n.2

To described the isolation and the genomic characterization of PRV originated from swine in Italy from 1984 to 2010. To carry out the phylogenetic and molecular analysis of this strains by comparing gC and gE sequences with selected PRV obtained from GenBank.

Study n.3

To reported the results of an AD survey based on PRV genome detection in samples from dogs clinically suspected of AD and from wild boars collected during four consecutive hunting seasons, 2010–2014. Dogs in which AD was clinically suspected were examined by using histopathological and virological methods, resulting in the isolation of 13 PRV strains from dead dogs. One strain isolated from a wild boar during the same period and region was also genetically characterized. The phylogenetic analysis was based on a partial sequence of the gC gene, and the results were compared with the sequences available in GenBank.

Study n.1

Aujeszky Disease:

serological and virological surveillance

in Italy during 2012-2014

Introduction

Pseudorabies virus (PRV) or suid herpesvirus 1 (SHV-1), a member of the Alphaherpesvirinae subfamily, is the causative agent of Aujeszky's disease (AD), an economically important disease of pigs (Aujeszky 1902). Its host range includes a wide spectrum of mammals, although domestic and wild members of the Suidae family are the only hosts capable of surviving a productive infection and can serve as reservoirs for the virus (Pensaert and Kluge 1989). As a general pattern, wildlife can both maintain and spread infections to domestic species (Gortazar *et al*. 2007), and the wild boar–domestic pig interface represents an example of this interaction, as both species have mutual transmission risks for their parasitic and infectious diseases (Boadella *et al*. 2012). AD is such a disease, since the presence of the PRV infection in wild boar populations has been reported worldwide with variable prevalence rates (Ruiz-Fons *et al*. 2008a). Although reports of PRV transmission from wild boar to domestic pigs are surprisingly rare, the success of disease eradication programmes in the domestic species could be influenced by wildlife reservoirs (Muller *et al*. 2011). Since the early 1980s, AD has spread globally due to the appearance of more virulent PRV strains and to changes in swine production systems, such as increases in animal density and the total confinement of the animals (Muller *et al*. 2011). Today, the virus has spread worldwide and causes economic losses in the pig industry due to increased mortality rates, depending on the age of the host and the virulence of the virus strain involved. PRV is currently the focus of eradication programmes almost worldwide, which include large-scale vaccination with gE-deleted vaccines. This strategy, together with increased control efforts, has decreased the incidence of the disease in several European Union (EU) member states (Pannwitz *et al*. 2012). In Italy, an AD national monitoring programme has begun in 1997 (Decreto Ministeriale 1997); it includes the application of direct prophylaxis, biosecurity measures and vaccination programmes. Although AD has not yet been eradicated from Italian pig herds, a considerable reduction in the spread of the

virus has occurred. Similar to the observations in many European countries, where AD was eradicated in domestic pigs but not in free-living wild boar populations (Boadella *et al*. 2012), PRV has been continuously detected in wild boar in Italy (Lari *et al*. 2006; Montagnaro *et al*. 2010; Verin *et al*. 2014). Although wild boar can serve as reservoirs for PRV (Ruiz-Fons *et al.* 2008b), limited data are available on the long-term epidemiology of PRV in free-ranging populations. The analysis of these data may provide baseline information on PRV infection dynamics under natural conditions indicating those factors most influential on the spread and maintenance of the virus into the wild populations. Therefore, the aim of this study, through targeted surveillance, and using serological and molecular testing, was to describe the temporal dynamics of PRV infection and to define the role of wild boar population and of domestic pig farms on spread and maintenance of AD.

Materials and methods

Study Area and Sampling

During the periods 2012–2013 and 2013–2014, wild boar tissue samples of lungs and tonsils were voluntarily collected by hunters in several provinces of North Italy. Tonsil samples were immediately processed and analyzed for the presence of PRV DNA. Clinical samples originated from domestic pigs were collected during the same period from routine diagnostic. Serum samples were obtained from routine diagnostic and from wild boars during the wildlife program.

Laboratory Analysis

Serological analyses were performed using an ELISA test for the detection of anti-gE antibodies with the Pseudorabies Virus gpI Antibody test kit (IDEXX PRV/ADV gI). The ELISA test was carried out according to the manufacturer’s instructions (Idexx, EK Hoofddorp, The Netherlands). Genomic

DNA extractions from wild boar samples were performed using an RNeasy kit (Qiagen, Hilden, Germany). The presence of PRV DNA was routinely determined using real-time PCR tests based on the specific detection of the gB and gE genes (Ma *et al*. 2008; Yoon *et al.* 2005).

Results

The serological tests for the detection of antibodies against the gE of Aujeszky disease virus carried out in 2012-2013 showed a percentage of positive samples for antibodies to gE by 7.5%, lower than in 2011-2012 (11.4%), confirming the downward trend shown in recent years. The serological testing to detect the presence of antibodies to gB performed in the same period in order to verify the effectiveness of vaccination, it showed a positive rate of 82.9% a slight increase over the previous period (82.7%). The virological analysis were conducted on the viscera of pigs through search of the viral genome without finding of positivity to PCR. The results of serological and virological pigs are summarized in Table 1.

Table n. 1**:** results of serological and virological in pigs for the period 11/01/2012 - 31/10/2013 performed by laboratories IZSLER

	tested	**positive**	**%**
gB antibodies	43235	35823	**82,9**
gE antibodies	129687	9708	**7,5**
virological	176	0	**0**

Below we are also reported results of serological and virological performed for Aujeszky's disease in wild boar (Table n. 2).

	tested	positive	%
gE antibodies	5783	1127	**19,5**
PCR	405	4	**0.1**

Table n. 2**:** Results of serological and virological in wild boar for the period 1/11 / 2011-31 / 10/2012

The seropositive results mainly concerns samples of wild boars in Emilia Romagna (97.3%), while in Lombardy was found to be 2.7%. Virological examinations were conducted on wild boar killed in Lombardy during the hunting season (90%), in particularly in the province of Brescia, while the remaining 10% consisted of wild boars from the province of Forli. Analyses were conducted on the viscera of wild boar through research of the viral genome and were found four wild boars positive from Brescia province.

The serological tests for the detection of antibodies against the gE of Aujeszky Disease virus performed in 2013-2014 in the course of activities under the control plan, revealed a percentage of positive samples for antibodies to gE by 9.3% . In fact the data observed in 2014 shows a slight increase in the percentage of positive samples that went from 7.5% in 2012-2013 to 9.3% in this period. This slight increase is likely due to the significant increase of serological tests (from 129.687 in 2012-2013 to 176.956 in 2013-2014), carried out mainly in Lombardy as a result of the approval of the new plan to control and mainly targeting the control of farms for fattening with unknown health status.

The serological testing to detect the presence of antibodies to gB, performed in the same period, in order to verify the effectiveness of vaccination, showed a positivity rate of 85.5%, an increase over the previous period (82.9%), then sign of increased use of vaccination.

The virological analysis were conducted on the viscera of pigs through search of the genome. The results of serological and virological pigs are summarized in Table 3.

	tested	**positive**	**%**
gB antibodies	42.131	36.002	**85,5%**
gE antibodies	176.956	16.504	**9,3%**
Virological	113	2	**1,8%**

Table n. 3: Results of serological and virological analysis in pigs for the period 01/11/2013 - 31/10/2014 performed by laboratories IZSLER.

The following also shows the results of serological and virological analysis performed for Aujeszky's disease in wild boar (Table n. 4).

	tested	**positive**	**%**
gE antibodies	7412	1836	**24,8%**
PCR	92	1	**1,1%**

Table n. 4: Results of serological and virological analisys in wild boar for the period 1/11 /2013-31/ 10/2014.

The seropositive results mainly concerns samples of wild boars in Emilia Romagna (77.2%), while this in Lombardy was 22.8%. It 'been a considerable increase in the percentage of positive boars in Lombardy (from 2.7% in 2012-2013 to 22.8% in 2013-2014), which is mainly caused by the high

percentage of positive wild boars in the province of Pavia. In fact, 81.1% of positive findings in Lombardy is due to boars from Pavia. Figures 1 and 2 shows the percentage of positivity on province.

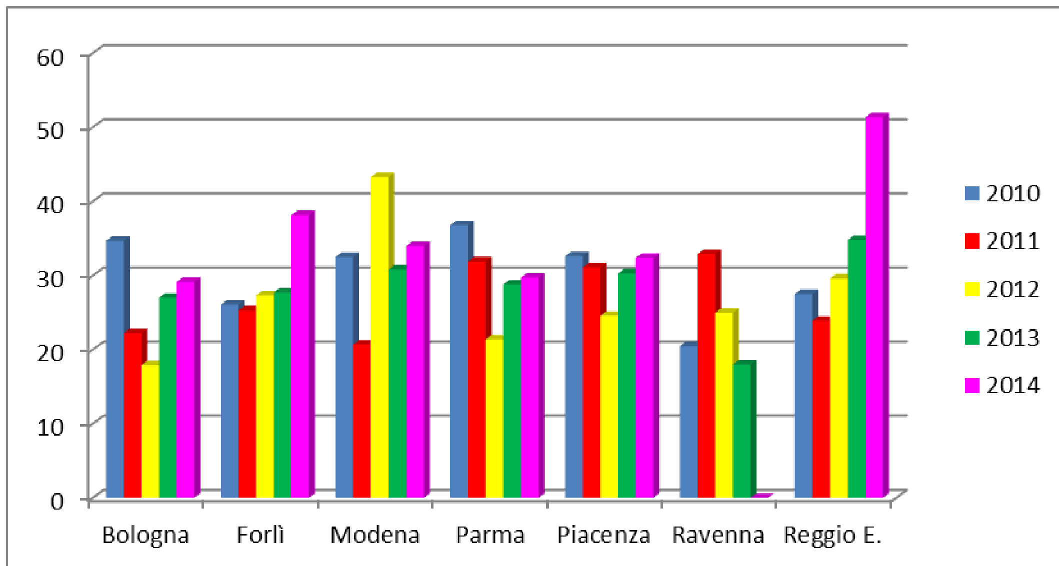


Figure n. 1: Positive serology in wild boar in Emilia Romagna from 2010 to 2014 (data updated to 10/31/14).

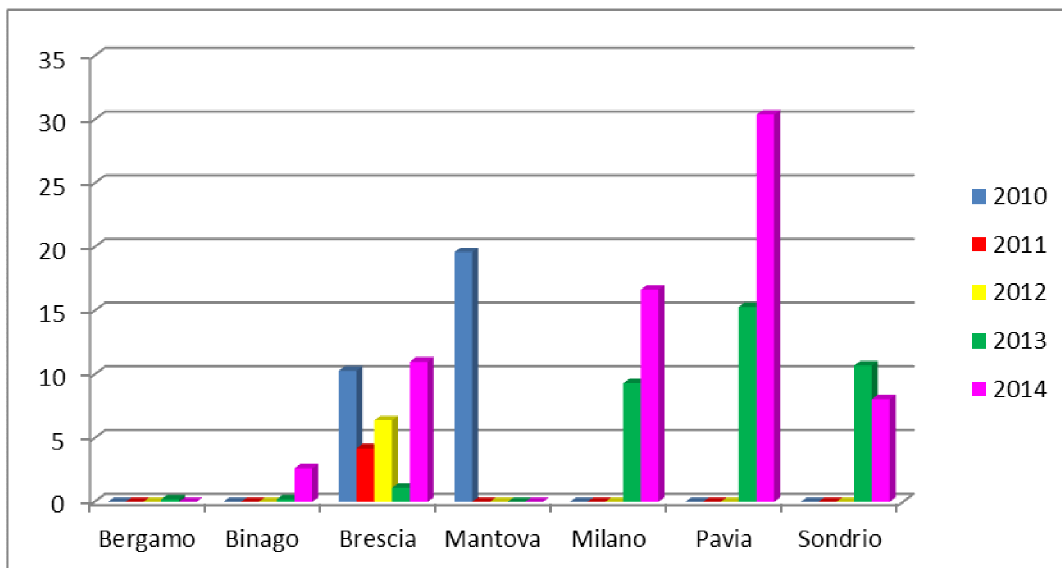


Figure n. 2: Positive serology in wild boar in Lombardia from 2010 to 2014 (data updated to 10/31/14).

Discussion

The present long-term surveillance of AD in the wild boar and pigs of the Norther Italy showed an increase in seroprevalence during the years, also in the wild boars, with a higher probability of being seropositive in older individuals, while the presence of domestic pigs had no effect on their seropositivity. In particular, the total AD seroprevalence in the study area increased, showing that the infection could persist as an endemic disease at low prevalence values in wild boar populations. This was also supported by the results of the molecular analyses, where the PCR positive animals confirmed the presence of ADV, even with a low diffusion, inside the wild boar population. Domestic pigs and wild boar have a reciprocal transmission risk for their infections, including AD, as demonstrated by experimentally infecting domestic pigs with AD strains of wild boar origin, suggesting the possible AD transmission between both sides (Muller e al. 2001). Although the presence of ADV in wild boar already posed concerns for AD control in pigs (Corn *et al*. 2009), it has been shown that the AD prevalence in wild boar was not a significant risk factor for the AD prevalence in the coexisting pig farms (Ruiz-Fons *et al*. 2008b).

Study n.2

Genomic characterization of pseudorabies virus strains isolated from swine in Italy

Introduction

PRV is currently the focus of worldwide eradication programmes that have decreased the incidence of the disease dramatically. An AD national monitoring programme, which has been running in Italy since 1997, involves the application of direct prophylactic and bio-security measures and vaccination programmes. Vaccination includes the administration of marker vaccines, an inactivated form in breeder pigs and a live attenuated form in fattening pigs, which enabled a substantial reduction of serum prevalence in the first 5 years; however, PRV is still present in the Italian pig population and is causing economic loss via a reduction in reproductive performance and body weight. Genomic characterization of PRV has been performed routinely using restriction fragment length polymorphism (RFLP) analysis. Recently, however, there have been some studies based on sequencing genes US8 and UL44, encoding the gE and gC proteins, respectively (Fonseca *et al*., 2010; Muller *et al*., 2010; Serena *et al*., 2011). To better understand the epidemiology of PRV in Italy, we undertook genomic characterization of 44 PRV strains, based on the partial sequencing of genes US8 and UL44. These PRV strains originated from pigs isolated between 1984 and 2010.

Materials and Methods

Forty-four PRV strains isolated between 1984 and 2010 were used in this study. The 44 porcine isolates were from animals of different ages and from various types of farm production (farrow-to-finish, farrow-to-weaning, fattening). The majority of these farms were examined serologically (sera were from the Italian compulsory plan for swine vesicular disease) to investigate the immune status. Swine samples were collected from farms with respiratory or reproductive problems. Data for materials, origin of strains and immune status of animals are given in Table 1. The porcine viral strains were isolated in primary cell culture and PK15. The extraction of genomic DNA was carried

out with one cell culture supernatant using an RNeasy kit (Qiagen, Hilden, Germany), and PCR was performed as described (Fonseca *et al*., 2010).

Primer	Sequenza primer (5'-3')	glicoproteina	regione	amplificati (bp)
gC-2U	GTTTCCTGATTCACGCCCACGC	gC	11-32	788
gC-1L	GAAGGGCTCACCGAAGAGGAC		821-841	
gE-nF	CCGCGGGCCGTGTTCTTTGT	gE	592-611	493
gE-nR	CGTGGCCGTTGTGGGTCAT		1066-1084	

The PCR products were purified using a QIAquick Gel Extraction kit (Qiagen, Inc., Valencia; CA, USA). DNA sequencing was carried out with a Big-Dye Terminator Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) with the same primers as those used for amplification. Sequencing reactions were run by capillary electrophoresis on an automatic sequencer (ABI 3130 Genetic Analyser; Applied Biosystems). Sequences were edited using the SeqMan program (DNASTAR, Madison, WI, USA), and sequence alignments were performed with the ClustalW method (DNASTAR); phylogenetic analysis was carried out with MEGA 5.0 (Tamura *et al*., 2011) using the neighbour-joining method with the Kimura two-parameters model; bootstrap analysis was carried out with 1000 replicates. The results were verified using maximum likelihood and maximum parsimony analysis, which showed similar topologies. The sequences were identified by species, state, sample number and year of isolation. The sequences of PRVs isolated from other countries (available through GenBank) were used for comparison with the Italian isolates. Deduced amino acid sequences were aligned using ClustalW, and the alignment was optimized manually. The sequences described here are deposited in the GenBank database.

Results

Phylogenetic analysis based on gE

Phylogenetic analysis of the US8 gene encoding the gE protein (Figure n.1) showed that sequences obtained from the Italian PRV isolates were divided into two large clusters, B and C (Fonseca *et al*., 2010). Thirty-eight of the 44 porcine strains formed a homogeneous group located in cluster B, which is closely related to the 89V87 and Nova Prata reference strains from Belgium and Brazil, respectively. Five swine isolates (swine/It/97897/2008, swine/It/56/1987, swine/It/900/1991, swine/It/26940/2010 and swine/It/2106/1996) were placed in cluster C. This cluster also grouped the reference strains NS374 and 75V19 isolated in Belgium in the 1970s. The remaining strain, swine/It/3779-1/1997, showed a high degree of homology (95.7%) with the Shope strain and was placed into a different cluster.

Phylogenetic analysis based on gC

Phylogenetic analysis of the UL44 gene encoding the gC protein demonstrated wide variation within the Italian strains. All except two (swine/It/32501/2008 and swine/It/4742/2000) of the Italian porcine strains belonging to cluster B in the US8 tree were placed into the same cluster in the UL44 tree together with the Nova Prata reference strain. Cluster C is a homogeneous group that contains the strain ITA 561 (96%) isolated from a wild boar in Italy in 1993 (Capua *et al*., 1997). A separate group within this cluster contained three porcine strains (swine/It/97897/2008, swine/It/26940/2010 and swine/It/32501/2008), that showed a high degree of homology to the Brazilian field strain IB341/86 (Figure n.2). The remaining porcine strains showed interesting features: (i) one porcine isolate (swine/It/4742/2000) located in cluster E has 100% homology to the NiA3 reference strain isolated from Northern Ireland; (ii) porcine strain swine/It/3779-1/1997 in cluster D has 100% homology to the Illinois strain PRV8044; and (iii) three porcine strains isolated in the 1980–1990s (swine/It/56/1987, swine/It/900/1991 and swine/It/2106/1996) appear genetically divergent and are clustered with the Asian strains as Ea and Fa in cluster F. An

alignment of the deduced amino acid sequences of the gC protein showed several insertions and deletions. Strains belonging to cluster B exhibited deletions at positions 24 and 181 according to the numbering introduced by Muller *et al*. (2010). Finally, the three porcine strains closely related to the Asian strains showed interesting features: (i) an insertion of seven amino acids after position 72, present also in Asian strains; and (ii) a deletion of amino acids 50–52 found only in the Italian strains.

Discussion

The results of this study show that the majority of the Italian PRV strains are grouped into two clusters. Most (36/44) porcine PRV strains are closely related to those isolated from domestic pigs in Europe and America in the last 20 years and belong to cluster B in the phylogenetic trees of the gE and gC genes. Two other porcine strains have the gE gene belonging to cluster B, whereas the gC gene shows a different pattern; the gene was related to old PRV strains, one strain (swine/It/4742/2000) was placed into cluster C, and the other strain (swine/It/32501/2008) was placed into a different group, related to an NIA reference strain. The gE of the remaining six porcine strains, includes five isolates related to old porcine PRV strains and one (swine/It/3779-1/1997) related to the Shope strain. The gC gene of three strains shows great variability and is placed into three different clusters; one into cluster D, two into cluster C and three into cluster F. These three strains are phylogenetically divergent, clustering with the geographically unrelated Ea and Fa strains from China. The divergent position might reflect an international origin for these strains. During the 1980s, a common procedure was to use some Chinese breeds characterized by a high degree of prolificacy to accelerate genetic progress (Legault, 1985), which could explain the origin of these strains; however, investigations based on complete sequencing of the gC and gE genes are in progress to investigate the genomic characteristics of these strains more fully. Two

molecular techniques commonly used for defining genetic relationships among pathogenic organisms are RFLP analysis and direct nucleotide sequencing. As described by Goldberg *et al*. (2001), both techniques indicate a lack of correlation between genetic and geographic distance but significant correlation between genetic and temporal distance and between genetic distance and host species of origin. PRV isolates representative of genomic type I are found in the United States and Central Europe, while isolates showing genomic type II are found predominantly in Central Europe and Japan. Groups III (genome type III) and IV (genome type IV) are limited strictly to isolates originating from Northern Europe and Thailand, respectively (Herrmann *et al*., 1984). On the basis of earlier genomic characterization based on sequencing the gC and gE genes and comparison with RFLP analysis, a good correlation has been shown between genotype II and cluster B (Schaefer *et al*., 2006; Fonseca *et al*., 2010). Furthermore, it is reported that genotype II is predominant in the pig population (Capua *et al*., 1997; Piatti *et al*., 2001), probably owing to its high virulence, and thus, pig transmissibility is higher with respect to genotype I (Glorieux *et al*., 2009). Similarly, the present study shows that the most predominant group in Italy, with 36 of the 44 PRV isolates belonging to cluster B, is referable to genotype II. Another group, cluster C, consists of isolates closely related to wild boar strains isolated in Europe. These strains showing a high degree of homology to PRV strains circulating in the 1970s and 1980s have almost disappeared. By contrast, strains similar to the old PRV strains are still circulating in the wild boar population, demonstrating that replacement with the recently discovered PRV strains has not taken place. The other clusters, except cluster B, were classified as genomic type I isolates. Similar results were reported by Muller *et al*. (2010), who investigated several PRV strains isolated from wild boar in different European countries. Amino acid analysis revealed insertions and deletions. All strains belonging to cluster B show deletion of the amino acids at positions 24 and 181. There is

an insertion of seven amino acids in all the strains in the group formed by the Italian and Asian strains and a deletion of three amino acids in the Italian strains.

AD has virtually disappeared from domestic pigs in many parts of Europe, but it is still a problem in some countries, including Italy. The genomic characterization of SHV-1 strains originating from swine and other mammals might help us to better understand the population diversity and facilitate tracing the infection chain back to its origin. Finally, the molecular and phylogenetic analysis of PRV strains in this study contributes to a better understanding of the distribution of the virus clusters and their evolution.

	Year of isolation	Sample n°	Province of origin	Type of farm	Immune status	Species	Material	Accession number gC	Accession number gE
1	2010	28617	RE	fattening	vaccinated	Swine	brain	JQ768137	JQ619738
2		26940	LO	Farrow-to-weaning	vaccinated	Swine	lung	JQ768112	JQ619735
3	2008	7145	BS	fattening	vaccinated	Swine	brain	JQ768139	JQ619741
4		29652	MN	Farrow-to-weaning	vaccinated	Swine	pool of organs*	JQ768140	JQ619742
5		32501	BS	Farrow-to-weaning	vaccinated	Swine	pool of organs	JQ768110	JQ619743
6		35155	BS	Farrow-to-weaning	vaccinated	Swine	pool of organs	JQ768141	JQ619770
7		97897	MN	Farrow-to-weaning	vaccinated	Swine	pool of organs	JQ768111	JQ619771
8		280666	BS	Farrow-to-weaning	vaccinated	Swine	pool of organs	JQ768138	JQ619773
9	2006	252504	BS	fattening	vaccinated	Swine	pool of organs	JQ768126	JQ619747
10		291822	BS	fattening	vaccinated	Swine	lung	JQ768128	JQ619750
11	2003	32754	CR	Farrow-to-weaning	vaccinated	Swine	foetus	JQ768129	JQ619751
12		137181	BS	Farrow-to-weaning	vaccinated	Swine	foetus	JQ768123	JQ619752
13	2002	285	MO	fattening	vaccinated	Swine	lung	JQ768130	JQ619753
14		1317	LO	Farrow-to-finish	vaccinated	Swine	lung	JQ768131	JQ619754
15		24939	BG	Farrow-to-weaning	vaccinated	Swine	foetus	JQ768132	JQ619755
16		47586	BS	fattening	vaccinated	Swine	lung	JQ768133	JQ619756
17	2001	1993	BG	fattening	vaccinated	Swine	lung	JQ768134	JQ619758
18		2945	BS	fattening	vaccinated	Swine	lung	JQ768135	JQ619759
19		4028	MO	Farrow-to-finish	vaccinated	Swine	lung	JQ768136	JQ619760
20		8225	BN	fattening	vaccinated	Swine	heart	JQ768146	JQ619761
21		15142	LO	fattening	vaccinated	Swine	foetus	JQ768147	JQ619762
22		13038	BS	Farrow-to-finish	vaccinated	Swine	foetus	JQ768148	JQ619763
23		14082	CN	fattening	vaccinated	Swine	lung	JQ768124	JQ619764
24		14096	BS	fattening	vaccinated	Swine	lung	JQ768149	JQ619765
25		14754	BG	fattening	vaccinated	Swine	pool of organs	JQ768150	JQ619766
26	2000	2580	MN	fattening	vaccinated	Swine	lung	JQ768143	JQ619767
27		4058	BS	Farrow-to-weaning	vaccinated	Swine	pool of organs	JQ768144	JQ619768
28		4742	MI	Farrow-to-finish	vaccinated	Swine	pool of organs	JQ768113	JQ619769
29		11719	FO	fattening	vaccinated	Swine	lung	JQ768145	JQ619744
30		12022	MO	fattening	vaccinated	Swine	lung	JQ768127	JQ619748
31	1999	12455	PG	n.a. ^	vaccinated	Swine	pool of organs	JQ768162	JQ619734
32	1998	361	MO	fattening	vaccinated	Swine	lung	JQ768161	JQ619733
33	1997	3779-1	MN	fattening	n.a.	Swine	pool of organs	JQ768114	JQ619732
34	1996	2106	MI	Farrow-to-finish	n.a.	Swine	liver	JQ768118	JQ619737
35	1994	1369	RE	fattening	n.a.	Swine	pool of organs	JQ768160	JQ619731
36		1712	MN	n.a.	n.a.	Swine	lung	JQ768159	JQ619730
37		426	PC	fattening	n.a.	Swine	pool of organs	JQ768158	JQ619724
38		182	BS	Farrow-to-weaning	n.a.	Swine	foetus	JQ768157	JQ619723
39		111	FO	Farrow-to-weaning	n.a.	Swine	foetus	JQ768155	JQ619736

40	1991	900	FO	Farrow-to-weaning	n.a.	Swine	foetus	JQ768117	JQ619727
41	1989	36	-	n.a.	n.a.	Swine	foetus	JQ768152	JQ619726
42	1988	5658	BS	n.a.	n.a.	Swine	tonsils	JQ768151	JQ619725
43	1987	56	CR	n.a.	n.a.	Swine	brain	JQ768116	JQ619721
44	1984	54	BS	n.a.	n.a.	Swine	brain	JQ768142	JQ619720

Tabella n.1: Origine e descrizione dei ceppi di SHV-1

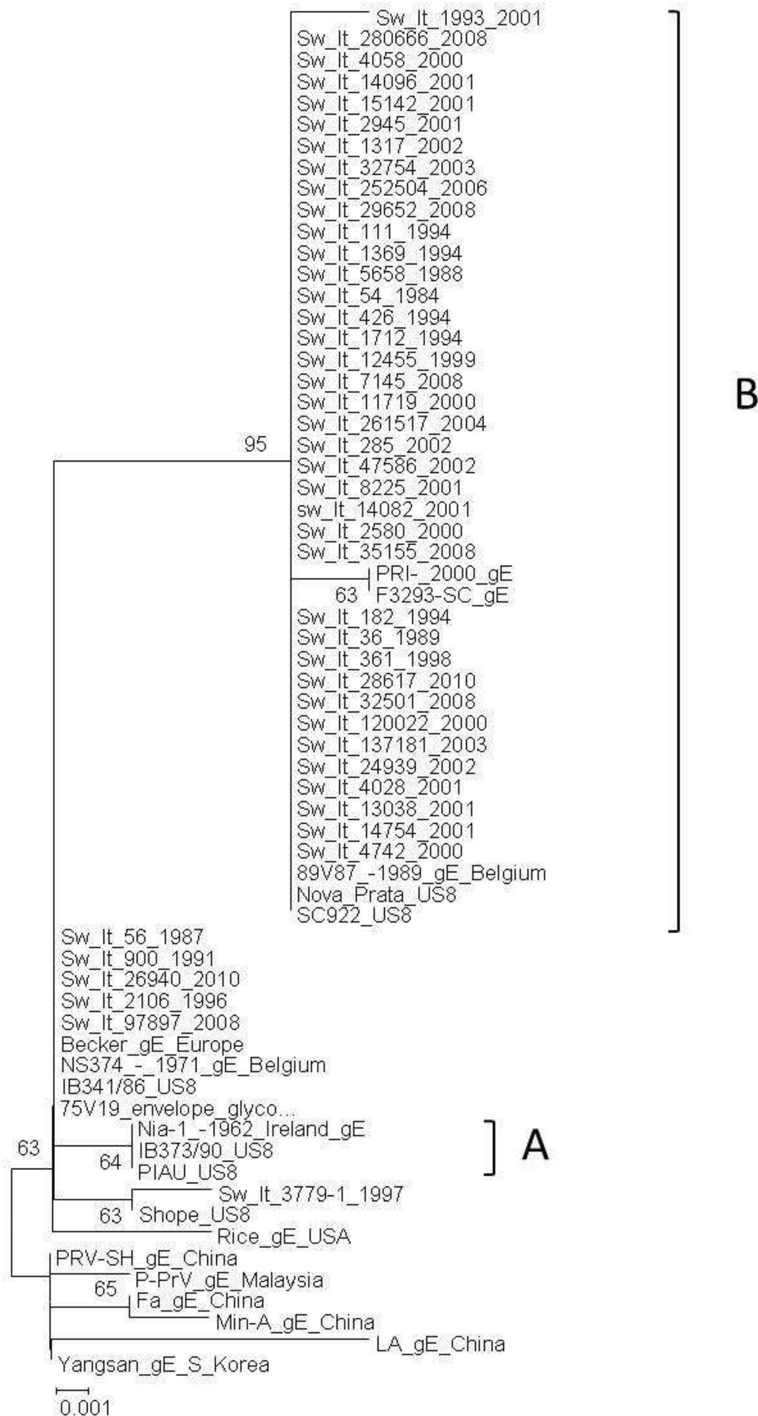


Figure n. 1: The phylogenetic tree based on the partial nucleotide sequence of the gE gene of Italian pseudorabies virus (PRV) isolates and corresponding sequences of PRV strains selected from GenBank. The tree was obtained by the neighbour-joining method and the Kimura two-parameters model with 1000 bootstrap replicates. Bootstrap percentage values (>60) are indicated at the nodes.

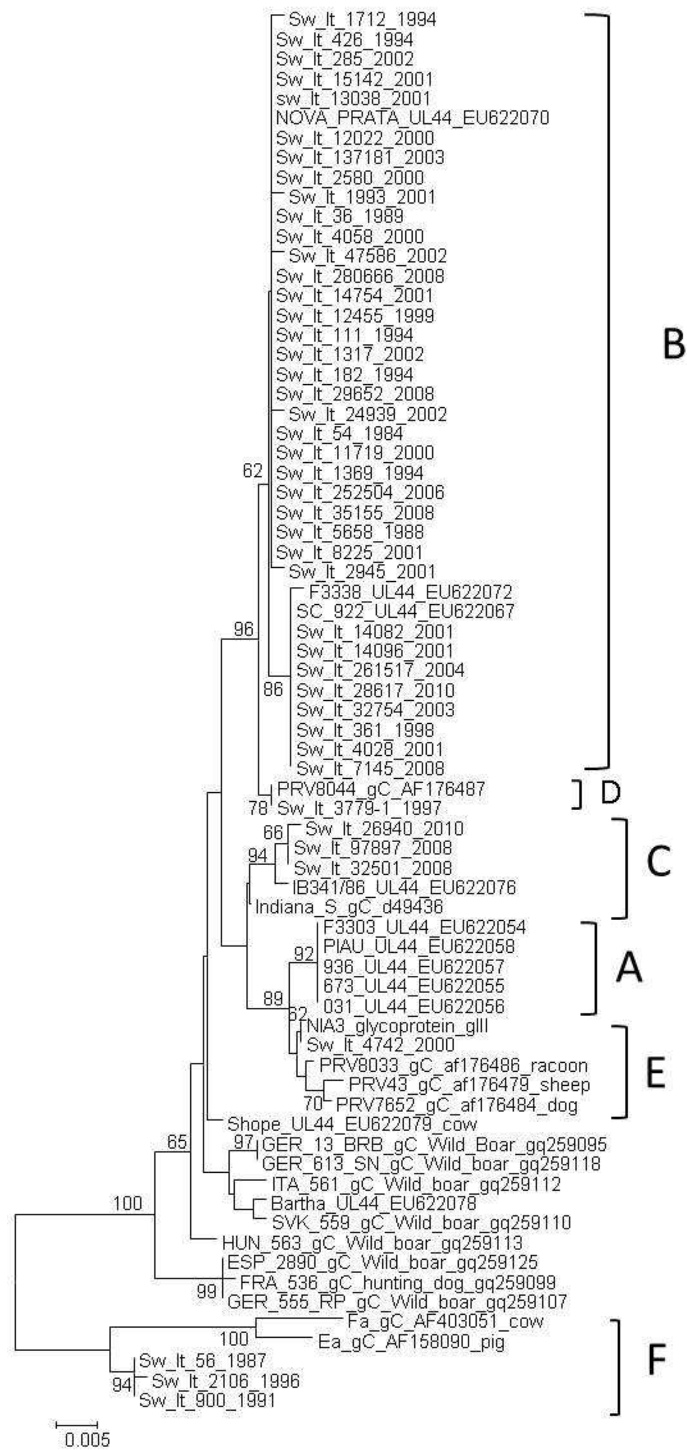


Figure n. 2: The phylogenetic tree based on the partial nucleotide sequence of the gC gene of Italian pseudorabies virus (PRV) isolates and corresponding sequences of PRV strains selected from GenBank.

Transboundary and Emerging Diseases

Transboundary and Emerging Diseases

ORIGINAL ARTICLE

Genomic Characterization of Pseudorabies Virus Strains Isolated in Italy

E. Sozzi, A. Moreno, D. Lelli, S. Cinotti, G. L. Alborali, A. Nigrelli, A. Luppi, M. Bresaola, A. Catella and P. Cordioli

Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna "B. Ubertini" (IZSLER), Brescia, Italy

Keywords:
pseudorabies virus; glycoprotein C; glycoprotein E; molecular characterization; Italy

Correspondence:
E. Sozzi. Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna "B. Ubertini" (IZSLER), via Bianchi 7/9, 25124 Brescia, Italy.
Tel.: +390302290289;
Fax: +390302290535;
E-mail: enrica.sozzi@izsler.it

Received for publication May 4, 2012

doi:10.1111/tbed.12038

Summary

In this study, we undertook the genomic characterization of 54 pseudorabies virus (PRV) strains isolated in Italy during 1984–2010. The characterization was based on partial sequencing of the UL44 (gC) and US8 (gE) genes; 44 strains (38 for gene gE and 36 for gC) were isolated on pig farms; 9 originated from dogs and 1 from cattle. These porcine PRV strains, which were closely related to those isolated in Europe and America in the last 20 years, and the bovine strain bovine/It/2441/1992 belong to cluster B in both phylogenetic trees. Six porcine strains that do not belong to cluster B are related in both gE and gC phylogenetic trees to the 'old' porcine PRV strains isolated in the 1970s and 1980s. In the last two decades, the presence of these strains in domestic pig populations has been reduced drastically, whereas they are prevalent in wild boar. The two remaining strains have an interesting genomic profile, characterized by the gC gene being closely related to the old porcine PRV strains, and the gE gene being similar to that of recently isolated strains. Three strains originating from working dogs on pig farms are located in cluster B in both phylogenetic trees. Five strains isolated from hunting dogs have a high degree of correlation with PRV strains circulating in wild boar. The last isolate has a gC gene similar to that in the two porcine strains mentioned previously, and the gE gene is correlated with the strains isolated from hunting dogs. These results provide interesting insight into the genomic characterization of PRV strains and reveal a clear differentiation between the strains isolated from hunting dogs that are related to the wild boar strains and those originating from domestic pigs.

Introduction

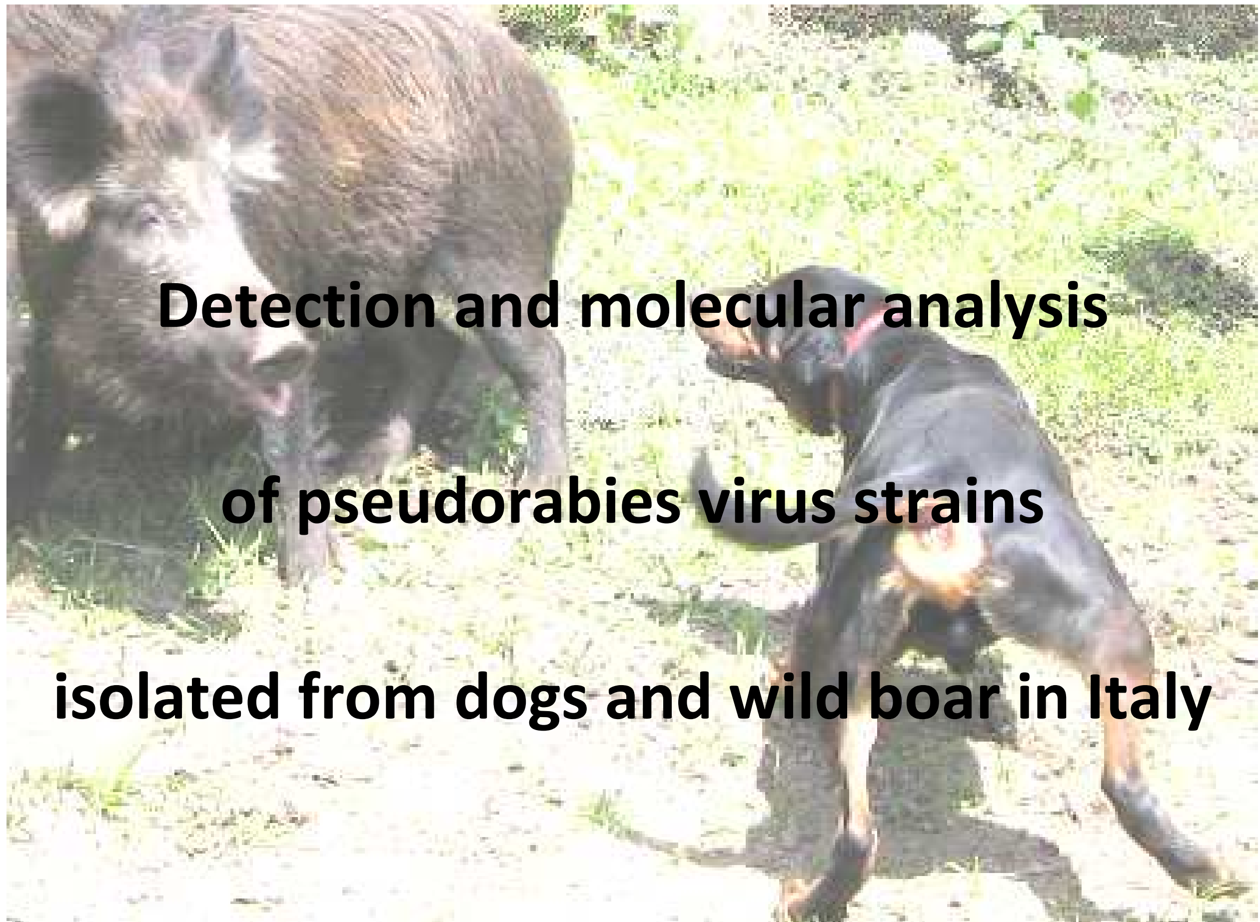
Pseudorabies virus (PRV) is the agent of Aujeszky's disease (AD), a neurological porcine disorder that was first described over a century ago (Aujeszky, 1902). AD occurs worldwide and causes great economic losses in the pig industry, owing to respiratory distress, nervous system disorders, genital disorders and consequent high mortality rate, according to the age of the host and the virulence of the virus strain involved. Despite its broad host range, which includes nearly all mammals except higher primates and humans, members of the Suidae family (domestic pigs and wild boar) are the only hosts capable of surviving a productive infection and serve as a reservoir of the virus.

PRV belongs to the Alphaherpesvirinae subfamily of the Herpesviridae family; it has a double-stranded, linear DNA genome of approximately 150 kb and belongs to the D class. This is characterized by two regions, UL and US, with the US region flanked by the internal repeat sequences (IRS and TRS, respectively). The viral genome encodes many proteins; 11 are glycosylated membrane proteins involved in virus entry, egress, cell-to-cell spread and modulation of immune response.

The glycoprotein C is a non-essential component of the virion envelope and is involved in the attachment of the virion to the cell (Mettenleiter, 1999). Immunologically, gC is a major target for both cellular and humoral immunity in pigs (Zuckermann et al., 1990; Katayama et al., 1997). It

 © 2013 Blackwell Verlag GmbH

Study n.3



Detection and molecular analysis of pseudorabies virus strains isolated from dogs and wild boar in Italy

Introduction

Although PRV has been eliminated in domestic pigs in many European countries, AD is being continuously reported in wild boar populations and in related hunting dogs (Albina *et al*., 2000; Gortazar *et al*., 2002; Lari *et al*., 2006; Leuenberger *et al*., 2007; Lutz *et al*., 2003; Muller *et al*., 2010; Pannwitz *et al*., 2012; Roic *et al*., 2012; Steinrigl *et al*., 2012; Verin *et al*., 2014; Vengust *et al*., 2005; Verpoest *et al*., 2014). Consequently, the possible impact of wild boars on the application and success of AD eradication programs and the risk they pose to the PRV-free status should be taken into account (Boadella *et al*., 2012). The role of wild boars as potential reservoirs of PRV has become increasingly important; thus, a deeper investigation on the distribution of PRV strains in wild boars and their genomic characterization at a regional level became necessary. AD surveillance plans were carried out (a) by testing for the presence of anti-PRV antibodies in sera samples collected within the wildlife national monitoring program in different regions of Italy (Lari *et al*., 2006, Montagnaro *et al*., 2010, Verin *et al*., 2014) and (b) by attempting PRV genome detection in wild boar samples voluntary submitted by hunters of some provinces of North Italy. In a previous study conducted on swine strains isolated before 2010, we showed a clear distinction between the strains isolated from wild boars and those originated from domestic pigs. In the following years, we continued to monitor the epidemiology of AD in Italy to better understand the distribution of the virus clusters and their evolution. In this study, we report the results of an AD survey based on PRV genome detection in samples from dogs clinically suspected of AD from 1993 to 2015 and from wild boars collected during four consecutive hunting seasons, 2010– 2014. Dogs in which AD was clinically suspected were examined by using histopathological and virological methods, resulting in the isolation of 22 PRV strains from dead dogs. One strain isolated from a wild boar during the same period and region was also genetically characterized. The phylogenetic

analysis was based on a partial sequence of the gC gene, and the results were compared with the sequences available in GenBank.

Materials and methods

Animals

A total of 20 dogs of different breeds, which were conferred to IZSLER laboratories in 2004–2015, and two dogs collected in 1993 and 1994 were included in the study. Of the dogs, fourteen were hunting dogs, six were dogs living in or close to pig farms (farm dogs), and although the last two dog's exposure was unknown, the dogs were unable to hunt. The origins and years of the PRV strain identifications are reported in Table 1. All of the hunting dogs had been used for hunting in the days immediately before the onset of clinical signs. Epidemiological investigations were conducted by the field veterinary services, and in all cases, the dogs' owners declared that animals had direct contact with wild boars or had been fed PRV-infected meat and/or offal. The clinical signs in the dogs included neurological signs, such as tremor, trismus, spasms of the muscles of the larynx and pharynx, dyspnea, vomiting and pruritus. Death occurred within 24–48 h. Wild boar tissue samples of lungs and tonsils were voluntarily collected by hunters in several provinces of North Italy during the period 2011–2014. The sampling sites were divided according two different ecologic areas, the Alps (AP) and the Apennines (AN). A total of 176 samples originated from AP and 155 from AN with the following per annum distribution: 2011- 86 AP, 3 AN; 2012- 89 AP, 3 AN; 2013- 1 AP, 99 AN; 2014- 48 AN. 2.2.

Laboratory investigations

Complete necropsies followed by virological examinations of selected organs were performed. The presence of PRV DNA in the field samples was systematically determined by real-time PCR tests

based on the specific detection of the gE gene as described by Yoon *et al*., 2005. Virus isolation was performed on PCR-positive samples as previously described. Histological and immunohistochemical investigations were completed only on fresh dog samples taken from well-preserved carcasses. A pool of three MAbs (1F2, 2E12 and 3D5) was used. Of these, MAb 1F2 recognized the gC protein, whereas the other two recognized the gE protein (Grieco *et al*., 1997).

Phylogenetic and molecular analysis

Partially sequencing of the gC genes was performed. Genomic DNA extraction from the cells culture supernatants was performed using an RNeasy kit (Qiagen, Hilden, Germany), and the PCR protocol described by Fonseca *et al*. (2010) was employed. The PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen, Inc., Valencia; CA, USA). DNA sequencing was performed using a Big-Dye Terminator Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) with the same primers that were used for amplification. The sequencing reactions were run by capillary electrophoresis on an automatic sequencer (ABI 3130 Genetic Analyser; Applied Biosystems, Foster City, CA, USA). The sequences were edited using the SeqMan program (DNASTAR, Madison, USA). The sequence alignments were performed using the ClustalW W method (DNASTAR, Madison, USA). The best-fit model of the nucleotide substitution was determined using the jModelTest v.0.1.1 (Posada, 2008). All of the models were compared using two criteria; i.e., the Akaike's information criterion (AIC) and the Bayesian information criterion (BIC). The preferred model was the HKY85 + I + G model. The phylogenetic tree was constructed with the maximum likelihood (ML) method within the IQ-tree software with bootstrap analyses involving 1000 replicates (Minh *et al*., 2013). The topologies were verified with the neighbor-joining method and the Kimura two-parameter model using MEGA 5.0 (Tamura *et al*., 2011). The Italian isolates were compared with the sequences of the references and field PRV strains that originated from other countries and are available at GenBank. The deduced amino acid sequences were aligned using

ClustalW, and the alignment was manually optimized. For the molecular analyses, the presence of aa deletions or insertions relative to the gC prototype sequence YP068347 was analyzed. The predicted glycosylation sites on the gC proteins of the Italian viruses were identified with NetNGlyc 1.0. A threshold value of a 0.5 average potential score was set to predict the glycosylated sites. The sequences described in this report have been deposited in the GenBank database under the accession numbers reported in Table 1.

Results

Laboratory investigations

During the necropsies, the external examinations of the carcasses revealed that the majority of animals had periorbital and facial abrasions that likely resulted from intense pruritus. Relevant internal gross lesions were not observed with the exception of mild to diffuse acute pulmonary alveolar emphysema and edema. Twenty two PRV strains were obtained from dog samples through inoculation onto PK-15 cells at the first passage, and the virus isolation was confirmed by PRV real-time PCR. Ten wild boar samples tested PCR-positive with the following spatiotemporal distribution: 2011- 5 AP, 2012- 2 AP, 2013- 2 AN, 2014 -1 AN. PCR-positive wild boar samples were also inoculated onto PK-15 cells, but the virus isolation and PCR for sequencing was attempted only in one case (WB-It-309516-2-2011). Immunohistochemistry performed in some dog samples revealed viral antigens in the cytoplasm of both neurons and glial cells within inflamed brain tissue.

Phylogenetic and molecular analysis

The phylogenetic analysis revealed that the Italian strains were divided into the following three clades. Clade 1 included fourteen strains from hunting dogs and one strain from a wild boar, and

they were closely related to another strain (ITA561) that was previously isolated from Italian wild boar in 1993 (Capua *et al*., 1997). This clade formed a separate Italian group (Italian clade 1) that was clearly distinguishable within Muller's clade A, which included the PRV European feral strains. Clade 2 was formed by three strains that were from three farm dogs and two non-hunting dogs of unknown exposure; these strains were closely related to Italian clade 2, field strains that were isolated in 2008–2011 from pigs and other farm dogs. This group exhibited a high percentage of similarity (99%) to the reference strain S66 that was isolated in Sweden and a Brazilian strain (IB341/86) that had not previously been included elsewhere. Clade 3 included the three strains isolated in the nineties from farm dogs which were closely related to pig strains isolated in Europe and America within the last 20 years (Fig. 1). The analysis of deduced amino acid (aa) sequences revealed that the Italian strains showed three different patterns of aa deletions or insertions that corresponded to the three phylogenetic clades. Italian clade 1 was characterized by only a single aa deletion at position 25. Italian clade 2 carried two deletions at positions 25 and 39. Clade 3 contained two aa deletions at positions 25 and 39 and, relative to the prototype sequence, and contained one insertion between positions 184 and 185 (184A). The analysis was then focused on the hot spot region located in the gC protein between residues 180 and 185, which showed the largest aa variations and were associated with changes in the hydrophobicity profile (Fonseca *et al*., 2012). Two different aa profiles were identified in the Italian strains; the first profile (VVVE) was related to the wild boar strains and was present in Italian clades 1 and 2. Interestingly, Italian clade 2 included only strains isolated recently from domestic pigs and from dogs unable to hunt and hence not exposed to the wild boar population. The second profile (ALDDD) was present in clade 3 and was closely related to the pig strains. The analysis of the predicted glycosylation sites in the gC protein showed a highly conserved pattern in all of the PRV strains. It was characterized

by three glycosylation sites at positions 40, 84 and 192 according to the prototype sequence YP068347 numbering (Fig. 2).

Discussion

Based on the gC analysis, the Italian PRV strains were primarily distributed into three different clusters, and this distribution revealed interesting findings. There was a clear distinction between both the wild boar strains (and those isolated from dogs that were used for hunting and subsequently traced back to wild boars) and the strains isolated from working farm dogs (and found to be closely related to strains in domestic pigs). The genetic results also indicated that the hunting dog and wild boar strains exhibited high homology to the PRV strains that were circulating in the 70 s and 80 s in pig farms. Indeed, these strains have nearly completely disappeared from commercial pig herds but are still circulating in feral pig populations and thus demonstrate that, in wild populations, the replacement of older strains with more recent PRV swine strains has not yet taken place. The Italian epidemiology was also found to be different from those of other European countries and was characterized by the presence of two typical Italian clades (Figure n.3). Strains originated from wild boars and epidemiologically related to hunting dogs belonged to clade A (Muller *et al*., 2010), although these formed a clearly distinguishable group (Italian clade 1). No strains included in clade B were found. These two clades, A and B, seem to overlap geographically in Central Europe but not in Italy, as isolates of both clades were found in German, Belgium and France (Muller *et al*., 2010; Verpoest *et al*., 2014). Strains related to those found in domestic pigs were conversely distributed in two different clades. One of these grouped strains, isolated until 2010, were related to pig strains isolated in Europe and America within the last 20 year. Studies previously conducted in Italy detected a high PRV seroprevalence mainly in the in wild boar populations of the Apennine Mountains (Lari *et al*., 2006; Montagnaro *et al*., 2010; Verin *et al*.,

2014). In contrast to this seroprevalence, PCR-positive samples were detected in a very few cases originating only from the Alps. PCR examination was performed on pooled wild boar tissues and in some cases only lung tissue was available. Similar to our results, the detection of a low number of PCR positive samples was previously reported (Steinrigl *et al*., 2012; Lutz *et al*., 2003) when non-CNS tissues were analyzed. Unfortunately, several samples were not well preserved and only one strain could be isolated and sequenced. PCR results observed in this study were biased by sampling, thus likely not representative of the AD prevalence, and were used only to perform a better genomic characterization of PRV strains circulating in our country. All of the other isolates were obtained from clinically affected dogs that were divided into hunting dogs (n. 14) epidemiologically related to wild boars and dogs living in pig farms (n. 6) and then related to domestic pigs. Moreover, for two dogs, no data were available, but it were unable to hunt and thus not exposed to wild boars. Sequence analysis further supported these two different epidemiologic linkages, showing a clear relationship between hunting dogs and wild boars and a distinct distribution for strains related to domestic pigs. AD infections could be transmitted from domestic pigs to wild boars and vice versa as demonstrated by the presence of some domestic pig and wild boar isolates that cluster together (Muller *et al*., 2010; Verpoest *et al*., 2014). Moreover, the transmission between both populations was confirmed in experimental infections (Muller *et al*., 2001). Despite these considerations, the role of wild boars as AD reservoirs for domestic pigs has been rejected by Muller *et al*. (2011) because genomic analysis evidenced genomic differences between virus strains in the two populations. Our results are in line with this hypothesis, suggesting the presence in Italy of two distinct infection cycles related to wild boars and domestic pigs. Although the region used for sequence analysis is a small part of the complete SHV1 genome and deeper studies of genomic characterization should be taken into account, our results suggested that Italian wild boars and domestic pigs could provide two distinct ecological niches for

the Italian AD strains. Molecular analysis and the presence of amino acid insertions/deletions were consistent with the phylogenetic analysis and confirmed the presence of two Italian clades, which were characterized by two patterns not described in other European strains. Additionally, the amino acid sequence in the hot spot region is different in the two Italian clades. It has been hypothesized that the complete substitution (from VVVE to ALDDD), evidenced in the recent domestic pig strains and in our group 3, is related to changes in the hydrophilicity profile and then the region could be more antigenically exposed (Fonseca *et al*., 2012). Interestingly, Italian clade 2, which included recent domestic pig strains and epidemiologically related dog strains, did not show these substitutions and showed a profile similar to the wild boar strains. Sequencing of more extended parts of genome should be taken into account to better characterize these Italian strains. Moreover, it would be interesting to perform additional in vivo infection experiments in wild boars and pigs using strains belonging to the Italian clades to deeper investigate their pathogenesis in different hosts. Although several regions of Northern Italy have implemented AD eradication programs in domestic pigs, the risk of transmission from wild boars to domestic pigs should not be completely ruled out and may be considered a constant threat. If preventive measures are disregarded, transmission between wild boars and free-range pigs or outdoor pigs might be possible. Therefore, the epidemiology in domestic pigs and wild boars should be continuously monitored and assessed.

Year	N°	Sample n.	Province of origin	Species (attitude) –Breed	Age (years)	gC clade	Accession number gC
1993	1	3718	Piacenza	Farm Dog- n.a.	n.a.	Clade 3	KP780805
1994	2	736	Piacenza	Farm Dog- n.a.	n.a.	Clade 3	KP780806
2004	3	249465	Bologna	Hunting Dog –n.a	n.a.	Italian Clade 1	JQ768122
2007	4	13814	Cremona	Farm Dog- n.a.	n.a.	Clade 3	JQ768125
2008	5	203379	Reggio Emilia	Hunting Dog –n.a.	n.a.	Italian Clade 1	JQ768119
2009	6	980	Reggio Emilia	Hunting Dog –n.a.	n.a.	Italian Clade 1	JQ768120
2010	7	294871	Piacenza	Hunting Dog –n.a.	n.a.	Italian Clade 1	KP862621
	8	325409	Bologna	Hunting Dog –n.a.	n.a.	Italian Clade 1	JQ768115
	9	325415	Bologna	Hunting Dog –n.a.	n.a.	Italian Clade 1	JQ768121
	10	101452	Brescia	Dog –n.a	n.a.	Italian Clade 2	JQ768109
2011	11	286509	Brescia	Hunting Dog- Maremma-Hound	4	Italian Clade 1	KP862611
	12	286672	Pavia	Hunting Dog - Mongrel	4	Italian Clade 1	KP862612
	13	290422	Brescia	Hunting Dog- Hound	3	Italian Clade 1	KP862613
	14	309516-2	Brescia	Wild Boar	n.a.	Italian Clade 1	KP893284
	15	310919/1	Brescia	Hunting Dog- Jura hound dog	>5	Italian Clade 1	KP862614
	16	310919/2	Brescia	Hunting Dog- Maremma - Hound	>5	Italian Clade 1	KP862615
2012	17	4966	Cremona	Farm Dog –n.a.	n.a.	Italian Clade 2	KP862616
	18	22640	Milano	Farm Dog –n.a.	<1	Italian Clade 2	KP862617
	19	160938	Napoli	Dog – n.a.	n.a.	Italian Clade 2	KP862618
2014	20	299424	Piacenza	Hunting Dog –n.a.	2	Italian Clade 1	KP862619
	21	360167	Forlì	Hunting Dog –n.a.	n.a.	Italian Clade 1	KP862620
2015	22	190449	Bologna	Farm Dog- n.a.	n.a.	Italian Clade 2	-
	23	147046	Parma	Hunting Dog –n.a.	n.a.	Italian Clade 1	-

Table n. 1 – Data of the Italian PRV strains used in the study

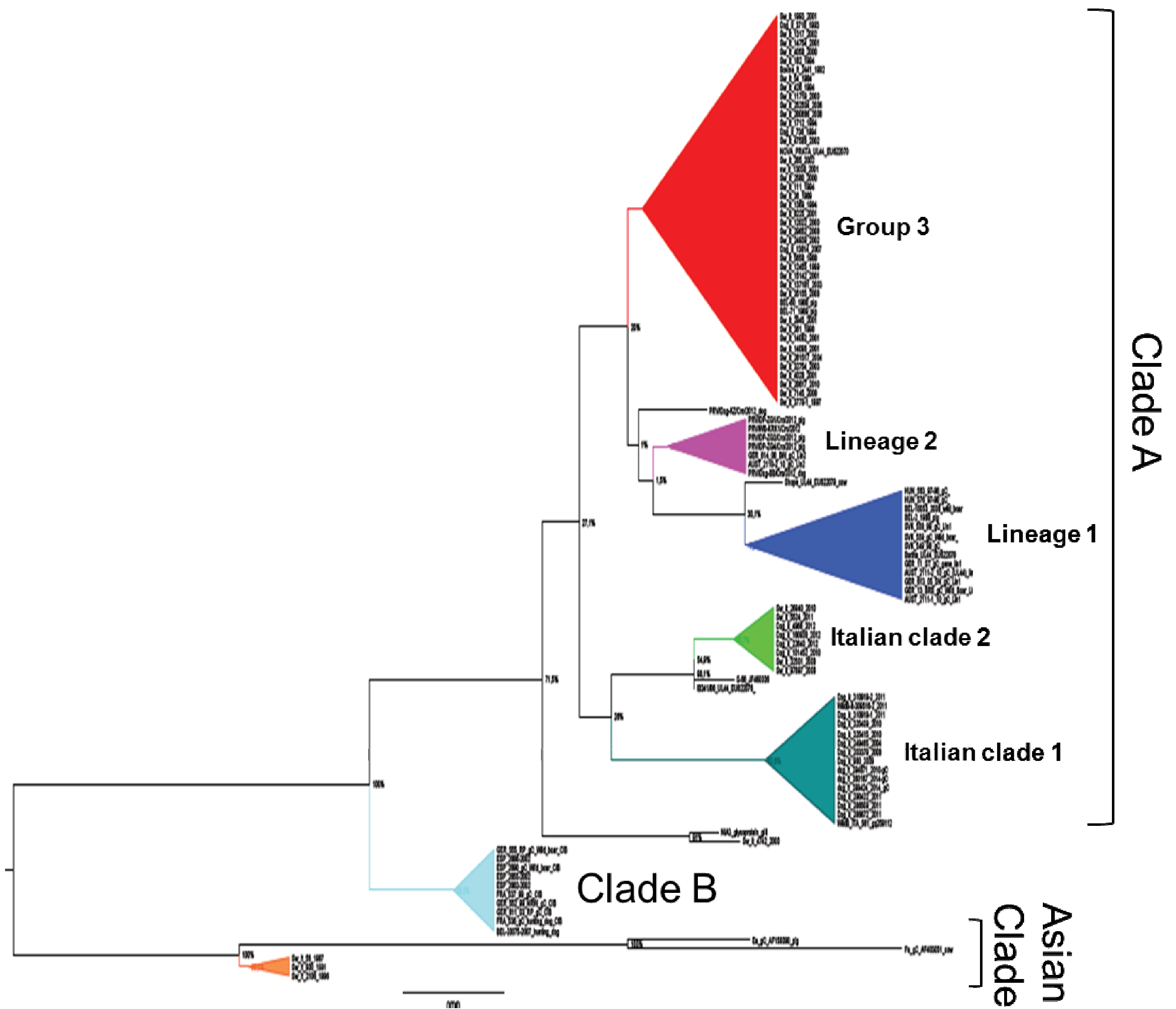


Figure n. 1: Phylogenetic tree based on the partial nucleotide sequences of the gC gene (690 bp) of the Italian pseudorabies virus (PRV) isolates (boldface) and the corresponding sequences of the selected PRV strains from GenBank. The tree was obtained using the maximum likelihood method and the HKY85 + I + G model with 1000 bootstrap replicates. The bootstrap percentage values are indicated at the nodes.

```
                                                           25             39
#YP068347_Prototype_sequence   MASLARAMLA LLALYAAAIA AAPSTTTALD TTPNGGGGGN SSEGELSPSP PPTPAPASPE AGAVSTPPVP PPSVSRRKPP  [ 80]
#NIA3_glycoprotein_gIII        .......... .......... .........G .......... .......... .......... .......... ..........  [ 80]
#WildB-It-309516-2_2011_It_cl1 .......... .......... ....-....G .......... .......... ........G. .......RA. ..........  [ 80]
#Dog_It_22640_2012_It_cl2      .......... .......... ....-....G ........-. .......... .......... V......RA. ..........  [ 80]
#Dog_It_3718_1993_clade_3      .......... .......... ....-....G ........-. .......... .......... .......RA. ..........  [ 80]

                                  84
#YP068347_Prototype_sequence   RNNNRTRVHG DKATAHGRKR IVCRERLFSA RVGDAVSFGC AVFPRAGETF EVRFYRRGRF RSPDADPEYF DEPPRPELPR  [160]
#NIA3_glycoprotein_gIII        .......... .......... .......... .......... .......... .......... .......... ..........  [160]
#WildB-It-309516-2_2011_It_cl1 .........S .......... .......... .......... .......... .......... .......... .....SD...  [160]
#Dog_It_22640_2012_It_cl2      .......... .......... ....G..... .......... .......... .......... .......... ..........  [160]
#Dog_It_3718_1993_clade_3      .......... .......... .......... .......... .......... .......... .......... ..........  [160]

                                                  180   185       192
#YP068347_Prototype_sequence   ERLLFSSANA SLAHADALAP VVVE-GERAT VANVSGEVSV RVAAADAETE GVYTWRVLSA NGT [223]
#NIA3_glycoprotein_gIII        .......... .......... ....-..... .......... .......... .......... ... [223]
#WildB-It-309516-2_2011_It_cl1 .......... .......... ....-.G... .......... .......... .......... ... [223]
#Dog_It_22640_2012_It_cl2      .......... .......... ....-..... .......... .......... .......... ... [223]
#Dog_It_3718_1993_clade_3      .......... .......... ALDDD.G... .......... .......... .......... ... [223]
```

Figure n. 2: Amino acid alignment of deduced gC protein sequences from three strains representatives of the Italian clades 1 and 2 and group 3. The position of deletions and insertions are reported respect to the prototype sequence YP068347. The hot spot region is indicated by a box. N-glycosylation sites are shown in bold face.

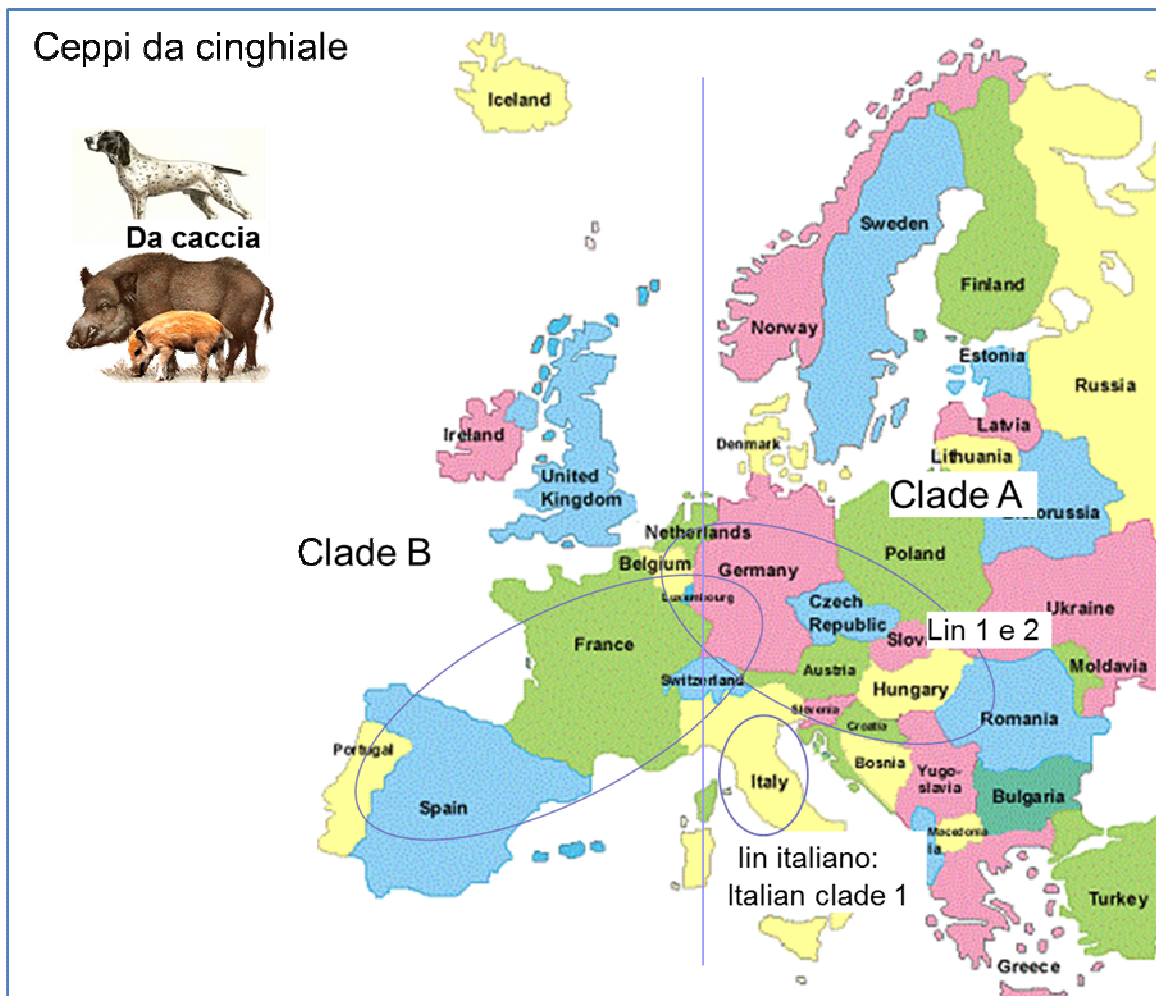
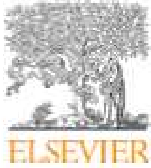


Figure n. 3: Geographical distribution of PRV isolated

Veterinary Microbiology 177 (2015) 359–365



Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Short Communication

Detection and molecular analysis of Pseudorabies virus strains isolated from dogs and a wild boar in Italy

Ana Moreno [*,2], Enrica Sozzi [2], Guido Grilli [1], Lucia Rita Gibelli, Daniela Gelmetti, Davide Lelli, Mario Chiari, Paola Prati, Giovanni Loris Alborali, Maria Beatrice Boniotti, Antonio Lavazza, Paolo Cordioli

National Reference Center for Aujeszky Disease - Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER), via Bianchi 7/9, 25124 Brescia, Italy

ARTICLE INFO

Article history:
Received 14 November 2014
Received in revised form 27 March 2015
Accepted 1 April 2015

Keywords:
Pseudorabies virus
Dogs
Wild boar
Diagnosis
Molecular characterization
Italy ;

ABSTRACT

Aujeszky's disease (AD) is one of the most economically important diseases of farmed pigs. Wild boars can act as reservoirs and might represent a potential threat for domestic animals, including dogs. The aim of this study was to report the results of an AD survey based on the Pseudorabies virus (PRV) genome detection in samples of dogs clinically suspected of AD and of wild boars collected during four consecutive hunting seasons in the period 2010–2014. Genomic characterization was based on the partial gC sequence of the Italian strains and the comparison with those from domestic pigs and European PRV strains circulating in wild boars. The Italian PRV strains were mainly distributed into three different clusters and revealed two interesting findings. First, there was a clear distinction between the viral strains that were isolated from dogs used for hunting and subsequently traced back to wild boars and the strains that were isolated from working dogs and subsequently found to be closely related to domestic pigs. Second, the Italian epidemiological situation was found to be different from those of European countries in that the Italian situation was characterized by the presence of both the typical Italian clades 1 and 2 and supported by new patterns of aa deletions/insertions. Italian clade 1 included strains from hunting dogs and two Italian wild boars, and Italian clade 2 grouped with recent strains from dogs that were unable to hunt and domestic pigs that were related to one old reference strain (S66) and not included elsewhere. Molecular and phylogenetic analyses of PRV strains are therefore necessary to improve the understanding of the distribution of the PRV clusters and their evolution.

© 2015 Elsevier B.V. All rights reserved.

1. Introduction

Pseudorabies virus (PRV) is the causal agent of Aujeszky's disease (AD), an animal disease primarily affecting pigs but also known to occur occasionally in cattle, sheep, goats, horses, dogs and cats. Suids are the natural reservoir of the virus, and the disease is self-limiting in the other species. AD is a contagious infection and is mainly transmitted by direct and indirect contact between pigs.

The causative agent is an enveloped DNA virus, which belongs to the Alphaherpesvirinae subfamily of the

* Corresponding author. Tel.: +39 0302290361; fax: +39 0302290535.
E-mail addresses: anamaria.morenomartin@izsler.it (A. Moreno), enrica.sozzi@izsler.it (E. Sozzi), guido.grilli@unimi.it (G. Grilli), luciarita.gibelli@izsler.it (L.R. Gibelli), daniela.gelmetti@izsler.it (D. Gelmetti), davide.lelli@izsler.it (D. Lelli), mario.chiari@izsler.it (M. Chiari), paola.prati@izsler.it (P. Prati), giovanni.alborali@izsler.it (G.L. Alborali), mariabeatrice.boniotti@izsler.it (M.B. Boniotti), antonio.lavazza@izsler.it (A. Lavazza), paolo.cordioli@izsler.it (P. Cordioli).
[1] Dipartimento di Scienze Veterinarie e Sanità Pubblica, – Università degli Studi di Milano, Via Celoria 10, 20121 Milano, Italy.
[2] Both authors contributed equally.

http://dx.doi.org/10.1016/j.vetmic.2015.04.001
0378-1135/© 2015 Elsevier B.V. All rights reserved.

General conclusion

Pseudorabies virus (PRV) is the agent of Aujeszky's disease (AD), a neurological porcine disorder that was first described over a century ago (Aujeszky, 1902). AD occurs worldwide and causes great economic losses in the pig industry, owing to respiratory distress, nervous system disorders, genital disorders and consequent high mortality rate, according to the age of the host and the virulence of the virus strain involved. Despite its broad host range, which includes nearly all mammals except higher primates and humans, members of the *Suidae* family (domestic pigs and wild boar) are the only hosts capable of surviving a productive infection and serve as a reservoir of the virus. PRV belongs to the *Alphaherpesvirinae* subfamily of the *Herpesviridae* family; it has a double-stranded, linear DNA genome of approximately 150 kb and belongs to the D class. This is characterized by two regions, UL and US, with the US region flanked by the internal repeat sequences (IRS and TRS, respectively). The viral genome encodes many proteins; 11 are glycosylated membrane proteins involved in virus entry, egress, cell-to-cell spread and modulation of immune response. The glycoprotein C is a non-essential component of the virion envelope and is involved in the attachment of the virion to the cell (Mettenleiter, 1999). Immunologically, gC is a major target for both cellular and humoral immunity in pigs (Zuckermann *et al*., 1990; Katayama *et al*., 1997). It is considered as the more variable part of the PRV genome owing to genetic alterations as a result of antibody selection. Sequence-level genetic variation in gC was reported by Ishikawa *et al*. (1996) as 2–3% among strains from diverse geographic areas. The glycoprotein E appears to have a prominent role in the spread of PRV within the nervous system. Deletion of the non-essential envelope glycoprotein E leads to a significant decrease in the virulence of some PRV strains. Glycoproteins E and I form a complex via a non-covalent bond that is expressed on the surface of PRV-infected cells (Jacobs *et al*., 1994). Pseudorabies virus primarily affecting pigs but also known to occur occasionally in cattle, sheep, goats, horses, dogs and cats. AD is a notifiable disease that causes substantial economic losses to the swine industry and has major economic

impact due to trade implications and income losses for farmers. Italian pig production is concentrated (over 80%) in the four Northern Italian regions of Lombardia, Emilia-Romagna, Piemonte and Veneto. The Lombardia region is the major producer, with 50% of the national pig production (Maiorano, 2009). Compared with the pig production of other countries, Italian pig production differs in the high live weight of pigs at slaughter; they are slaughtered at 10–12 months of age when they reach a live weight of 150–170 kg and provide carcasses of 125–140 kg maintaining proper adiposity. The European general policy is to eradicate AD in order to support free intra-EU trade. However, eradication may take several years depending on the epidemiological situation in countries where the disease is endemic. In Italy, an AD national monitoring program was implemented in 1997. However, since 2011, with the issuing of Decree 30/12/2010 and the latest update in 2014, the National Authority has put in place more strict measures to ensure a substantial reduction in virus circulation on pig farms. Thereafter, extraordinary regional control plans were implemented, especially in the Northern Italian regions with the highest concentrations of pig industry (Lombardia Region, D.d.s. 9/05/2014 n. 3822; Emilia Romagna Region, Delibera Giunta Regionale n. 1588–13/10/2014; Veneto Region, Delibera Giunta Regionale n. 2061–11/10/2012), to achieve AD eradication status and to be included in annex two of the EU Decision 2008/185/EC. Important tools in AD eradication plans included the use of DIVA gE-deleted vaccines, movement restrictions, more intensive serological testing and the application of stamping out or slaughter policies to remove infected animals. Only the Bolzano province was classified in this annex that includes member states or regions that applied disease controls programs and have already eradicated AD or are in an advanced stage of eradication. In this thesis I present three studies. The first study report the results of a Aujeszky Disease monitoring programme performed in Northern Italy during the period 2012-2014. This was based on control of all respiratory, nervous and reproductive forms, genome detection and virus

isolation, serological investigations. To analyse the distribution of the Aujeszky Disease and evaluate the epidemiological situation in the area of IZSLER. Today, the virus has spread worldwide and causes economic losses in the pig industry due to increased mortality rates, depending on the age of the host and the virulence of the virus strain involved. The serological tests for the detection of antibodies against the gE of Aujeszky Disease virus performed in 2013-2014 in the course of activities on the control plan, revealed a percentage of positive samples for antibodies to gE by 9.3% with a slight increase. This increase is likely due to the significant increase of serological tests carried out mainly in Lombardy as a result of the approval of the new plan to control and mainly targeting the control of farms for fattening with unknown health status.

The long-term surveillance of AD in the wild boar of the Norther Italy showed an increase in seroprevalence during the years, with a higher probability of being seropositive in older individuals, while the presence of domestic pigs had no effect on their seropositivity. In particular, the total AD seroprevalence in the study area increased, showing that the infection could persist as an endemic disease at low prevalence values in wild boar populations. This was also supported by the results of the molecular analyses, where the PCR positive animals confirmed the presence of ADV, even with a low diffusion, inside the wild boar population. The second study report the genomic characterization of PRV based on sequencing genes US8 and UL44, encoding the gE and gC proteins, respectively of 44 PRV strains, originated from pigs isolated between 1984 and 2010. The results shows that the most predominant group in Italy, with 36 of the 44 PRV isolates belonging to cluster B, is referable to genotype II. Another group, cluster C, consists of isolates closely related to wild boar strains isolated in Europe. These strains showing a high degree of homology to PRV strains circulating in the 1970s and 1980s have almost disappeared. By contrast, strains similar to the old PRV strains are still circulating in the wild boar population, demonstrating that replacement with the recently discovered PRV strains has not taken place. The other clusters,

except cluster B, were classified as genomic type I isolates. Similar results were reported by Muller *et al*. (2010), who investigated several PRV strains isolated from wild boar in different European countries. Finally, the third study report the results of an AD survey based on phylogenetic analysis was based on the gC in samples from dogs clinically suspected of AD (22 PRV strains) and from wild boars collected during four consecutive hunting seasons, 2010– 2014 (one strain isolated from a wild boar). The Italian PRV strains were primarily distributed into three different clusters, and this distribution revealed interesting findings. There was a clear distinction between both the wild boar strains (and those isolated from dogs that were used for hunting and subsequently traced back to wild boars) and the strains isolated from working farm dogs (and found to be closely related to strains in domestic pigs). The genetic results also indicated that the hunting dog and wild boar strains exhibited high homology to the PRV strains that were circulating in the 70 s and 80 s in pig farms. Indeed, these strains have nearly completely disappeared from commercial pig herds but are still circulating in feral pig populations and thus demonstrate that, in wild populations, the replacement of older strains with more recent PRV swine strains has not yet taken place. The Italian epidemiology was also found to be different from those of other European countries and was characterized by the presence of two typical Italian clades. Strains originated from wild boars and epidemiologically related to hunting dogs belonged to clade A (Muller *et al*., 2010), although these formed a clearly distinguishable group (Italian clade 1). No strains included in clade B were found. These two clades, A and B, seem to overlap geographically in Central Europe but not in Italy, as isolates of both clades were found in German, Belgium and France. Strains related to those found in domestic pigs were conversely distributed in two different clades. One of these grouped strains, isolated until 2010, were related to pig strains isolated in Europe and America within the last 20 year. The present long-term surveillance may provide baseline information on the dynamics of AD infections under natural conditions. In addition, these results

together with the absence of evidence of epidemiological association of ADV between the domestic pig and the wild boar in the study area corroborate the hypothesis that AD maintenance in the wild boar population is independent of the occurrence of AD in pigs (Muller *et al*. 1998; Ruiz-Fons *et al*. 2008b; Pannwitz *et al*. 2012). Nevertheless, since spillovers cannot completely be ruled out and due to the fact that these results reinforce the idea that wild boar is able to maintain the virus at natural condition also at low density values, open-air pig systems, in particular, might be at risk if preventive measures are disregarded (Pannwitz *et al*. 2012; Ruiz-Fons *et al*. 2008a, b). These findings may be considered when implementing ADV eradication programmes in livestock, in particular, in areas where the wild boar population is maintained at a low density, as in the Alps.

Reference

- Akkermans J.P., Aujeszky's disease in the Netherlands, Pig Vet. Soc. Proc. (1980) 5:59–67.
- Albina E, Mesplede A, Chenut G, Le Potier MF, Bourbao G, Le Gal S, Leforban Y (2000) A serological survey on classical swine fever (CSF), Aujeszky's disease (AD) and porcine reproductive and respiratory syndrome (PRRS) virus infections in French wild boar from 1991 to 1998. Veterinary Microbiology 77:43–57
- Aujeszky A (1902) Uber ein neue Infektionskrankheit bei Haustieren. Zentralbl Bakteriol Parasitenkd Infektionskr Hyg Abt 32:353–357
- Baskerville A., The histopathology of pneumonia produced by aerosol infection of pigs with a strain of Aujeszky's disease virus, Res. Vet. Sci. (1971) 12:590–592.
- Ben-Porat T., Kaplan A.S., Molecular biology of pseudorabies virus, in: Roizman B. (Ed.), The Herpesviruses, Plenum press, 1985, Vol. 3, pp. 105–173.
- Ben-Porat, T., DeMarchi, J.M., Lomniczi. B. and Kaplan, AS., 1986. Role of glycoproteins of pseudorabies virus in eliciting neutralizing antibodies. Virology, 154: 325-334.
- Berthomme H., Monahan S.J., Parris D.S., Jacquemont B., Epstein A.L., Cloning, sequencing and functional characterization of the two subunits of the pseudorabies virus DNA polymerase holoenzyme: evidence for specificity of interaction, J. Virol. (1995) 69:2811–2818.
- Bitsch V., Correlation between the pathogenicity of field strains of Aujeszky's disease virus and their ability to cause cell fusion – syncytia formation – in cell cultures, Acta Vet. Scand. (1980) 21:708–710.
- Boadella M, Gorta´zar C, Vicente J, Ruiz-Fons F (2012) Wild boar: an increasing concern for Aujeszky's disease control in pigs? BMC Veterinary Research 8:7.

- Brideau A.D., Banfield B.W., Enquist L.W., The Us9 gene product of pseudorabies virus, an alphaherpesvirus, is a phosphorylated tailanchored type II membrane protein, J. Virol. (1998) 72:4560–4570.
- Brittle, E. E., A. E. Reynolds, and L. W. Enquist. 2004. Two modes of pseudorabies virus neuroinvasion and lethality in mice. J. Virol. 78:12951–12963.
- Brockmeier, S. L., K. M. Lager, and W. L. Mengeling. 1993. Comparison of in vivo reactivation, in vitro reactivation, and polymerase chain reaction for detection of latent pseudorabies virus infection in swine. J. Vet. Diagn. Investig. 5:505–509.
- Capua, I., C. Casaccia, G. Calzetta, and V. Caporale, 1997: Characterisation of Aujeszky's disease viruses isolated from domestic animals and from a wild boar (Sus scrofa) in Italy between 1972 and 1995. Vet. Microbiol. 57, 143–149.
- Chang Y.Y., Lin H.W., Wong M.L., Chang T.J., Regulation of the vhs gene promoter of pseudorabies virus by IE180 and EP0, and the requirement of a Sp1 site for the promoter function, Virus Genes (2004) 28:247–258.
- Cheung, A. K., J. Fang, and R. D. Wesley. 1994. Characterization of a pseudorabies virus that is defective in the early protein 0 and latency genes. Am. J. Vet. Res. 55:1710–1716.
- Coe, N.E. and Mengeling, W., 1990. Mapping and characterization of neutralizing epitopes of glycoproteins gII1 and gp50 of the Indiana-Funkhauser strain of pseudorabies virus. Arch. Viral., 110: 137-142.
- Corn JL, Cumbee JC, Barfoot R, Erickson GA (2009) Pathogen exposure in feral swine populations geographically associated with high densities of transitional swine premises and commercial swine production. Journal of Wildlife Disease 45:713–721.
- Davies, E. B., and G. W. Beran. 1980. Spontaneous shedding of pseudorabies virus from a clinically recovered postparturient sow. J. Am. Vet. Med. Assoc. 176:1345–1347.

- De Wind N., Berns J., Gielkens A.L., Kimman T.G., Ribonucleotide reductasedeficient mutants of pseudorabies virus are avirulent for pigs and induce partial protective immunity, J. Gen. Virol. (1993) 74:351–359.
- Elford W.J., Galloway I.A., The size of the virus of Aujeszky's disease ("pseudorabies", "infectious bulbar paralysis", "mad itch") by ultrafiltration analysis, J. Hyg. (Cambr.) (1936) 36:536–539.
- Eloit. M., Bouzghaia. H. and Toma, B.. 1990. Identification of antigenic sites on pseudorabies virus
- Enquist, L. W. 1999. Life beyond eradication: veterinary viruses in basic science. Arch. Virol. Suppl. 15:87–109.
- Field, H. J., and T. J. Hill. 1974. The pathogenesis of pseudorabies in mice following peripheral inoculation. J. Gen. Virol. 23:145–157.
- Fonseca Jr., A.A., Camargos, M.F., Sales, M.L., Heinemann, M.B., Leite, R.C., Reis, J.K., 2012. Pseudorabies virus can be classified into five genotypes using partial sequences of UL44. Braz. J Microbiol. 43 (4), 1632–1640.
- Fonseca, A. A. Jr., F. M. Camargos, A. Macedo de Oliveira, J. Ciacci-Zanella, M. A. C. Patricio, A. C. Braga, E. S. Cunha, R. D'Ambros, M. B. Heinemann, R. C. Leite, and J. K. Pimenta dos reis, 2010: Molecular epidemiology of Brazilian pseudorabies viral isolates. Vet. Microbiol. 141, 238–245.
- Fuchs W., Granzow B.G., Klupp B.G., Kopp M., Mettenleiter T.C., The UL48 tegument protein of pseudorabies virus is critical for intracytoplasmic assembly of infectious virus, J. Virol. (2002a) 76:6729–6742.

- Fuchs W., Klupp B.G., Granzow H., Hengartner C., Brack A., Mundt A., Mettenleiter T.C., Physical interaction between envelope glycoproteins E and M of pseudorabies virus and the major tegument protein UL49, J. Virol. (2002c) 76:8208–8217.
- Fuchs W., Klupp B.G., Granzow H., Mettenleiter T.C., Essential function of the pseudorabies virus UL36 gene product is independent of its interaction with the UL37 protein, J. Virol. (2004) 78:11879–11889.
- Fuchs W., Klupp B.G., Granzow H., Osterrieder N., Mettenleiter T.C., The interacting UL31 and UL34 gene products of pseudorabies virus are involved in egress from the host-cell nucleus and represent components of primary enveloped but not of mature virions, J. Virol. (2002b) 76:364–378.
- Fuchs, W., Rziha, H-J., Lukaes, N., Braunscheiger, I., Visser, N., Liitticken, D., Schreurs, C.S., Thiel, H-J. and Mettenleiter, T.C.. 1990. Pseudorabies virus glycoprotein g1: in vitro and in viva analysis of
- gI-deleted mutants of Aujeszky's disease virus in the maxillary nerve and trigeminal ganglion of pigs after intranasal inoculation. J. Gen. Viral.. 76: 2063-2066.
- Glass, C. M., R. G. McLean, J. B. Katz, D. S. Maehr, C. B. Cropp, L. J. Kirk, A. J. McKeirnan, and J. F. Evermann. 1994. Isolation of pseudorabies (Aujeszky's disease) virus from a Florida panther. J. Wildl. Dis. 30:180–184.
- Glorieux, S., H. M. Favoreel, G. Meesen, W. de Vos, W. Van den Broeck, and H. J Nauwynck, 2009: Different replication characteristic of historical pseudorabies virus strains in porcine respiratory nasal mucosa explants. Vet. Microbiol. 136, 341–346.
- glycoprotein gp50 implicated in virus penetration of the host cell. J Gen Virol. 1990 Sep;71 (Pt 9):2179-83.

- Goldberg, T. L., R. M. Weigel, E. C. Hahn, and G. Scherba, 2001: Comparative utility of restriction fragment length polymorphism analysis and gene sequencing to the molecular epidemiological investigation of a viral outbreak. Epidemiol. Infect. 126, 415–424.
- Gortazar C, Ferroglio E, Hofle U, Frolich K, Vicente J (2007) Diseases shared between wildlife and livestock: a European perspective. European Journal of Wildlife Research 53:241–256.
- Gortazar, C., Vicente, J., Fierro, Y., Leon, L., Cubero, M.J., Gonzalez, M., 2002. Natural Aujeszky's disease in a Spanish wild boar population. Ann. N Y Acad. Sci. 969, 210–212.
- Grieco, V., Gelmetti, D., Finazzi, G., Brocchi, E., Finazzi, M., 1997. Immu-nohistologic diagnosis of pseudorabies (Aujesky's disease) using monoclonal antibodies. J. Vet. Diagn. Invest. 9, 326–328.
- Gustafson, D. P. 1986. Pseudorabies, p. 209–223. *In* A. D. Leman, R. D. Glock, W. L. Mengeling, R. H. C. Penny, E. Scholl, and B. Straw (ed.), Diseases of swine, 6th ed. Iowa State University Press, Ames, Iowa.
- Hanson R.P., The history of pseudorabies in the United States, J. Am. Vet. Med. Assoc. (1954) 124:259–261.
- Hanssens F.P., Nauwynck H.J., Pensaert M.B., Involvement of membrane-bound viral glycoproteins in adhesion of pseudorabies virus-infected cells, J. Virol. (1993) 67:4492–4496.
- Herrmann, S. C., B. Heppner, and H. Ludwig, 1984: Pseudorabies viruses from clinical outbreaks and latent infections grouped into four major genome types. Curr. Top. Vet. Med. Anim. Sci. 27, 378–401.
- Hoffman B., Beer M., Reid S.M., Mertens P., Oura, C.A., Van Rijn P.A., Slomka M.J., Banks J., Brown I.H., Alexander D.J. & King D.P. (2009). A review of RT-PCR technologies used in

veterinary virology and disease control: sensitive and specific diagnosis of five livestock diseases notifiable to the World Organisation for Animal Health. Vet. Microbiol., 139 (1–2), 1–23.

- Iglesias G., Pijoan C., Molitor T., Interactions of pseudorabies virus with swine alveolar macrophages I: virus replication, Arch. Virol. (1989) 104:107–115.
- Iglesias, Cl.. Molitor, T., Reed, D. and L'Italien. J., 1990. Antibodies to Aujeszky's disease virus in pigs immunized with purified virus glycoproteins. Vet. Microbial.. 24: l-10.
- Ishikawa, K., M. Tsutsui, K. Taguchi, A. Saitoh, and M. Muramatsu, 1996: Sequence variation of the gC gene among pseudorabies virus strains. Vet. Microbiol. 49, 267–272.
- Jacobs, L., W. A. Mulder, J. Priem, J. M. Pol, and T. G. Kimman, 1994: Glycoprotein I of pseudorabies virus (Aujeszky's disease virus) determines virulence and facilitates penetration of the virus into the central nervous system of pigs. Acta Vet. Hung. 42, 289–300.
- Jacobs. L.. Mulder. W.A.M., Van Oirschot, J.T.. Gielkens, A.L.J. and Kimman, T.G., 1993. Deleting two amino acids in glycoprotein gI of pseudorabies virus decreases virulence and neurotropism for pigs, but does not affect immunogenicity. J. Gen. Viral.. 74: 2201-2206.
- Jöns A., Gerdts V., Lange E., Kaden V., Mettenleiter T.C., Attenuation of dUTPase deficient pseudorabies virus for the natural host, Vet. Microbiol. (1997) 56:47–54.
- Kaliman A.V., Boldogkoi Z., Fodor I., Large and small subunits of the Aujeszky's disease virus ribonucleotide reductase: nucleotide sequence and putative structure, Biochim. Biophys. Acta (1994) 1219:151–156.
- Kaplan A.S., Vatter A.E., A comparison of herpes simplex and pseudorabies viruses, Virology (1959) 7:394–407.

- Katayama, S., N. Okada, K.-I. Yoshiki, T. Okab, and Y. Shimizu, 1997: Protective effect of glycoprotein gC-rich antigen pseudorabies virus. J. Vet. Med. Sci. 59, 657–663.
- Kimman T.G., Pol J.M., deWind N., Oei-Lie N., Berns A.J., Gielkens A.L., Role of different genes in the virulence and pathogenesis of Aujeszky's disease virus, Vet. Microbiol. (1992) 33:45–52.
- Kimman, T. G., G. J. Binkhorst, T. S. van den Ingh, J. M. Pol, A. L. Gielkens, and M. E. Roelvink. 1991. Aujeszky's disease in horses fulfils Koch's postulates. Vet. Rec. 128:103–106.
- Kimman, T.G., 1994b. Inactivation of glycoprotein gE and thymidine kinase or the US3-encoded protein kinase synergistically decreases in viva replication of pseudorabies virus and the induction of protective immunity. Viral., 205: 5 1 l-5 18.
- Kimman. T.G., 1994a. Immunological protection against pseudorabies virus. Proc. O.I.E. Symp. on Aujesrky's Disease, Bangkok, Thailand, pp. 1 l-22.
- Kit S., Kit M., Bartkoski M.J., Dees G., Genetically engineered pseudorabies virus vaccine with deletions in thymidine kinase and glycoprotein genes, Cold Spring Harbor Symposium Vaccines 87, Pseudorabies Vaccine 1987, 345-349.
- Kit, S., Kit, M. and Pirtle, CC.. 1985. Attenuated properties of thymidine kinase-negative deletion mutant of pseudorabies virus. Am. J. Vet. Res., 46: 1359-1367.
- Kluge, J. P., G. W. Beran, H. T. Hill, and K. B. Platt. 1999. Pseudorabies (Aujeszky's disease), p. 233–46. *In* B. E. Straw, S. D'Allaire, W. L. Mengeling, and T. J. Taylor (ed.), Diseases of swine, 8th ed. Iowa State University Press, Ames, Iowa.
- Klupp B.G., Altenschmidt J., Granzow H., Fuchs W., Mettenleiter T.C., Identification and characterization of the pseudorabies virus UL43 protein, Virology (2005) 334:224–233.

- Klupp B.G., Fuchs W., Granzow H., Nixdorf R.,Mettenleiter T.C., The pseudorabies virus UL36 tegument protein physically interacts with the UL37 protein, J. Virol. (2002) 76:3065–3071.
- Klupp B.G., Fuchs W., Weiland E., Mettenleiter T.C., Pseudorabies virus glycoprotein L is necessary for virus infectivity but dispensable for virion localization of glycoprotein H, J. Virol. (1997) 71:7687–7695.
- Klupp B.G., Hengartner C.J., Mettenleiter T.C., Enquist L.W., Complete, annotated sequence of the pseudorabies virus genome, J. Virol. (2004) 78:424–440.
- Köhler M., Köhler W., Zentralblatt für Bakteriologie – 100 years ago Aladar Aujeszky detects a "new" disease – or: It was the cow and not the sow, Int. J. Med. Microbiol. (2003) 292:423–427.
- Kritas, S.K., Nauwynck, H.J. and Pensaert. M.B., 1995. Dissemination of wild-type and gC-, gE- and gI-deleted mutants of Aujeszky's disease virus in the maxillary nerve and trigeminal ganglion of pigs after intranasal inoculation. J Gen Virol. 1995 Aug;76 (Pt 8):2063-6.
- Kritas, S.K., Pensaert, M.B. and Mettenleiter, T.C., 1994b. Role of envelope glycoproteins gI, gp63 and gII1 in the invasion and spread of Aujeszky's disease virus in the olfactory nervous pathways of the pig. J. Gen. Viral., 75: 2319-2327.
- Kritas, S.K., Pensaert. M.B. and Mettenleiter, T.C., 1994a. Invasion and spread of single glycoprotein deleted mutants of Aujeszky's disease virus in the trigeminal nervous pathway of pigs after intranasal inoculation. Vet. Microbial.. 40: 323-334.
- Lari A, Lorenzi D, Nigrelli D, Brocchi E, Faccini S, Poli A (2006) Pseudorabies virus in European wild boar from Central Italy. Journal of Wildlife Disease 42:319–324.
- Legault, C., 1985: Selection of breeds, strains and individual pigs for prolificacy. J. Reprod. Fertil. Suppl. 33, 151–166.

- Lehman I.R., Boehmer P.E., Replication of herpes simplex virus DNA, J. Biol. Chem. (1999) 274:28059–28062.
- Leuenberger R, Boujon P, Thur B, Miserez R, Garin-Bastuji B, Rufenacht J, Stark KDC (2007) Prevalence of classical swine fever, Aujeszky's disease and brucellosis in a population of wild boar in Switzerland. Veterinary Record 160:362–368.
- Lutz W, Junghans D, Schmitz D, Mu¨ller T (2003) A long-term survey of pseudorabies virus infections in European wild boar of western Germany. Zeitschrift fu¨r Jagdwissenschaft 49:130–140.
- Ma W, Lager KM, Richt JA, Stoffregen WC, Zhou F, Yoon KJ (2008) Development of real-time polymerase chain reaction assays for rapid detection and differentiation of wild-type pseudorabies and gene-deleted vaccine viruses. Journal of Veterinary Diagnostic Investigation 20:440–447.
- Maes, R. K., M. D. Sussman, A. Vilnis, and B. J. Thacker. 1997. Recent developments in latency and recombination of Aujeszky's disease (pseudorabies) virus. Vet. Microbiol. 55:13–27.
- Maiorano, G., 2009. Swine production in Italy and research perspectives for the local breeds. Slovak. J Anim. Sci. 42 (4), 159–166.
- Marchioli, C.C.. Yancey, R.J., Timmins. J.G., Post, L.E., Young, B.R. and Povendo. L.A., 1988. Protection of mice and swine from pseudorabies virus-specific mouse monoclonal antibodies. Am. J. Vet. Res.. 49:860-864.
- Masic, M., M. Ercegan, and M. Petrovic. 1965. The significance of the tonsils in the pathogenesis and diagnosis of Aujeszyk's disease in pigs. Zentralbl. Veterinarmed. B. 12:398–405. (In German.)

- McCracken, R. M., J. B. McFerran, and C. Dow. 1973. The neural spread of pseudorabies virus in calves. J. Gen. Virol. 20:17–28.
- McFerran J.B., Dow C., The excretion of Aujeszky's disease virus by experimentally infected pigs, Res. Vet. Sci. (1964) 5:405–410.
- Mengeling W.L., Lager K.M., Volz D.M. & Brockmeier S.L. (1992). Effect of various vaccination procedures on shedding, latency and reactivation of attenuated and virulent pseudorabies virus in swine. Am. J. Vet. Res., 53 (11), 2164–2173.
- Mettenleiter T.C., Aujeszky's disease (pseudorabies) virus: the virus and molecular pathogenesis – State of the art, June 1999, Vet. Res. (2000) 31:99–115.
- Mettenleiter T.C., Brief overview on cellular virus receptors, Virus Res. (2002) 82:3–8.
- Mettenleiter T.C., Intriguing interplay between viral proteins during herpesvirus assembly or not: The herpesvirus assembly puzzle, Vet. Microbiol. (2006) 113:163–169.
- Mettenleiter T.C., Zsak L., Zuckermann F., Sugg N., Kern H., Ben-Porat T., Interaction of glycoprotein gIII with a cellular heparinlike substance mediates adsorption of pseudorabies virus, J. Virol. (1990) 64:278–286.
- Mettenleiter, T. C., 1999: Aujeszky's disease (pseudorabies) virus: the virus and molecular pathogenesis–state of the art, June 1999. Vet. Res. 31, 99–115. Review.
- Mettenleiter. T.C.. Lomniczi, B., Zsak. L., Medveczky. 1.. Ben-Porat. T. and Kaplan, AS., 1989. J.T. Van Oirschot (Editor) Analysis of the factors that affect virulence of pseudorabies virus. Proc. CEC Seminar on Vaccination and Control of Aujeszky's Disease, pp. 3-l 1.
- Minh, B.Q., Nguyen, M.A.T., von Haeseler, A., 2013. Ultrafast approxima-tion for phylogenetic bootstrap. Mol. Biol. Evol. 30, 1188–1195.

- Miry, C. and Pensaert. M., 1989. Aujeszky's disease virus replication in tonsils and respiratory tract of non-immune and immune pigs. In: J.T. van Oirschot (Editor), Vaccination and Control of Aujeszky's Disease, pp. I63- 173.
- Moennig V., Woldesenbert P., Fey H.R., Liess B., Dopotka H.D. & Behrens F. (1982). Comparative evaluation of ELISA and neutralization test for the diagnosis of Aujeszky's disease. *In:* Current Topics in Veterinary Medicine and Animal Science 17. Aujeszky's Disease, Wittmann G. & Hall S.A., eds. Martinus Nijhoff, The Hague, The Netherlands, 51.
- Montagnaro S, Sasso S, De Martino L, Longo M, Lovane V, Ghlurmino G, Plsanelli G, Nava D, Baldl L, Pagninl U (2010) Prevalence of antibodies to selected viral and bacterial pathogens in wild boar (Sus scrofa) in Campania Region, Italy. Journal of Wildlife Disease 46:316–319.
- Mulder W., Pol J., Kimman T., Kok G., Priem J., Peeters B., Glycoprotein D-negative pseudorabies virus can spread transneuronally via direct neuron-to-neuron transmission in its natural host, the pig, but not after additional inactivation of gE or gI, J. Virol. (1996) 70:2191–2200.
- Mulder. W.A.M.. Pol. J.M.A.. Kimman, T.G.. Kok. G.L.. Priem. J. and Peeters, B.P.H., 1995. gD-negative pseudorabies virus can spread transneuronally via direct cell-to-cell transmission in its natural host, Abstract No. 52. 20th bit. Herpesvirus Workshop, Groningen. The Netherlands.
- Muller T, Hahn EC, Tottewitz F, Kramer M, Klupp BG, Mettenleiter TC, Freuling C (2011) Pseudorabies virus in wild swine: a global perspective. Archives of Virology 156:1691–1705.
- Muller, T., B. G. Klupp, C. Freuling, B. Hoffmann, M. Mojcicz, I. Capua, V. Palfi, B. Toma, W. Lutz, F. Ruiz-fon, C. Gortarzar, A. Hlinak, U. Schaarschmidt, K. Zimmer, F. J. Coraths, E. C. Hahn, and T. C. Mettenleiter, 2010: Characterization of pseudorabies virus of wild boar origin from Europe. Epidemiol. Infect. 138, 1590–1600.

- Muller, T., H. J. Batza, H. Schluter, F. J. Conraths, and T. C. Mettenleiter. 2003. Eradication of Aujeszky's disease in Germany. J. Vet. Med. B Infect. Dis. Vet. Public Health 50:207–213.
- Muller, T., Teuffert, J., Zellmer, R., Conraths, F.J., 2001. Experimental infection of European wild boars and domestic pigs with pseudora-bies viruses with differing virulence. Am. J Vet. Res. 62 (29), 252–258.
- Nakamura, T., Ihara. T., Nagata, T., Ishihama, A. and Ueda. S., 1990. A complement-dependent neutralizing monoclonal antibody against glycoprotein II of pseudorabies virus. Vet. Microbial., 24: 193-198.
- Nauwynck H.J., Pensaert M.B., Abortion induced by cell-associated pseudorabies virus in vaccinated sows, Am. J. Vet. Res. (1992) 53:489–493.
- Nauwynck H.J., Pensaert M.B., Cell-free and cell-associated viremia in pigs after oronasal infection with Aujeszky's disease virus, Vet. Microbiol. (1995a) 43:307–314.
- Nauwynck, H.J. and Pensaert, M.B., 1995b. Effect of specific antibodies on the cell-associated spread of pseudorabies virus in monolayers of different cell types. Arch. Virol., 140: I137- 1146.
- Nauwynck, H.J., 1993. The cell-associated spread of Aujeszky's disease virus and its possible role in the pathogenesis of abortion in vaccinated sows. Ph.D. thesis, Gent, Belgium.
- Newcomb W.N., Homa F.L., Thomsen D.R., Trus B.L., Cheng N., Steven A., Booy F., Brown J.C., Assembly of the herpes simplex virus procapsid from purified components and identification of small complexes containing the major capsid and scaffolding proteins, J. Virol. (1999) 73:4239–4250.
- Ono E., Watanabe S., Nikami H., Tasaki T., Kida H., Pseudorabies virus (PrV) early protein 0 activates PrV gene transcription in combination with the immediate-early protein IE180 and enhances the infectivity of PrV genomic DNA, Vet. Microbiol. (1998) 63:99–107.

- Ou C.J., Wong M.L., Chang T.J., A TEF-1-element is required for activation of the promoter of pseudorabies virus glycoprotein X gene by IE180, Virus Genes (2002) 25:241–253.
- Pannwitz, G., Freuling, C., Denzin, N., Schaarschmidt, U., Nieper, H., Hlinak, A., Burkhardt, S., Klopries, M., Dedek, J., Hoffmann, L., Kramer, M., Selhorst, T., Conraths, F.J., Mettenleiter, T., Mu ller, T., 2012. A long-term serological survey on Aujeszky's disease virus infections in wild boar in East Germany. Epidemiol. Infect. 140 (2), 348–358.
- Pensaert M.B., De Smet K., De Waele K., Extent and duration of virulent virus excretion upon challenge of pigs vaccinated with different glycoprotein-deleted Aujeszky's disease vaccines, Vet. Microbiol. (1990) 22:107–117.
- Pensaert M.B., Labarque G., Favoreel H., Nauwynck H., Aujeszky's disease vaccination and differentiation of vaccinated from infected pigs, Dev. Biol. (Basel) (2004) 119:243–254.
- Pensaert M.B., Miry C., Biront P., Castryck F., De Roose P., Robijns J., Segers E., Evolution of Aujeszky's disease in Belgium (1974–1987), Flemish Vet. J. (1987) 56:425–432.
- Pensaert, M. B., and J. P. Kluge. 1989. Pseudorabies virus (Aujeszky's disease), p. 39–64. *In* M. B. Pensaert (ed.), Virus infections of porcines. Elsevier Science Publishers, Amsterdam, The Netherlands.
- Piatti, R. M., A. A. Ikuno, E. S. Cunha, R. D'Ambros, F. Gregori, R. M. Soares, A. Cortez, and L. J. Richtzenhain, 2001: Characterization of Aujeszky's disease virus isolates from south and southeast Brazil by RFLP analysis. Braz. J. Microbiol. 32, 144–146.
- Pittler M., The occurrence and control of Aujeszky's disease in the Federal Republic of Germany, in: Wittmann G., Hall S.A. (Eds.), Current topics in veterinary medicine and animal science, Martinus Nijhoff, vol. 17, 1982, pp. 259–265.

- Pol J.M., Broekhuysen-Davies J.M., Wagenaar F., La Bonnardière C., The influence of porcine recombinant interferonalpha 1 on pseudorabies virus infection of porcine nasal mucosa in vitro, J. Gen. Virol. (1991) 72:933–938.
- Pol J.M.A., Gielkens A.L.J., van Oirschot J.T., Comparative pathogenesis of three strains of pseudorabies virus in pigs, Microb. Pathog. (1989) 7:361–371.
- Posada, D., 2008. jModelTest: phylogenetic model averaging. Mol. Biol. Evol. 25 (7), 1253–1256.
- Rauh I., Mettenleiter T.C., Pseudorabies virus glycoproteins gII and gp50 are essential for virus penetration, J. Virol. (1991) 65:5348–5356.
- Reagan R.L., Schenk D.M., Harmon M.P., Brueckner A.L.,Morphological observations by electron microscopy of pseudorabies virus (Aujeszky strain) after propagation in the rabbit, Am. J. Vet. Res. (1952) 13:577–578.
- Roic, B., Jemersic, L., Terzic, S., Keros, T., Balatinec, J., Florijancic, T., 2012. Prevalence of antibodies to selected viral pathogens in wild boars (Sus scrofa) in Croatia in 2005-06 and 2009-10. J. Wildl. Dis. 48 (1), 131–137.
- Ruiz-Fons F, Segales J, Gortazar C (2008a) A review of viral diseases of the European wild boar: effects of population dynamics and reservoir role. The Veterinary Journal 176:158–169.
- Ruiz-Fons F, Vidal D, Vicente J, Acevedo P, Fernandez-de-Mera IG, Montoro V, Gortazar C (2008b) Epidemiological risk factors of Aujeszky's disease in wild boars (Sus scrofa) and domestic pigs in Spain. European Journal of Wildlife Research 54:549–555.
- Rziha, H. J., T. C. Mettenleiter, V. Ohlinger, and G. Wittmann. 1986. Herpesvirus (pseudorabies virus) latency in swine: occurrence and physical state of viral DNA in neural tissues. Virology 155:600–613.

- Rziha, H.-J., V. F. Ohlinger, D. Lu¨ttiken, and N. Visser. 1989. Latency characteristics of pseudorabies vaccine virus, p. 79–85. *In* J. T. Van Oirschot (ed.), Vaccination and control of Aujeszky's disease. Kluwer Academic Publishers, Boston, Mass.
- Sabin A.B., Studies on the B virus. I. The immunological identity of a virus isolated from a human case of ascending myelitis associated with visceral necrosis, Br. J. Exp. Pathol. (1934) 15:248–268.
- Sabin, A. B. 1938. Progression of different nasally installed viruses along different nervous pathways in the same host. Proc. Soc. Exp. Biol. Med. 38:270–275.
- Sabo A., Rajcani J., Blaskovic D., Studies on the pathogenesis of Aujeszky's disease. I. Distribution of the virulent virus in piglets after peroral infection, Acta Virol. (1968) 12:214–221.
- Sabo A., Rajcani J., Blaskovic D., Studies on the pathogenesis of Aujeszky's disease. III. The distribution of virulent virus in piglets after intranasal infection, Acta Virol. (1969) 13:407–414.
- Sabo, A., and J. Rajcani. 1976. Latent pseudorabies virus infection in pigs. Acta Virol. 20:208–214.
- Schaefer, R., J. Ciacci-Zanella, N. Mores, A. Kleitton, R. M. F. Dambros, M. F. Schiochet, and M. Coldebella, 2006: Characterization of Aujeszky's disease virus isolates from South Brazil in the last twenty years by restriction enzyme analysis. Braz. J. Microbiol. 37, 390–394.
- Schmiedhoffer J., Beiträge zur Pathologie der infektiösen Bulbär paralyse (Aujeszkyschen Krankheit), Z. Infekt. Krankh. Parasit. Hyg. Haustier (1910) 8:383–405.
- Schoenbaum M.A., Beran G.W. & Murphy D.P. (1990). A study comparing the immunologic responses of swine to pseudorabies viral antigens based on the ELISA, serum virus neutralization, and latex agglutination tests. J. Vet. Diagn. Invest., 2 (1), 29–34.

- Schwartz J.A., Brittle E.E., Reynolds A.E., Enquist L.W., Silverstein S.J., UL54-null pseudorabies virus is attenuated in mice but productively infects cells in culture, J. Virol. (2006) 80:769–784.
- Serena, M. S., G. E. Metz, E. C. Mortola, and M. G. Echeverrıa, 2011: Phylogenetic analysis of Suid Herpesvirus 1 isolates from Argentina. Vet. Microbiol. 154, 78–85.
- Shope R.E., An experimental study of "mad itch" with especial reference to its relationship to pseudorabies, J. Exp. Med. (1931) 54:233–248.
- Shope R.E., Pseudorabies is a contagious disease in swine, Science (1934) 80:102–103.
- Smith G.A., Enquist L.W., Break ins and break outs: viral interaction with the cytoskeleton of mammalian cells, Annu. Rev. Cell Dev. Biol. (2002) 18:135–161.
- Spear P.G., Eisenberg R.J., Cohen G.H., Three classes of cell surface receptors for alphaherpesvirus entry, Virology (2000) 275:1–8.
- Stegeman, A., M. C. de Jong, A. van Nes, and A. Bouma. 1997. Dynamics of pseudorabies virus infections in vaccinated pig populations: a review. Vet. Q. 19:117–122.
- Steinrigl, A., Revilla-Ferna´ ndez, S., Kolodziejek, J., Wodak, E., Bago´ , Z., Nowotny, N., Schmoll, F., Kofer, J., 2012. Detection and molecular characterization of Suid herpesvirus type 1 in Austrian wild boar and hunting dogs. Vet. Microbiol. 157 (3-4), 276–284.
- Strebel M., Eine eigenthümliche Juckkrankheit beim Rinde, Schweiz. Arch. Tierheilk. (1889) 31:125–129.
- Taharaguchi S., Inoue H., Ono E., Kida H., Yamada S., Shimizu Y., Mapping of transcriptional regulatory domains of pseudorabies virus immediate-early protein, Arch. Virol. (1994) 137:289–302.

- Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar, 2011: MEGA 5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.
- Thawley, D. G., R. F. Solorzano, and M. E. Johnson. 1984. Confirmation of pseudorabies virus infection, using virus recrudescence by dexamethasone treatment and in vitro lymphocyte stimulation. Am. J. Vet. Res. 45:981–983.
- Toma B., Le Turdu Y., Ursache R., Bernard F., Rose R., David C., Metianu T., Renault L., Gilbert Y., Duée J.P., Épidémiologie descriptive de la maladie d'Aujeszky en France en 1977, Rec. Méd. Vét. (1985) 154:681–685.
- Van de Walle G.R., Favoreel H.W., Nauwynck H.J., Mettenleiter T.C., Pensaert M.B., Transmission of pseudorabies virus from immune-masked blood monocytes to endothelial cells, J. Gen. Virol. (2003) 84:629–637.
- Van Oirschot J.T., Kaashoek M.J., Rijsewijk F.A., Stegeman J.A., The use of marker vaccines in eradication of herpesviruses, J. Biotechnol. (1996) 44:75–81.
- van Oirschot, J. T., and A. L. Gielkens. 1984. In vivo and in vitro reactivation of latent pseudorabies virus in pigs born to vaccinated sows. Am. J. Vet. Res. 45:567–571.
- van Oirschot, J. T., H. J. Rziha, P. J. Moonen, J. M. Pol, and D. van Zaane. 1986. Differentiation of serum antibodies from pigs vaccinated or infected with Aujeszky's disease virus by a competitive enzyme immunoassay. J. Gen. Virol. 67:1179–1182.
- Van Rijn P.A., Wellenberg G.J., Hakze-Van Der Honing R., Jacobs L., Moonen P.L. & Feitsma H. (2004). Detection of economically important viruses in boar semen by quantitative real time PCR technology. J Virol. Methods,120 (2),151–160.

- Vannier P., Hutet E., Bourgueil E., Cariolet R., Level of virulent virus excreted by infected pigs previously vaccinated with different glycoprotein deleted Aujeszky's disease vaccines, Vet. Microbiol. (1991) 29:213–223.
- Vannier. P., Hutet, E., Bourgueil, E. and Cariolet, R., 1990. Vaccins contre la maladie d'Aujeszky et souches vaccinales delttees. Rec. Med. Vet., 166: 1127-l 133.
- Vengust, G., Valencak, Z., Bidovec, A., 2005. Presence of antibodies aganist Aujeszky's disease virus in wild boar (Sus scrofa) in Slovenia. J. Wildl. Dis. 41 (4), 800–802.
- Verin R, Varuzza P, Mazzei M, Poli A (2014) Serologic, molecular, and pathologic survey of pseudorabies virus infection in hunted wild boars (Sus Scrofa) in Italy. Journal of Wildlife Diseases 50:559–565.
- Verpoest, S., Cay, A.B., de Regge, N., 2014. Molecular characterization of Belgian pseudorabies virus isolates from domestic swine and wild boar. Vet. Microbiol. 172, 72–77.
- Wathen, L.M.K., Platt, K.B., Wathen, M.W., van Deusen, R.A., Whetstone, C.A. and Pirtle, E.C., 1985. Production and characterization of monoclonal antibodies directed against pseudorabies virus. Virus Res., 19-29.
- Wheeler, J. G., and F. A. Osorio. 1991. Investigation of sites of pseudorabies virus latency, using polymerase chain reaction. Am. J. Vet. Res. 52:1799–1803.
- Whitley, R. J. 2001. Herpes simplex viruses, p. 2461–2509. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology, 4th ed., vol. 2. Lippincott Williams & Wilkins, Philadelphia, Pa.
- Wittmann G., Jakubik J., Ahl R., Multiplication and distribution of Aujeszky's disease (pseudorabies) virus in vaccinated and non-vaccinated pigs after intranasal infection, Arch. Virol. (1980) 66:227–240.

- Wittmann, G., V. Ohlinger, and H. J. Rziha. 1983. Occurrence and reactivation of latent Aujeszky's disease virus following challenge in previously vaccinated pigs. Arch. Virol. 75:29–41.
- Wong M.L., Ho T.Y., Huang J.H., Hsiang C.Y., Chang T.J., Stimulation of type I DNA topoisomerase gene expression by pseudorabies virus, Arch. Virol. (1997) 142:2099–2105.
- Wu L., Berk A.J., Transcriptional activation by the pseudorabies virus immediate early protein requires the TATA box element in the adenovirus 2 E1B promoter, Virology (1988) 167:318–322.
- Yoon H.-A., Eo S.-K., Aleyas A.G., Cha S.-Y., Lee J.-H., Chae J.-S., Jang H.-K., Cho J.-G. & Song H.-J. (2006). Investigation of pseudorabies virus latency in nervous tissues of seropositive pigs exposed to field strain. J. Vet Med. Sci., 68(2), 143–148.
- Yoon, H.A., Eo, S.K., Aleyas, A.G., Park, S.O., Lee, J.H., Chae, J.S., Cho, J.G., Song, H.J., 2005. Molecular survey of latent pseudorabies virus infec-tion in nervous tissues of slaughtered pigs by nested and real-time PCR. J. Microbiol. 43 (5), 430–436.
- Zanin, E., I. Capua, C. Casaccia, A. Zuin, and A. Moresco. 1997. Isolation and characterization of Aujeszky's disease virus in captive brown bears from Italy. J. Wildl. Dis. 33:632–634.
- Zhang, G., *Stevens,* R. and Leader, D.P., 1990. The protein kinase encoded in the short unique region of pseudorabies virus: Description of the gene and identification of its product in virions and in infected cells. J. Gen. Viral., 71: 1757-1765.
- Zuckermann, F. A., L. Zsak, T. C. Mettenleiter, and T. Ben-Porat, 1990: Pseudorabies virus glycoprotein gIII is a major target antigen for murine and swine virus-specific cytotoxic T lymphocytes. J. Virol. 64, 802–812.

Legal norms

DM 1/04/1997. Piano nazionale di controllo della malattia di Aujeszky nella specie suina. http://www.izsler.it/izs_bs/allegati/396/DM01041997%20%20Piano%20naz%20controllo%20Aujeszky.pdf

Ministero della Salute. Piano Nazionale Integrato 2011-2014, 4/03/2014. Malattia di Aujeszky, cap 3C.http://www.salute.gov.it/pianoNazionaleIntegrato/paginaInternaSottomenuPianoNazionaleIntegrato. jsp?id=3918&menu=capitolo3&sottomenu=2144

Lombardia Region .D.d.s. 9/05/2014 - n. 3822 - - Approvazione del piano regionale di controllo ed eradicazione della malattia di Aujeszky e contestuale revoca del d.d.u.o.n. 10784/2011. http://217.56.218.163/TESTI/BURLDDSDGSRL090520143822.pdf

Emilia Romagna Region. Delibera Giunta Regionale n. 1588–13/10/2014. Linee guida per l'attuazione dei controlli inerenti la malattia di Aujeszky per gli allevamenti suini della Regione Emilia Romagna. http://bur.regione.emilia-romagna.it/dettaglio-inserzione?i=e16bd4c2aa0d-41d4866e88fd857a5de9

Veneto Region. Delibera Giunta Regionale n. 2061–11/ 10/2012. Piano di controllo finalizzato all'eradicazione della malattia di Aujeszky nella Regione Veneto. http:// bur.regione.veneto.it/BurvServices/pubblica/DettaglioDgr.aspx?id=243209

Commission Decision 2008/185/EC regarding additional guarantees in intra-Community trade of pigs related to Aujeszky's disease and criteria for listing a Member State or a region thereof as free from Aujeszky's disease or as having an approved disease control program. http://ec.europa.eu/food/animal/diseases/controlmeasures/docs/guidance_2008-185-EC_en.pdf

RINGRAZIAMENTI

CON QUESTE POCHE RIGHE DESIDERO RINGRAZIARE COLORO CHE HANNO COLLABORATO ALLO SCOPO DI PERMETTERMI DI APPROFONDIRE LE TEMATICHE ANALIZZATE IN QUESTA TESI.

UN PARTICOLARE RINGRAZIAMENTO VA AL TUTOR DOTT. GUIDO GRILLI CHE SI È PRODIGATO CON L'APPORTO DELLA SUA ESPERIENZA E PRATICA PER DARMI CONSIGLI PREZIOSI NELL'ITER DI STESURA DI QUESTO STUDIO.

PREZIOSO È STATO L'INTERVENTO DELLA DOTT.SSA ANA MORENO, RESPONSABILE DEL CENTRO DI REFERENZA DELLA MALATTIA DI AUJESZKY, CHE HA EFFETTUATO NUMEROSE RICERCHE, METTENDOMI A DISPOSIZIONE MATERIALE BIBLIOGRAFICO E DI ANALISI BIOMOLECOLARE INDISPENSABILI PER SVILUPPARE IL LAVORO COMPLETATO IN QUESTA TESI.

LA MIA RICONOSCENZA VA ANCHE AL DOTT. ANTONIO LAVAZZA, RESPONSABILE DEL REPARTO VIROLOGIA, PER IL SUPPORTO E L'AIUTO A RAGGIUNGERE QUESTO TRAGUARDO.

RINGRAZIO ANCHE IL PROF GIUSEPPE SIRONI; COORDINATORE DEL DOTTORATO DI RICERCA, PER LA GENTILEZZA E LA CORTESIA ACCORDATAMI IN OGNI MOMENTO.

A TUTTI I MIEI PIÙ SENTITI RINGRAZIAMENTI.