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Detection of swine emerging viruses by Real-time PCR and histopathological examination of tissue lesions in domestic pigs (*Sus scrofa domesticus*) in Sardinia

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Abstract

Population growth is the main factor causing an increase in global meat consumption, and pork production is a relevant sector in agricultural economies, occupying more than 30% of the overall meat demand. Pork production plays a very important role in the Italian economy representing 6% of the agri-food industry. In Sardinia, most pork-producing farms are for self-consumption, but intensive farms are present with a limited distribution in some specific areas. Intensive pig farms are stress-inducing systems that negatively impact animal welfare, and lead to an increased susceptibility to diseases. Swine pathologies affecting pig farms are a serious concern causing death in pig herds and leading to very important losses. Porcine Reproductive and Respiratory Syndrome (PRRS), Foot and Mouth Disease (FMD), and Porcine Circovirus 2 (PCV2) are some of the most common viruses reported in swine-intensive rearing systems. Although emerging viruses are omnipresent in the swine population, they are still scarcely investigated leading to limited knowledge of the pathogenesis and pathological aspects of implicated diseases. In Sardinia, pork production has suffered from African Swine Fever (ASF) for many years, however, studies regarding emerging swine viruses are limited. A multidisciplinary approach is advised to investigate the swine emerging viruses'presence and pathogenic effect to prevent and control diseases.

To accomplish this goal, we evaluated lesions in swine organs by gross and histopathology examinations and investigated the presence of the most common viruses (PRRS and PCV2), and of the emerging viruses such as Torque Teno Sus virus (TTSuV), Senecavirus A (SVA), Atypical Porcine Pestivirus (APPeV), LINDA virus, Porcine Circovirus 3 (PCV3), Parvoviruses (PPV) 2, 3, 4, 5, 6, 7, Swine Enteric Alphacoronavirus (SEACoV), and Porcine Deltacoronavirus (PDCoV), by RT-PCR. Additionally, we evaluated viral co-infections in pig tissues.

Two hundred thirty-two samples from 29 pigs of different age categories (10 adult pigs, 10 post-weaning pigs, and 9 piglets) were collected from a Sardinian intensive farm. Moderate to severe chronic lymphoplasmacytic bronchitis and interstitial pneumonia were found in all swine categories. Adult pigs and piglets were characterized by more severe bronchiolar lesions than post-weaning pigs, whereas this last category showed more severe lesions in the lung interstitium than adults. PCV2 was detected in adult, post-weaning pigs and piglets' organs showing, in the lung, a higher viral load than PRRSV. This data suggested that interstitial pneumonia in our cases could be more related to PCV2 infections than PRRSV. Moving on to the emerging viruses, PCV3 was detected in all swine tissues with no statistically significant differences between organs suggesting a broad tropism of PCV3. Notably, TTSuV was detected in 100% of the three age categories of swine, with the highest viral load in the lung and spleen suggesting that TTSuV may play a role in swine lung lesions, enhancing the development of disease caused by other pathogenic agents. Among PPVs, PPV2 was found in most examined tissues of adults and piglets, and to less extent in post-weaning pigs. Moreover, since interstitial pneumonia is the most representative lung lesion, and all cases are 100% positive for PPV2, we can propose that this virus could be an important contributor to the development of lung lesions in swine. Also, PPV3-7 emerging viruses have been detected. Moreover, APPeV was detected in adult pigs' mediastinal and mesenteric lymph nodes with a high viral load, whereas SVA, LINDAV, PDCoV, and SEACoV were not detected in swine tissues. Co-infections were found, with PPV2, TTSuV, and PCV2 in lung samples from all pig categories. We propose including emerging viruses such as TTSuV and PPV2 in the routine diagnostic investigation of respiratory swine disease.

Despite the challenges facing our work, related to the number of intensive farms selected, and the missing tests investigating the presence of mixed bacterial infections, it offered the first report of several newly emerging viruses, which haven't been previously reported in the domestic pig population farmed in Sardinia (PPV2-7, APPeV), and of the co-infection between

emerging and common viruses (PPV2, TTSuV, and PCV2) in lungs of pigs with pneumonia, regardless of age categories. Further investigations are needed to localize specific viruses in the organs of co-infected pigs, to better clarify the role of each pathogen and its impact on swine health and industry.

Chapter 1

Introduction

1. Global swine sector overview

1.1. Pork meat consumption

Meat consumption registered an increase in most countries from the 60s till nowadays due to the major protein source shift from plant to animal-derived products [1]. Important factors affecting the level and the type of meat consumption are demographic growth, incomes, prices, environmental norms, animal welfare, and animal health. These determinants change continuously and considerably over the decades and between countries [2].

Population growth is the main factor causing an increase in meat consumption, and global growth of 14% is expected in meat consumption by 2030, accounting for 33% of the total meat consumption increase [2]. Another critical issue of meat consumption is the lesser affordability of meat than other alimentary sources since meat purchase is related to income growth. In 2021 the top pork consumer was China, and the consumption was expected to grow by 4% in 2022. Belarus, the European Union, South Korea, and Vietnam are the second, third, fourth, and fifth consumers, respectively [3]. Pork consumption worldwide is shown in Figure 1.1 [4].

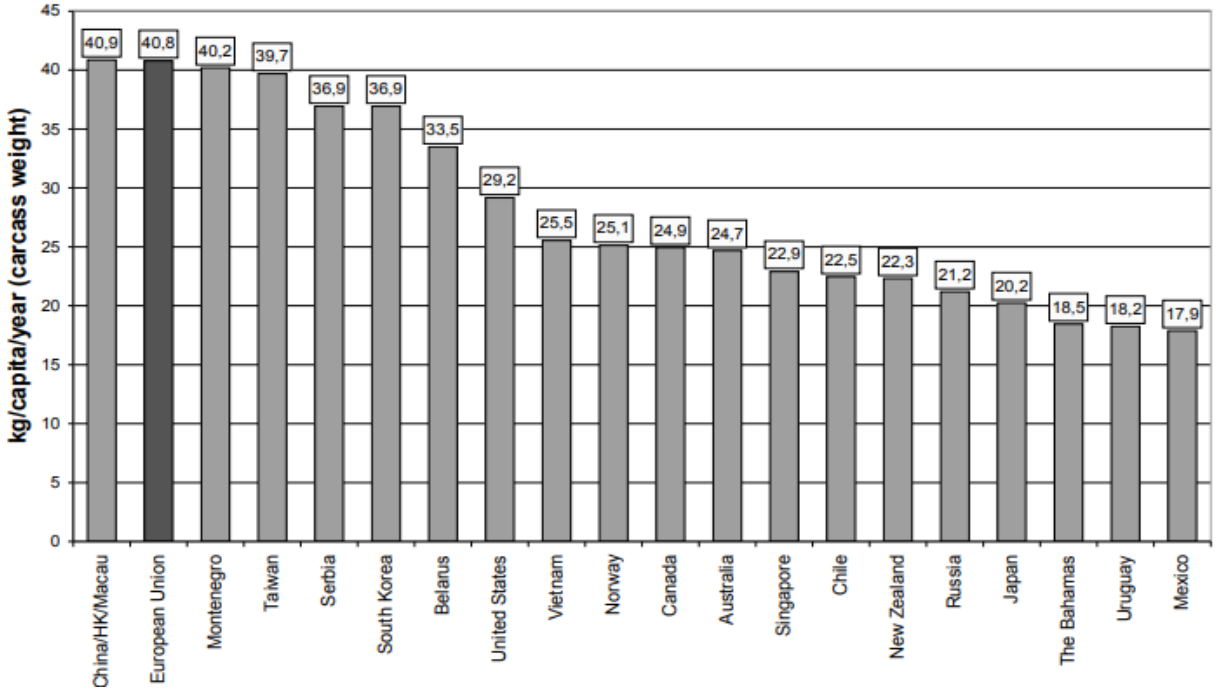


Figure 1.1. Pork consumption per capita in the top twenty countries in 2017 (Kg/capita/year) [4].

Pork meat dominates meat consumption in the center and south of Italy (119g/week/person) whereas reaches its lowest consumption in the northwest (59 grams/week/person). The Italian pork industry produces cold cuts including several products labeled as "Protected Designations of Origin" (PDO), and "Protected Geographical Indication" (PGI) products, representing the greatness of food products in Europe [5, 6]. Between 2016 and 2020 pig meat showed a change in the Italian diet since a decrease of 2.5 kg of pork meat consumption was registered [7]. Pork consumption in Italy is reported in Figure 1.2 [7].

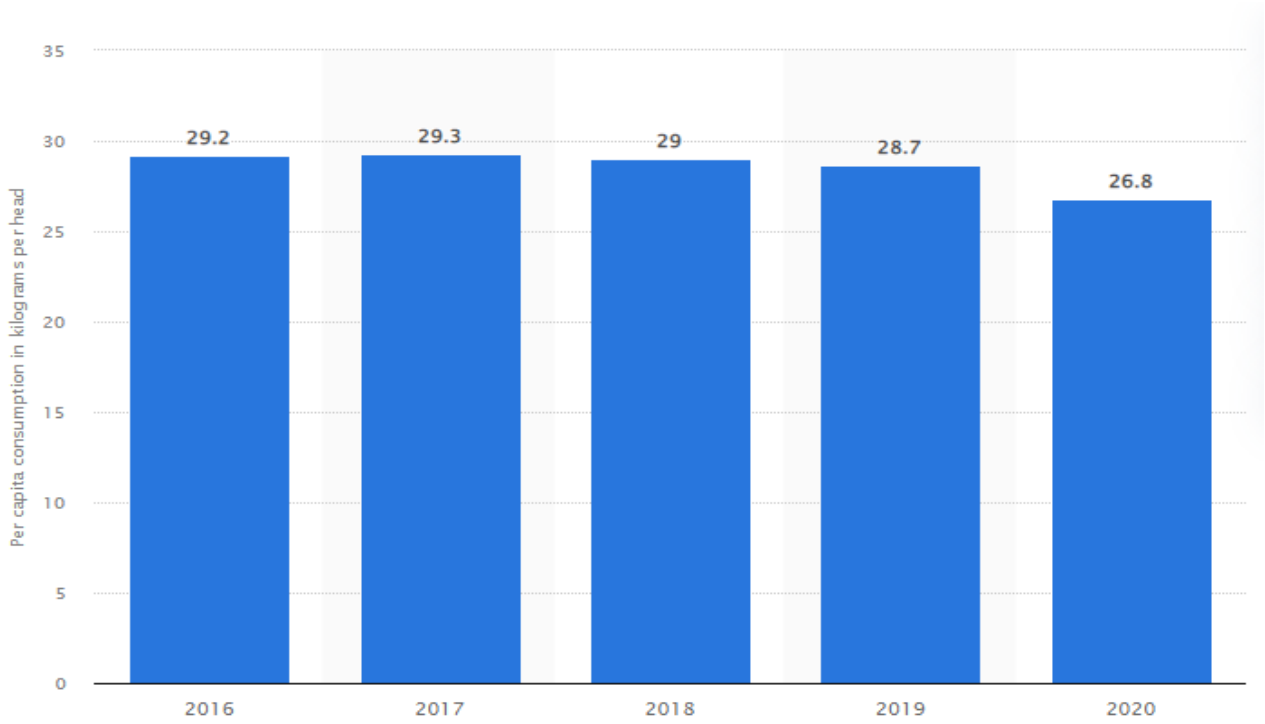


Figure 1.2. Diagram demonstrating Italian pig meat consumption per capita from 2016 to 2020 (kg/head) [7].

1.2. Global pig farming and pork meat production

1.2.1. Historical background

Pigs belong to the order Ungulates, suborder Artiodactyla, family Suidae, genus *Sus*, species "*Sus scrofa domestica*". The origin of domestic pigs is not univocal as it can be derived from Asian (*Sus scrofa vittatus*, *verrucosus*, *barbatus*) or European wild boar (*Sus scrofa ferus*) [8]. The earliest depictions of pigs' ancestors are dated back 40,000 years before the present,

painted on the cave wall of Altamira, Spain, southern Rhodesia, and Scotland [9]. The domestication of pigs dates back to 5-6 millennium BC in China, while in Europe it occurred during the Neolithic period [10].

1.2.2. Pig farming worldwide

Several criteria play important roles in the choice of type and management of pig farms: farm location, feed availability, and the industrial process [11]. Farm types are generally extensive (outdoor) or intensive (indoor) farms due to the rapid pork industry development. Intensive farms are focused on good pig breeding, high-value feed, increased growth rates with high biosecurity measures, and very good expertise [12]. Pigs are raised worldwide [10] and stocks at the global level [13] have increased in the decade 2000-2010 from 898 to 971 million heads (+8%). However, in the decade 2010-2020, the number of heads became stable (952 million heads in 2020). Nowadays, the leading countries in pig production are China (406 million heads in 2022), the USA (77 million heads), and Brazil (41 million heads). Regarding the European Union, the 27 member countries have a total number of 146 million heads, with Spain (32 million heads), Germany (26 million heads), France (13 million heads), Denmark (13 million heads), Poland (11 million heads), and Netherlands (11 million heads) being the leading countries [13].

1.2.3. Pig farming in Italy

Pig farming in Italy has ancient origins, as the Greek historian Polybius reported the abundance of pigs in the Po Valley in the middle of the second century BC [8]. Italian pig-rearing systems are mostly located in the North (Lombardia, Emilia-Romagna, Piemonte, Veneto) and slaughterhouses (82%) are concentrated within these regions [12,14]. Reared pigs (50%) are focused in Lombardia, and a total of about 89% in all of Northern Italy [14], containing livestock of more than 9 million pigs [12]. In 2018, the pig population reached 8.5 million heads, with an average number of 287 pigs per farm, which ranked Italy in seventh place in European pig breeding [14].

Italian pig farming is characterized by a very high number of small pig farms managed at the familiar level. However, only 10% of the total heads are reared in those farms. Intensive industrial farms comprise about 50% of the total heads reared in Italy [8]. Intensive rearing systems usually use stables during the whole production cycle of pigs and are classified as open and closed systems when these are respectively based or not on the purchase of pigs from other farms. Commercial pigs, which are cross-bred, also called hybrids, are used in intensive farms to improve productivity performance.

Intensive farms in Italy are commonly specialized to produce two pork types: the "heavy pig" which is slaughtered once it reaches a live weight of 130 kg to produce the local "prosciutto crudo" (ham) and "salumeria" products (cured pork), and the "light pig" slaughtered at the live weight of 90-110 kg to produce pork meat [8]. On the other side, open-air farms are even more common and are based on pigs reared in outdoor pens.

Autochthonous breeds of Italy are Casertana, Mora Romagnola, Apulo-Calabrese, Nero Siciliano, Cinta Senese, and Sarda [15], whereas the most common cosmopolitan breeds are Large White, Landrace, Pietrain, Duroc, and Hampshire [8].

1.2.4. Pork meat production

The global pork production was 122 million tons in 2021, and it is expected to reach almost 127 million tons by 2030 [16]. Asia produces 112.5 million tons of carcass, representing 56% of pork production worldwide, and China remained the first pork producer, despite important losses of pig's heads caused by African swine fever (ASF) between 2018 and 2020 [16, 17]. USA and Canada produce 12%, whereas the European Union accounts for 21% of the global pork industry with France, Spain, and Germany ranked as the main producers. The EU pig meat production is expected to increase yearly by around 3%. Brazil and Vietnam will rise in pig production rate by around 5.5% yearly, and the Russian Federation by about 2.4% to satisfy consumer demands [16, 17]. However, pig meat production declined in 2021 in the Philippines, the Republic of Korea, and Myanmar.

Global pork production is shown in Figure 1.3 [14].

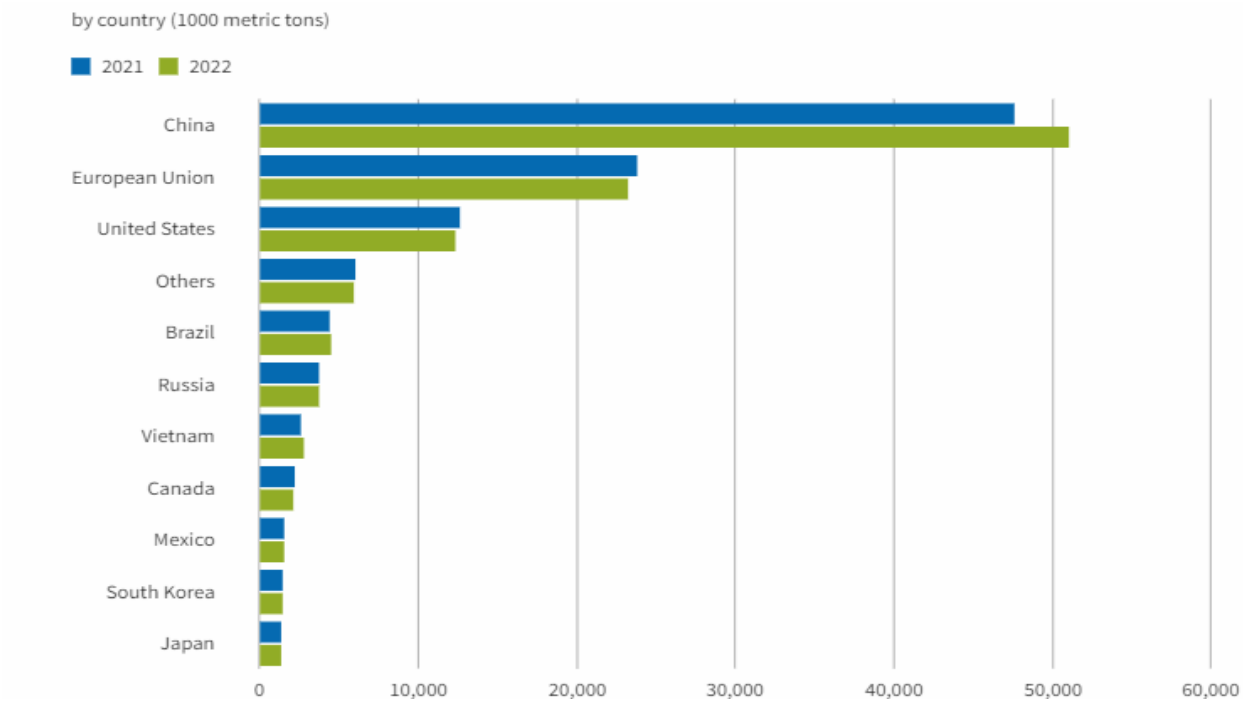


Figure 1.3. Diagram demonstrating the global pork production in 2021 and 2022 [14].

The EU pork production represents almost half of the European meat production, and swine is the largest livestock with 150 million reared pigs. This significantly important meat production sector represents about 8,5% of the total EU-27 agricultural achievements [18]. EU follows China in the pork-producing industry, but it is the first exporter of pig-derived products [19]. The pork meat demand's continuous increase, especially in the EU, pushed the prices to an expected increase of 32% in 2022 and improved the economic status of pig rearing in EU member countries [18, 20]. However, studies done by the European Commission on pig meat production, showed that this sector is facing several challenges, mainly related to the COVID-19 pandemic which slowed down exports to China, the diffusion of African Swine Fever in the EU countries, and the invasion of Ukraine by Russia related to animal feed scarcity [19]. In Italy, pig production has a very important impact on the Italian economy, since it represents 6% of the agri-food industry, equivalent to about 8 billion euros.

European agricultural food production labels products by “Protected Designation of Origin” (PDO) and it is characteristic of each country [21]. To fit these labels, the product processing cycle must fulfill specific rules according to the EU Regulations 1151/2012. [22] Cured hams are very well-known pork-based PDO products and Parma ham and San Daniele ham represent a remarkable part of the Italian pork industry. Parma ham production in Italy is the largest cured ham manufacturer since its value was assessed in 2020 at EUR 720 million, with the exportation of 29% of 8.7 million produced hams [22].

1.3. Factors causing losses in pork production

1.3.1. Relevant stressors in swine rearing.

The pork industry worldwide is affected by several factors, leading to important economic losses, such as animal transport, pig farming management, vectors, and diseases.

Losses caused by the transport of pigs from farms to slaughterhouses are affected mainly by the loading density and the handling of pigs [23,24]. This phase is crucial, and transport can cause “Pig stress syndrome”, also called malignant hyperthermia, which is a complex disease that causes physical effort leading to increased body temperature, dyspnea, cyanosis followed by cardiac arrest, and pig death. [23,24]. Post-mortem examination reveals rapid and complete animal rigor mortis, pulmonary edema, and pale soft and exudative aspect of musculature, known as PSE meat, that results in a devaluation of the carcasses. Moreover, non-ambulatory pigs, due to injuries at any time during transport, further affect the marketing process and lead to additional economic losses [23]. Stress induced by transport in pigs can cause the release of glucocorticoids, thus reducing the immune system efficiency, and increasing the susceptibility to numerous infectious diseases [24].

From the1960s till today, swine farms changed progressively from familiar to intensive rearing systems [25]. Intensive farming limits the contact of reared animals with wildlife, thus minimizing the risk of infectious diseases. However, Espinosa *et al.,* 2020 underlined that

intensively reared pigs are more susceptible to immunosuppression, which leads to the increase and the rapid spread of diseases in herds. Indeed, even if the diffusion of pathogenic agents within intensive farms is uncommon, because of limited access to infective agents, a more severe impact of diseases is expected due to the exposure risk [26]. Usually, the spread of infections is related both to direct contact between pigs and to indirect contact by vectors, facilities, water, feed, air, and staff. Direct contact with infected herds crucially affects the dynamics of the disease, whereas indirect contact leads to possible long-distance transmission, imposing a significant challenge for the pork production sector [27].

Swine-related pathogens are viral agents, bacteria, fungi, and parasites but although the coexistence of these agents is common in intensive systems, co-infection is scarcely reported in the pig population, [28]. One of the most important factors causing losses and high mortality rates in swine is the rapid spread of infectious agents, specifically viruses, mainly due to the intensification of production and the legal and illegal farm movement of swine. The prevention of this spread from farm to market is a crucial point in the pig production procedure and is important for food quality and safety, and the prevention of zoonotic diseases threatening public health [25].

1.3.2. Important swine viruses' characteristics and associated diseases

Numerous swine viruses that diffused years ago are still spreading nowadays causing outbreaks with very important economic losses in the pork industry. Some of these pathogens have been eradicated, and others are hard to be controlled [29]. Generally, viruses negatively affecting the pork industry in the last decades are Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), Foot and Mouth Disease Virus (FMDV), Porcine Circovirus 2 (PCV2), African Swine Fever Virus (ASFV), and several more [29, 30,31,32].

PRRSV was reported in Canada for the first time in the '80s and was detected later in the USA, Asia, and Europe [31]. This virus is enveloped with a single-stranded RNA of 15.4 kilobases (kb). It belongs to the Arteriviridae family with 10 open reading frames (ORFs), encoding for

structural and non-structural proteins. It causes reproductive failure in sows and respiratory infection of variable severity in piglets [29], leading to high mortality rates [33] and its control is challenging [34].

FMDV caused a devastating outbreak in China in 1997 which led to the death of about 38% of reared pigs, with a relatively high spread speed, thus infecting about a million pigs in the affected area [31]. This virus is a single-stranded RNA, non-enveloped, 8.4 kb in length, classified in the family Picornaviridae with a single ORF encoding a polyprotein for structural and non-structural proteins [29]. It causes fever, lameness, anorexia, and vesicles on the feet, tongue, and mouth [30]. It can also cause myocarditis in neonatal piglets leading to a high mortality rate [35].

PCV2 highly influences the swine industry, affecting pig production, and causing relevant economic losses [34,35]. It was discovered in 1998 and has spread worldwide in pig herds. This virus is single-stranded DNA, non-enveloped with a genome of 1.7 kb, classified in the Circoviridae family, and has only ORF1 and ORF2, encoding respectively for replicase protein and capsid protein, responsible for Porcine Circovirus-Associated Diseases (PCVAD) [31,35]. PCV2 targets the thymus and lymphoid cells leading to lymphoid depletion and then immunosuppression, thus causing the pigs to be more susceptible to other opportunistic viruses and bacteria. This mixed infection will then increase clinical signs severity and pathological lesions [36].

ASFV was first found in Kenya in 1921, persisted in Africa, and later reached other areas. Between the 50s and 80s, it spread to Russia, Europe, South America, and the Caribbean [37]. This virus is a double-stranded DNA, enveloped, with 170-193 kb, and is classified in the family Asfaviridae [29]. It causes 100% mortality with important economic losses worldwide [37] since it is highly resistant and can survive for a long time in the environment, pig-derived products, leading to very challenging disinfection [29]. It causes several clinical symptoms or

remains subclinical depending on the viral strain and causes hemorrhagic fever and peracute death [37].

1.3.3. Emerging viral diseases in pigs

Emerging viruses are related to the mutations in the RNA or single-stranded DNA of the implicated agent, the invasion of new hosts by a pathogen, and improper veterinary practices on farm and slaughterhouse premises [38]. Swine emerging viruses are, among others, Torque Teno Sus virus (TTSuV), Senecavirus A (SVA), Atypical Porcine Pestivirus (APPeV), LINDA virus, Porcine Circovirus 3 (PCV3), Parvoviruses (PPV) 2, 3, 4, 5, 6, 7, Swine Enteric Alphacoronavirus (SEACoV), and Porcine Deltacoronavirus (PDCoV), affecting pigs' health and recently causing a significant economic impact on swine industry worldwide [38,39].

While most emerging viruses are ubiquitous in global pig populations, they are still under investigation, because only some aspects, concerning mostly epidemiological or genetic issues, have been described. Therefore, there is a lack of knowledge of their biology, epidemiology, pathogenicity, and possible health threats [33,36,40], as well as the dynamics of co-infections and the usefulness of vaccines need deeper understanding [33].

1.3.3.1. Senecavirus A

Seneca Valley virus (SVV), known as SVA, is the only virus in the *Senecavirus* genus and belongs to the family Picornaviridae. This icosahedral linear virus is a non-enveloped, non-segmented, positive-sense, single-stranded RNA [41]. The length of the SVA genome is 7.2 kb with a 30 nm in diameter capsid [41,42]. It contains a leader (L) and various polypeptides: P1 and P3 including four polypeptides each, and P2 including three polypeptides [41,42]. The capsid protein comprises 60 protomers. Each protomer has four structural proteins with different lengths: VP1 (263 residues), VP2 (284 residues), VP3 (239 residues), and VP4 (72 residues) [43]. SVA genome includes 7300 nucleotides with one large ORF, which encodes for a 2181 amino acids polyprotein, and it is bordered by 5' untranslated regions (UTR) and 3' UTR. At the level of the 5' UTR, the type IV internal ribosome entry site (IRES) induces

RNA translation [41,42]. The 3' UTR has a poly-adenylated tail. Viral proteases ensure genome cleavage after translation, into the four structural proteins previously mentioned, and eight non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C, 3D, L) [41-44]. In 2002, SVA was described as a contaminant of PER.C6 (transformed cell retinoblast) in a laboratory of human genetics in the USA [41]. Additionally, since the 1980s, SVA was detected in the USA, after the isolation and sequencing, in pigs infected with picorna-like viruses [45]. In 2007, SVA was identified by PCR in vesicular lesions from the snout of a pig in Minnesota [46]. Singh *et al.*, 2012 reported the macroscopic and microscopic manifestations caused by SVA in a Chester White boar, suffering from lethargy and anorexia. Vesicles were found at the level of the snout and coronary bands, whereas ulcers were detected at the level of the hind and forelimbs. This virus was classified as a causative agent of vesicular disease. [47]. In 2014, several acute SVA outbreaks in Brazil were reported, with lesions on the sows' snouts and coronary bands and a death rate of 30-70% in neonatal piglets [48,49]. In an exhibition of pig breeding herds in Iowa in 2015, vesicular disease cases presenting acute lameness were observed, with no reported mortalities [50,51]. SVA was also reported in two Chinese farms in 2015, where affected animals suffered from lameness and vesicular lesions. The Chinese SVA strain was similar to the ones of the USA and Brazil [52,53]. In the following years, SVA spread in more than half of the Chinese provinces, and detected isolates were divided into five genetic branches after phylogenetic analysis [54], with several genetic recombinations between strains [55,56]. In 2016, SVA was detected in Colombia with around 98% nucleotide similarity to the US strain [57], and in Thailand, with also 98% nucleotide homology to the Canadian strain [58]. In 2018, SVA spread to Vietnam with 99% similarity to all previously mentioned strains [59]. The clinical presentation caused by SVA is similar to other vesicular-forming diseases, like FMD, vesicular exanthema, or swine vesicular disease [42,61,62]. Gross lesions were fluid-filled vesicles of different sizes, intact and ruptured, found at the level of the coronary band, snout, carpus, and tongue [42,63-65]. After

the rupture of vesicles, ulcerative lesions, and necrosis were detectable within digital spaces, on the snout, and the tongue leading to ulcerative glossitis [42,63–65]. Similarly, to other vesicular pathologies, these lesions started resolving in one week, and epithelium was completely repaired after around 14 days [66].

Neutrophilic infiltration, parakeratotic hyperkeratosis, and epidermal hyperplasia were commonly found on affected skin [47]. In the first week after experimental infection, pigs suffered from mild atelectasis, lung congestion, and hyperplasia of lymph nodes, tonsils, and spleen Also, infiltrates of inflammatory cells, associated with multifocal dermal separation, hemorrhage, and fibrin deposition were observed [61]. In piglets suffering from an epidemic, transient neonatal losses (ETNL) associated with SVA, lethargy, weakness, cutaneous hyperemia, diarrhea, neurological signs, and death were also reported [48], with the persistence of symptoms in piglets that survived. Leme *et al.,* 2016, described ulcers on the tongue and coronary bands and hemorrhage at the level of the kidneys. At histopathology, interstitial pneumonia and diphtheritic glossitis were reported, along with myocarditis, encephalitis, and degeneration of the ureters and urinary bladder [66]. A comprehensive study carried out in 2017, by Oliveira *et al.,* 2017 [67], on piglets from 23 Brazilian farms suffering from ETNL clinical manifestations, reported renal hemorrhage, pulmonary congestion, mesocolonic edema, and lymphadenopathy. Occasionally, hyperplasia was found at the level of Peyer's patches, along with ulcerative gingivitis, cheilitis, and glossitis. The most common histopathological lesions were interstitial pneumonia, necrotizing dermatitis of limbs and snout, intestinal atrophic villi, and ballooning degeneration of the urinary tract epithelium [66, 67,68]. SVA is diagnosed by clinical presentation, virus, and anti-SVA antibodies detection [69]. As recently reported by Houston *et al.*, 2020 [70], the diagnostic methods applied on vesicular material were polymerase chain reaction (PCR) and quantitative real-time PCR (qRT-PCR) [64,71–79], in situ hybridization (ISH) [67,70,86], and immunohistochemistry (IHC) [68–83]. The fastest, most sensitive diagnostic methods, reliable for SVA detection, are

conventional and qRT-PCR [70]. SVA can also be detected by neutralization assays, virus-competitive enzyme-linked immunosorbent assays (cELISA), indirect immunofluorescence (IF), and indirect ELISA targeting different proteins [61,63,64,69,81,82]. A recent, quick, and affordable diagnostic method has been developed and consists of a recombinase polymerase amplification (RPA), a reverse-transcription loop-mediated isothermal amplification (RT-LAMP), and a fluorescent hydrolysis probe-based insulated isothermal PCR (iiPCR) [84,85].

1.3.3.2. Torque Teno Sus Virus

Torque teno virus (TTV) is a virus non-enveloped belonging to the Anelloviridae family, genus *Anellovirus* and has a circular, small, single-stranded, negative-sense DNA genome [86,87]. The length of this genome is 2 to 3.9 kb [87]. The genus *Anellovirus* includes viruses infecting several animal hosts: swine, dogs, cats, and tupaia [88,89]. Torque Teno Sus virus (TTSuV) is the strain that infects pigs and wild boars. Currently, the International Committee on Taxonomy of Viruses genus accepted the Iotatorquevirus with TTSuV1a and the Kappatorquevirus genus, with TTSuV2a and TTSuV2b [88,89]. TTSuV has a 2.8 kb genome with a highly preserved UTR and three ORFs. ORF1 encodes the protein of capsid, ORF2 encodes a protein necessary for the replication of the virus, and ORF3 has an unknown function [86,90,91]. Anellovirus shows a horizontal transmission via the fecal-oral route [92,93], and a vertical route, plus a sexual route as demonstrated by the presence of TTSuV in boars' semen [94]. These characteristics allowed TTSuV spread worldwide [93,95,96]. Nowadays, TTSuV infection has no associated clinical signs in swine [97,98].

In 1999, TTSuV was first reported [99] and has been circulating since 1985 in pig farms [100], and TSuV1 and TTSuV2a were globally found in swine herds, showing a percentage of infection between 24 and 100% [101-108]. Regarding wild boars, limited data were reported [93,96,104,109,110]. On the other hand, highly variable infection frequencies of TTSuV2b have been recently reported in pig sera [105]. In Europe, TTSuV1 was reported in Spain [111,112], Sweden [113], Slovakia [114], and Italy [115]. TTSuV DNA has been found in

several swine organs (brain, heart, liver, lungs, hematopoietic tissues, and kidneys) showing a broad tissue tropism [92,108,112].

TTSuV has been found in combination with PCV2, PRRSV, and PPV, influencing the development of Porcine Dermatitis and Nephropathy Syndrome (PDNS), and Post-weaning Multi-systemic Wasting Syndrome (PMWS) [92,106,111–113,116,117].

The role and the pathogenicity of TTSuV regarding macroscopic and microscopic lesions are still unclear, with limited data reported in related studies [108,118–120]. Krakowka and Ellis, 2008, reported in their study on experimentally infected pigs with TTSuV1, progressive and severe interstitial pneumonia, moderate infiltration of lymphocytes and histiocytes in the liver, membranous glomerulonephropathy, and thymus atrophy [119]. Supplementary histopathological lesions were found in TTSuV1-infected pigs, such as non-suppurative encephalitis and myocarditis, mild lymphoid depletion, interstitial nephritis, hepatitis, hyperplasia of spleen follicles, bronchial interstitial pneumonia [108]. In a study done by Mei *et al.,* 2011, on experimentally infected pigs with TTSuV2, no gross lesions were reported, whereas histopathology showed hyperemia and congestion at the level of the endocardium and myocardium, membranous glomerulonephropathy, interstitial pneumonia, and mild portal hepatitis [120].

Detection of the TTSuV and its viral load was performed by PCR and qRT-PCR on serum samples [97,120-122], heart, liver, tonsils, spleen, and stillborn fetuses' blood [112]. Studies done by Krakowka and Ellis (2008) and Polster *et. al,* 2022 mentioned the probe used in ISH for TTSuV1 [108,119].

1.3.3.3. Atypical Porcine Pestivirus and LINDA virus

Pestiviruses, belonging to the Flaviviridae family, genus *Pestivirus*, is a small, single-stranded RNA virus. [123,124]. Classification (2017) revealed 11 species of Pestiviruses from A to K [125]. Classical Swine Fever Virus (CSFV), Bovine Viral Diarrhea 1 and 2 (BVDV1, BVDV2), and Border Disease Virus (BDV) are the classical pestiviruses corresponding to the

first four species from A to D. Species from E to K represent Pronghorn Antelope Pestivirus (PAPeV), Porcine Pestivirus (PPeV), Giraffe Pestivirus (GPeV), HoBi-like Pestivirus (HoBiPeV), Aydin-like Pestivirus (AydinPeV), Rat Pestivirus (RPePV), and Atypical Porcine Pestivirus (APPeV) [124]. Pestivirus K, known as APPeV, has a spherical viral structure with an approximate 60 nm diameter and a genome of 11 to 12 kb in length [126]. This virus consists of one large ORF encoding for 3635 amino acids, and a polyprotein divided into four structural proteins, [127,128,129] such as a capsid (C) and three envelope glycoproteins (Erns, E1, and E2), and eight non-structural proteins as follows: Npro, P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B [129,130]. The three-envelope glycoproteins help the virus invasion of host cells. Erns, E2, and the nonstructural protein NS3 are responsible for the host humoral immune response after viral invasion [130] The E2 protein in APPeV is shorter than other pestiviruses' E2 protein [129], and it is the main antigenic protein used to find a suitable vaccine [130,131]. Gonzalez *et al*., 2020 reported that APPeV was present in Switzerland in 1986 [132], but it was first described in 2015 as a major cause of congenital tremors of the head and extremities of piglets [130]. APPeV was frequently reported in Canada, Brazil, Europe (Austria, Germany, Hungary, Netherlands, Spain, Italy, Sweden, Switzerland, and the Republic of Serbia), and Asia (China, South Korea, and Taiwan) [133,135]. Gonzalez *et al*., 2020 reported that several new APPeV sequences were described since the first report done in 2015, leading to a division of the phylogeny into three, four, or even five different genotypes. They also reported that APPeV genome identity (99%) showed a high heterogeneity even within the same country, with difficulties in the deduction of the origin and the spread of different virus strains [132]. APPeV is present worldwide with a high prevalence in domestic pigs, and in wild boars which are the reservoir of APPeV, and this might have an epidemiological significance [130]. Gonzalez *et al.*, 2020 mentioned that research done by Postel *et al.*, 2022 revealed, after the analysis of 1460 samples from clinically healthy pigs from several countries, a fluctuation of APPeV prevalence from 10% to 60% of the seropositive tested

animals [132 and references therein]. The APPeV occurrence was higher than 70% in piglets suffering from congenital tremors and varies from 0% to 22% in apparently healthy pigs, implying that the novel virus can be considered a swine pathogenic agent of global importance [136]. Pestivirus K causes high pre-weaning mortality rates (up to 60%,) leading to relevant economic losses in the pig industry [130,137]. Nevertheless, an APPeV outbreak's economic impact on pork production remains uncertain, but it is assessed that this virus caused the decline of the number of weaned piglets/sows by more than 10% [135].

Gross findings were restricted to local dermatitis and mild suppurative omphalitis. From the histopathological side, the central, and peripheral nervous systems showed regular myelination [138]. However, hypomyelination in the white matter of naturally infected pigs, and the detection of oligodendrocytes, with mild, partially perivascular, focal gliosis, and small foci of extravasation at the level of the kidneys were found [139]. In piglets, necrosis of neurons and neuronophagia associated with satellitosis were observed in the cerebral cortex and spinal cord [126].

The virus should be detected in the samples from saliva, serum, central nervous system (CNS) material, and cerebrospinal fluid by PCR, IHC, and ISH [126,138,139]. Dessureault *et al.*, 2018 reported the detection of APPeV by ISH in the cerebellar inner granular layer, neurons of the spinal ganglia, and tonsils [138,140]. The pestivirus was also detected by PCR in the CNS, in lymphoid organs with the highest viral load [126,140,141], as well as in semen, saliva, nasal and rectal swabs [140,141].

Pestivirus L, also known as LINDA virus, was discovered in an Austrian piglet farm in 2015. The most important clinical signs appreciated during this outbreak were severe lateral shaking and suckling incompetence in piglets, with an increase in death rates. The novel pestivirus was called lateral shaking-inducing neurodegenerative agent (LINDA) [142]. The sequencing of LINDAV genomes showed a high similarity to the Bungowannah virus (BPuV) detected in Australia in 2005. [143]. Studies done in Switzerland and Germany didn't show the presence

of LINDAV in swine sera samples [144,145]. Nevertheless, in another study, it is reported that despite the absence of clinical signs, gross or microscopic lesions in the affected piglets, LINDAV infection showed viremia with rapid seroconversion, and the virus was detected in lymphoid organs after 21 days [146]. Recently, Kiesler *et al.*, 2021 mentioned a new LINDAV strain in an Austrian farrow-to-finish farm, where they reported reproductive failure in sows and gilts such as abortion, stillborn and mummified fetuses, neonatal deaths, and signs of congenital tremors in the litters [147,148]. There were no significant gross lesions, whereas, at histopathology, there were multifocal scattered and perivascular lymphoplasmacytic myelitis and encephalitis.

The detection of LINDAV could be performed by IHC, using specific monoclonal antibodies [148] as well as by RT-PCR [142,146-148].

1.3.3.4. Porcine Circovirus 3

Porcine circoviruses are circular, small, single-stranded DNA, capable of autonomously replicating viruses, belonging to the family Circoviridae, genus *Circovirus* including PCV1, PCV2, PCV3, and PCV4 [149]. PCV3 is a single-stranded DNA virus, non-enveloped, icosahedral with a genome of 2000 bp, which is the biggest genome in all PCVs. This genome contains two ORF1 and ORF2 in opposite directions, where ORF1 encodes a 296 amino acid replicase protein (Rep), and ORF2 encodes a 241 amino acid capsid protein (Cap), representing the structural proteins in circoviruses and inducing specific immune responses in hosts [150, 151,152,153]. PCV3 genome has also an ORF3 encoding for a 231 amino acid protein of an unknown action [154]. PCV3 genome is subdivided into different groups PCV3a and PCV3b with 99% and 97% sequence identities, and PCV3c [155]. Temeeyasen *et al.*, in 2021 reported the presence of some PCV3 amino acids mutations (24 and 27 of the protein Cap), useful for the classification of PCV3 into the three groups a, b, and c [156]. PCV3 was first identified in the USA (NC), in 2015, on a sow farm with PDNS and reproductive failure, [149,150,157,158,159], causing an increase in mortality rate [160]. This virus appeared later

in many countries in Asia, South America, and Europe [150,157,158,159]. Retrospective studies showed the presence of PCV3 in pig herds in the early 90s even if it was recently discovered [152,157]. PCV3 has been reported in several domestic and wild animal species, with the highest prevalence of this virus found in wild boars, thus playing a very important role in its epidemiology [161]. This virus was found in sows, mummified fetuses, and stillborn [162], with a high prevalence in weaning piglets [160]. Moreover, it was demonstrated that PCV3 can undergo cross-species transmission [158]. Chen *et al.*, 2021 mentioned in their study that PCV3 showed a broad histotrophism in several pig tissues and fluids, with different viral loads depending on the tissue origin. This virus may also be found in farrowed sows and colostrum, as well as in the gilt placenta, in the pre-colostrum sera of piglets, and semen samples, which means PCV3 is transmitted via horizontal and vertical routes [162].

In experimental studies, PCV3 also caused the enlargement of the inguinal and tracheobronchial lymph nodes whereas the kidney presented severe, multifocal to coalescing, chronic-active glomerulonephritis or scattered hemorrhages [150,158]. In addition, multifocal to lobular, hemorrhagic bronchopneumonia, necrotic hepatitis with gray-white nodules, and enlarged spleen with necrotic foci were also reported [150,158]. At histopathological examination, granulomatous lymphadenitis was often present in experimentally infected subjects, associated with multinucleated giant cells, necrosis, lymphocytic depletion, eosinophilic infiltration, and hemosiderin deposition [158]. In the kidneys, cortical tubules were dilated, and regeneration of tubular epithelium was observed, associated with diffuse membranoproliferative glomerulonephritis [150,158]. In the lungs, peribronchiolar lymphohistiocytic inflammation was reported, as well as thickening of the alveolar walls, and severe necrotizing and interstitial pneumonia, whereas the liver showed dilation and congestion of the hepatic central veins and sinusoids [150,158]. The small intestine presented the degeneration of epithelium, necrosis, and lymphocytic eosinophilic infiltration [150]. Local to systemic vasculitis was mainly reported in the heart, kidney, and intestines [163].

Histopathological examination in naturally infected pigs showed fibrinoid necrotizing vasculitis in the dermis, associated with lymphoplasmacytic dermatitis. The epidermis was hyperplastic with mild ortho keratotic hyperkeratosis, with palpable purpura and inflammation of the surrounding hair follicles [149,157]. Another study on naturally infected pigs reported lymphoplasmacytic infiltration in the external layers of arterioles of the heart [159], and lymphocytic periarteritis in the mesentery [157].

The possible pathology mechanisms induced by PCV3 are intricate and are divided into at least three categories: virus-induced, immune response-mediated, and immune-complex mediated mechanisms [163]. See the possible PCV3-induced pathology mechanisms in Figure 1.4 [163].

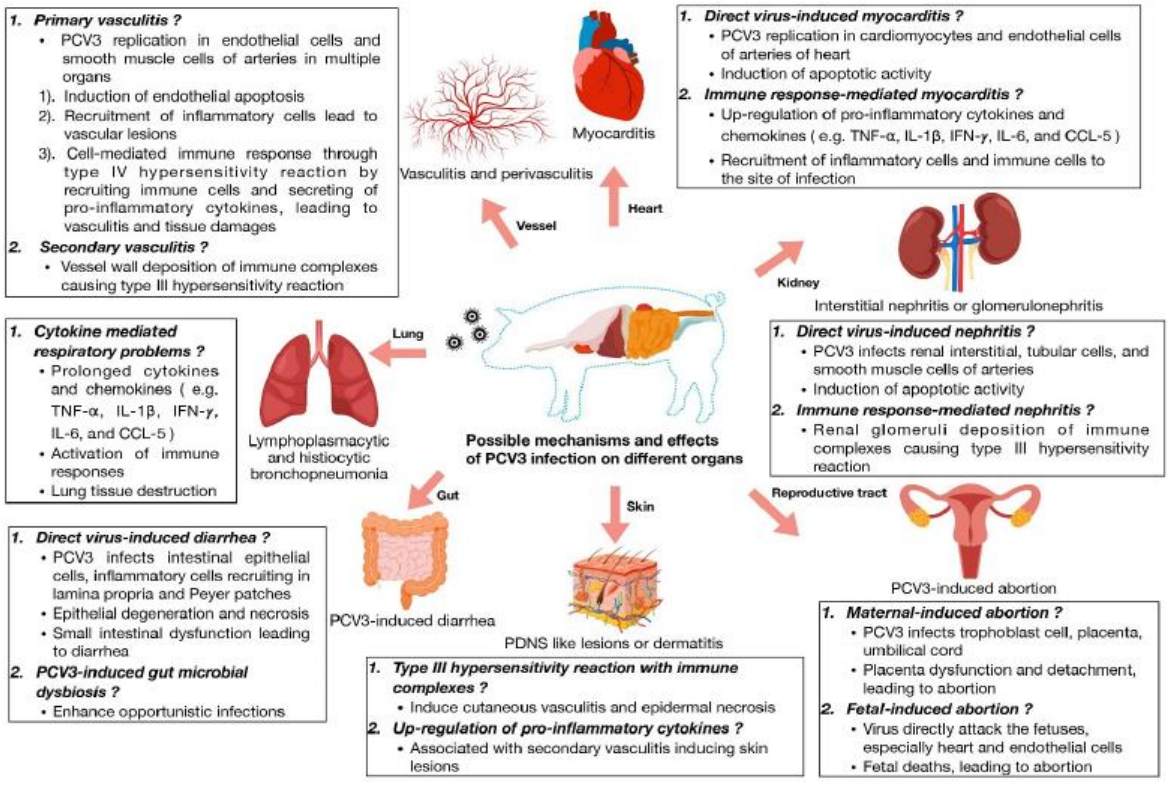


Figure 1.4. The possible PCV3-induced pathology mechanisms [163].

To detect the presence of the novel virus PCV3, in the affected organs of patients, several methods could be used. The diagnostic technics utilized are qPCR, IHC, ISH, iIFA, and indirect ELISA. PCV3 antigen was detected, considering the applied tests in different studies,

by qPCR and by IHC in the epithelial cells of bronchi, alveolar exudate, macrophages, and interstitium of the lungs. It was also detected in the renal tubular of the kidneys, in the hepatic sinus of the liver, and in the necrotic tissues of the heart [149,150,157,158,159]. The novel antigen was also detected by ISH, in the arteries of the heart's smooth muscle, kidneys, cerebrum, cardiac myocytes, CNS [157,159], also in adipocytes and large intestines, in hair follicle endothelial and epithelial cells of the dermis [157].

1.3.3.5. Parvoviruses 2, 3, 4, 5, 6, 7

Parvoviruses (PPVs) are members of the Parvoviridae family and are characterized by the two subfamilies *Parvovirinae,* infecting vertebrates, and *Densovirinae,* infecting arthropods [164]. PPVs are linear, small, non-enveloped, negative sense, single-stranded DNA viruses of approximately 4 to 6.3 kb in length [164,165]. Parvoviruses genome has two genes containing each P4 and P40 protomers. The P4 protomer is responsible for the transcription of the nonstructural proteins having a replicase activity from the left ORF, whereas the P40 protomer is responsible for the transcription of the structural proteins from the right ORF. There is an additional ORF3, which is peculiar to the PPV4, encoding for the translation of nuclear phosphoproteins, and is located between ORF1 and ORF2 [166].

Parvoviruses are divided into three genera: *Protoparvovirus* (PPV1), *Tetraparvovirus* (PPV2-3), and *Copiparvovirus* (PPV4-6). They are very important pathogens causing reproductive failure in swine leading to significant losses in the pork sector worldwide [167,168].

Lagan *et al.*, 2020 mentioned that PPV2-4 appeared in the 50s and has a high substitution rate in nucleotides, like RNA viruses. The capacity of parvovirus to adapt and spread in pig populations called for constant phylogenetic mapping and disease association study. These authors also mentioned that PPV2 was discovered in 2001, and PPV3, like PPV2, was identified in China in 2007 [169 and references therein]. PPVs 2, 3, and 4 were found globally in swine herds and were all described by Lau *et al.,* in 2011 [170]. PPV4 was detected in 2010

in lung lavages collected from wasting pigs during an outbreak of PCVAD in North Carolina [169 and reference therein] and then reported in Asia, Europe, and Africa [170,171].

PPV5 was identified in the USA in 2013, whereas PPV6 was discovered in China in 2014. Parvoviruses 5 and PPV6 are both very closely related to PPV4. Parvoviruses 7 was identified in 2016 by Next Generation Sequencing (NGS) and is most closely related to turkey parvovirus (TuPV) and was classified as the proposed Chapparvovirus genus [169 and references therein].

An additional parvovirus, under the name Bocavirus (PBoV), was first reported by Blomstrom *et al*., 2009 in piglets having PWMS and infected with PCV2. Bocavirus was found, in a high percentage, with other infections, suggesting that this pathogen could act as an opportunistic agent and be activated during co-infections [172,173].

The prevalence of each parvovirus varies, depending on the pig's health status, its age, and the presence of other pathogenic agents [174]. Unfortunately, data regarding the gross pathology related to the novel parvoviruses is understudied. In histopathology, Novosel *et al.*, 2018 reported the lesions caused by PPVs as severe congestion of the lungs, associated with T lymphocytic infiltration, around the bronchi and B lymphocytes in the blood vessels, necrosis of epithelial cells, and reduction of the alveolar spaces. Mucus and inflammatory cells in the bronchial lumen were also reported [175].

The detection of the different parvoviruses was performed by PCR, specifically duplex or multiplex RT-PCR, to detect more than one PPV strain. Kim *et al.*, 2022 mentioned the use of the conventional multiplex PCR protocol on the serum and lung homogenate tissue samples. Serum samples tested negative for PVVs, whereas different viral loads were found in lung samples of pigs of all ages [176].

Another study done by Lagan *et al.*, 2020 pointed out the use of the qPCR Quantitech SYBR Green protocol to detect the viral load of PCV2, PVV2, PPV3, and PPV4 in lung homogenate, colon, small intestine, thymus, and brain samples [169]. All PPVs were detected in fetuses

and abortion serum, but no positivity was found in the colon, small intestine, thymus, and brain samples. All lung samples, mediastinal lymph nodes, and nasal swabs were positive for all PPVs with the highest prevalence for PPV2 in the lungs and the bronchial lymph node. [169 and references therein].

1.3.3.6. Porcine Enteric Alphacoronavirus and Porcine Deltacoronavirus

Coronaviruses belong to the order Nidovirales, family Coronaviridae, and subfamily *Orthocoronavirinae*. They are enveloped, positive-sense, single-stranded RNA viruses, and are divided into 4 genera: Alpha, Beta, Gamma, and Delta coronaviruses [177-179]. Alphacoronaviruses include Transmissible Gastroenteritis virus (TGEV), it's variant Porcine Respiratory virus (PRV), Porcine Epidemic Diarrhea virus (PEDV), and Swine Enteric Alphacoronavirus (SeACoV). Betacoronaviruses include Porcine Hemagglutinating Encephalomyelitis virus (PHEV), and Porcine Deltacoronavirus (PDCoV) [180 and reference therein].

Coronaviruses infect mammals and birds, causing subclinical, and gastrointestinal diseases [177-179]. TGEV, PEDV, PDCoV, and SeACoV cause acute diarrhea in neonatal piglets and have high mortality rates, causing relevant losses in the pork industry [177 and references therein].

SeACoV also known as Porcine Enteric Alphacoronavirus (PEAV) or Swine Acute Diarrhea Syndrome (SADS-CoV) [177 and references therein] has a spike envelope protein (S) found on the surface of the virus and has the crown-like characteristics. PEAV particles are 100-120 nm in diameter, round, and covered with visible trimers of S protein, and its genome is 27,155 nucleotides in length with a 5' capsid and a 3' polyadenylated tail. This genome has nine ORF flanked by a 5' UTR and a 3'UTR. At the level of the 5' two/third of the genome, ORF1a and ORF1b encode for 16 nonstructural proteins (Nsp1-16), and at the level of the 3'third, there are ORFs encoding for four structural proteins which are as follows: S protein, envelope (E), membrane (M), and nucleocapsid (N), ORF3 which is an accessory ORF between the two

structural proteins S and E, and two conjoining ORFs (NSa, and NSb) following the N gene [180].

SeACoV was discovered in China, in February 2017, after the occurrence of diarrhea outbreaks in piglets, in four pig farms [177,178]. It caused severe acute diarrhea, and rapid weight loss, in newborn piglets [177-179,181,182]. Yang *et al.*, 2020 reported, in their recent review, that there no more SeACoV cases were registered in pig farms, from 2017 to 2019. Unfortunately, in February 2019, the novel enteric coronavirus reemerged in the city's pig farms, but it has not been reported in any other China provinces, or any other region worldwide [177].

Acute diarrhea caused by PEAV leads to moderate to high mortality [177], of more than 90%, in piglets aged less than 5 days, and about 5% in piglets older than 8 days [179]. It has been causing significant economic losses in Asia, and North America [177], and represents a continuing threat to the pig production market development [178]. PEAV targets the digestive system cell lines and causes the thinning of the wall of the small intestine as well as the cecum and colon, which get filled with yellow, watery feces and causes mesenteric congestion in some cases [181,182]. Histopathological lesions, caused by the new PEAV, are characterized by atrophy and severe adhesion of intestinal villi, associated with mucosal inflammation [181,182].

For the detection of the enteric coronavirus antigen, several tests could be performed, such as IFA, ELISA, and PCR [177,179,181,182]. The samples used for PEAV detection are small intestine tissue samples, rectal swabs [177,181,183], feces, tonsils, stomach, mesenteric lymph nodes, and other organs [181]. Most commonly, rectal swabs, feces, and small intestines were highly loaded with the virus, and majorly positive to the PCR testing, showing fluorescence in the serological assays.

Another novel coronavirus, Porcine Deltacoronavirus (PDCoV), shares the same characteristics as other coronaviruses. PDCoV is the smallest among all coronaviruses with a

genome of 25.4 kb long [184,185]. The genome contains common coronaviral genes and is organized as follows: 5'UTR-ORF1a-ORF1b-S-E-M-NS6-N-NS7-3'UTR. ORF1a and ORF1b are at the level of the 5' three/fourth and are responsible for two overlapping replicase polyproteins, then structural protein ORFs come next (S), (E), and (M), followed by the nonstructural proteins NS6 and NS7 at the level of the 3' UTR having in between the structural protein nucleocapsid (N) [180]. ORF1a and ORF1b encode for the viral replication, spike (S), envelope (E), and membrane (M) encoding for the viral assembly and the induction of the antibodies specific for virus neutralization. The receptor-binding protein S encodes for the viral entry and virus-host interaction. NSP6 nonstructural protein and Nucleocapsid (N) cooperate to achieve bonding activities, and the nonstructural NSP7 protein is responsible for accessory functions [186]. The N protein is responsible for the packaging of the genome into long, flexible, helical ribonucleoprotein complexes called capsids, which are responsible for the protection of the genome and the replication of the virus [185].

See the genome organization of alphacoronavirus and deltacoronavirus in Figure 1.5 [185].

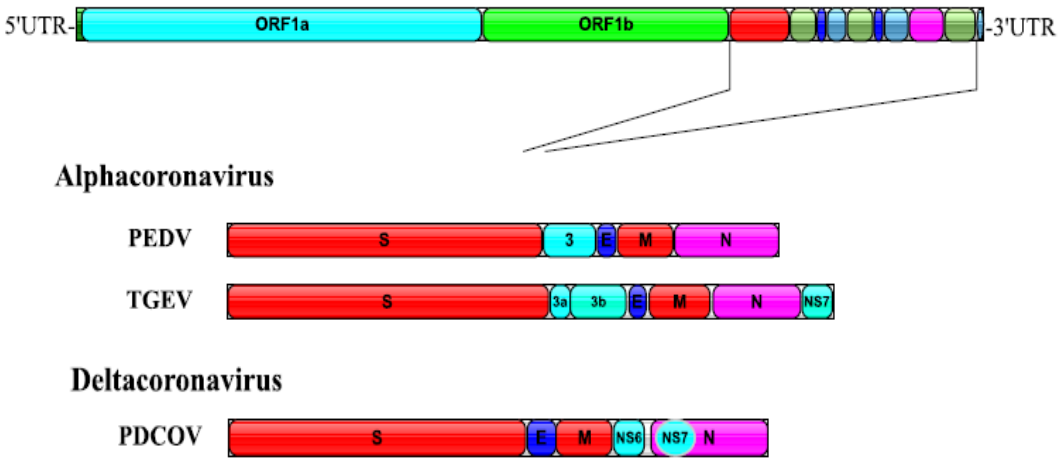


Figure 1.5. Diagram of the genome organization of alphacoronaviruses and PDCoV [185].

Porcine Deltacoronavirus was first identified in 2009 in several domestic and wild bird species in Hong Kong and then reported in a retrospective study in 2012, on rectal swabs, of affected pigs, presenting diarrhea, vomiting, and dehydration in piglets [184,187,188]. Porcine

Deltacoronavirus rapidly spread to the US, Canada, China, Thailand, Laos, and Japan [184,187-190,191]. After that, it spread to Canada, Asia, and Mexico [187-191], sharing notably very high similarity in strains' nucleotides [190]. A retrospective, epidemiological study, revealed the presence of the deltacoronavirus in the US since 2013, and in some areas of China in 2004 [191].

The clinical presentation of PDCoV is very similar to other coronaviruses, such as TGEV and PEDV, except for the mortality rate, especially in nursing pigs, which is lower for PDCoV than other coronaviruses but still results in huge losses in the pork industry [187,192].

The clinical signs of PDCoV similar to PEAV infection are vomiting, dehydration, anorexia, and watery diarrhea in piglets with gas-distended intestines, especially at 4 days post-infection, whereas no lesions were registered in other organs [188,190,191].

At histopathology, reported lesions are also restricted to the intestine, with a multifocal to diffuse swelling and intracytoplasmic vacuolation of enterocytes, and submucosal edema, with lymphocytic and neutrophilic infiltration [184,185,190-192]. Villi atrophy associated with mild to severe acute jejunitis and ileitis was also reported, characterized by lymphocyte infiltration, and scarce neutrophils, associated with blood vessel congestion [184,187-192].

For the detection of PDCoV, several tests could be performed, such as PCR, IHC, ISH, indirect IFA, and ELISA. Tonsil, heart, trachea, lung, liver, spleen, kidney, stomach, mesenteric lymph node, intestine, and fecal samples were used as samples [187,189,191,193]. The presence of the highest viral load of PDCoV was detected by PCR in the fecal samples and the apical part villi of the small intestines a few days' post-infection, as well as in the mesenteric lymph node. [184,187-189,193]. Immunofluorescence at ISH was reported in the epithelium of small and large intestines [193].

Chen *et al.,* 2015 as well as Dong *et al.,* 2016, reported that PDCoV was found in a very low quantity in several organs [190, 191].

2. Swine sector overview in Sardinia

2.1. Pig farming in Sardinia

In Sardinia, pig farming can be traced back to the 6th millennium BC, based on bone remains found in archaeological sites [194]. Later, during the Roman Empire, Sardinian pig breeding increased to fulfill taxes requirements of the central Rome government, and during the Middle Ages pig farming was even ruled in several chapters of laws collection, the Code of Mariano IV, issued by the Judge of Arborea (1317-1375), and the subsequent "Carta de Logu" issued by his daughter Eleonora (1340-1404). Sardinian farms comprise 163.000 pigs, reared in farms, mainly located in the historic provinces of Oristano, Cagliari, Sassari, and Nuoro [195], with an average number of 13 pigs per farm [196]. This means that intensive-industrial farms are extremely uncommon and that almost the totality of farms is limited at the family level for self-production and consumption. The extensive method, based on the use of large outdoor pastures, is very common in marginal areas [197].

Ninety percent of the Sardinian farms are housed and 10% are semi-wild, whereas wild farming is prohibited as prescribed by the Extraordinary Plan for the Eradication of African Swine Fever (Regional Law 22 December 2014, n.34). This type of farming was traditionally widespread in some areas of the region, where the exploitation of the resources of the territory was maximized. The organization of the breeding was limited, with few food supplements and without planning of mating and parts [195]. Semi-wild farms differ from the previous one, in the way the animals are hospitalized, by the organization of mating, and by the regulation of quantity and type of food supplements. Generally, for this type of breeding in the Sardinian territory, pre-existing fixed structures are used to shelter the sows in particular production stages, especially during farrowing, suckling, and mating. During gestation, however, the sows are reared outdoors in confined pastures [195]. Most semi-wild farms are in Olbia (36%) and Sassari (24%) provinces, while Sanluri and Cagliari areas are only marginally interested in semi-wild farming (4% and 7%, respectively).

As previously described, almost all Sardinian farms are of the stable type. About 96% of the farms are closed-cycle and, among the 191.537 animals surveyed by 31 December 2019, most of these are in the Cagliari area, which counts 2.804 closed-cycle farms for a total of 43.439 animals reared. The open-cycle farms are mainly concentrated in the Nuoro area, with a total of 47 farms, even if the Oristano area covers the highest number of animals in open-cycle farms, with a total of 3.490 animals reared [195].

2.2. Pig production and consumption in Sardinia

Sardinian companies are mainly oriented towards the production of suckling pigs (6-10 kg) and lean (90-110 kg), but also heavy pigs (130-160 kg) are produced to a minimum extent. Since 1978, the Sardinian pig economy has suffered a drastic block because of ASF, which affected domestic pigs, and wild boars [195]. Notably, the diffusion of ASF in the territory has prevented producers from guaranteeing food safety [195]. In the last decade, contrast measures against the illegal free-raging pigs, responsible for the infection persistence, have contributed to a sensible decline in outbreaks. In July 2022, due to the favorable results of the eradication plan, restrictions applied to the commercialization of swine products have been lifted, except in the Nuoro area, based on 2021/605 (EU) regulation. The plan for 2023, aimed to eradicate the disease, is articulated in 5 points, some of which involve, among others, active surveillance in the region and passive surveillance of domestic and wild swine populations [198].

2.3. Virus spread in Sardinia

Sardinia is the oldest Mediterranean widespread region of ASFV. Despite many eradication programs in this area, ASFV is the agent responsible for the ASF disease and has been first identified in Sardinia in 1978 causing important outbreaks in swine herds, free-ranging pigs, and wild boars. According to the Istituto Zooprofilattico Sperimentale (IZS) of Sardinia, all

Sardinian samples dating from 1978 to 2018 are classified under genotype 1, different from the remaining genotypes abundant in other countries. Studies reported that free-ranging pigs could represent a reservoir of ASFV in Sardinia [199, 200]. Another significant swine virus reported in the Sardinian region is known as the PCV2 virus, which has eight different genotypes from PCV2a to PCV2h. The analyses of domestic pigs and wild boar, sampled from 2009 to 2013 showed that PCV2b was the most frequently found strain within the Sardinian swine livestock. Another major alteration of genotype dominance from PCV2b to PCV2d was reported, noting that the first detection of PCV2d in Sardinia dated back to 2010 and 2011 [201]. Regarding emerging viruses, two recent works reported the presence of TTSuV and PCV3 in pig herds of Sardinia [115,154]. TTSuV was first reported in sera of otherwise healthy pigs, highlighting the presence of co-infections by multiple viral species, TTSuV1, and TTSuVk2ad. PCV3 was detected in swine herds, feral pigs, and wild boars of Sardinia by Dei Giudici *et al*., 2020 underlining that Sardinian strains shared similar genomic features to other PCV3 strains reported worldwide [115,154].

3. Aim of the thesis

The pork industry worldwide could be threatened by several factors such as emerging viral diseases. Emerging viral diseases in pigs may be completely new appearing agents or previous viruses presenting new strains; moreover, very little information is known about the presence of emerging swine viruses in Sardinian pig farms, as well as the presence of co-infections in these herds. Emerging viruses are found in pigs of all ages and co-infection could lead to the creation of recombinant viruses representing a new challenge in intensive pig farms. Additionally, it is difficult to differentiate pathological aspects caused by emerging viruses and other more common viruses, and recurrent lesions in pig organs, such as interstitial pneumonia and lymphoid depletion, are similar when emerging and common viruses are involved making it difficult to associate lesions to viruses.

A multidisciplinary approach must be performed to understand the prevalence and the pathogenic effect of emerging viruses and their impact on swine health status in Sardinia. To accomplish this goal, our work aimed to evaluate the organs of pigs from different age categories classifying lesions grossly and by histopathology, and detect the viral circulation of the most common and emerging viruses by RT-PCR in an intensively reared Sardinian pig herd. For this purpose, our research was articulated as follows:

- Evaluate and classify lesions from organs by gross pathology and histopathology
- Detect the presence and the viral load of the PRRS and PCV2 and PPV1 viruses in pig tissues by RT-PCR
- Detect the presence and the viral load of the emerging viruses in pig tissues: SVA, PCV3, PPV2, PPV3, PPV4, PPV5, PPV6, PPV7, TTSuV, APPeV, LINDAV, PDCoV, and SeACoV
- Highlight the presence of co-infections of several viruses in pig tissues.

Chapter 2

Materials and methods

2. Materials and methods

2.1. Sample collection

In November 2020, tissue samples of 29 pigs were collected from a North Sardinian slaughterhouse receiving pigs from one intensive farm located in the south of Sardinia. The swine samples belonged to three different age groups: 10 adult pigs (174 days), 10 post-weaning (65 days), and 9 piglets (40 days). Regarding the gender pigs were both male and female but we did not know the ratio between the number of females and males. Piglets were immunized against *Mycoplasma hyopneumoniae* and PCV2, post-weaning for *Mycoplasma hyopneumoniae*, Pseudorabies disease, and PCV2, whereas adult pigs were vaccinated against Mycoplasma hyopneumoniae, Pseudorabies disease, PCV2*, Erysipelothrix rhusiopathiae, Parvovirus, E. coli,* and Atrophic rhinitis disease. Each pig's visceral organs have been refrigerated and transported from the slaughterhouse to the necropsy room of the Department of Veterinary Medicine of Sassari University.

2.2. Necropsy

A macroscopic examination was accomplished on the organs of 29 pigs (lung, heart, liver, spleen, kidney, intestine, mediastinal lymph node, and mesenteric lymph node), to detect the presence of lesions. The lungs were observed for consistency and color variation and two horizontal cuts were applied in the lower part of each lobe to check for internal lesions. The trachea and bronchi were cut vertically to evaluate the presence of mucus and exudate. Mediastinal and mesenteric lymph nodes were carefully separated from the pleura and peritoneum, respectively, and cut open to check for variation in consistency and color.

The heart was opened by positioning its right side in the left hand and by applying a transversal incision at the apex of the heart, exposing the left ventricle. Then an incision was performed from the apex of the left ventricle to the right auricle and the aorta along the ventricular septum. Another incision was applied from the bottom center of the left ventricle to the left

auricle, and the heart was thoroughly checked for any lesions such as thrombosis, pericarditis, or others. The same technique was applied to the right side of the heart.

The intestine was incised along its anti-mesenteric side, for its entire length, to check the mucosa as well as its content. The mesenteric lymph nodes were also incised along their longitudinal axes and evaluated.

The capsule of the kidney was gently removed after a longitudinal incision along its lateral side, to check for the presence of signs of inflammation. The kidney was then completely incised along its longitudinal side to evaluate the cortex/medulla ratio and the presence of any lesions.

The liver and spleen were examined on the ventral and dorsal sides and multiple incisions were made to evaluate liver parenchyma and spleen white and red pulp.

From each organ two aliquots were collected: the first for histopathological examination was fixed in 10% neutral buffered formalin, and the second, intended for molecular biology analysis, was frozen at - 80°C and then submitted to the "Istituto Zooprofilattico della Sardegna" (IZS). Digital pictures of each organ were taken.

2.3. Histopathological examination and classification of lesions

2.3.1. Histopathological examination

A total number of 232 samples were 10% formalin-fixed for 48h, dehydrated in an automatic tissue processor (HISTO-PRO200) with increasing concentrations of alcohol and xylene (Figure 2.1), and paraffin-embedded (Figure 2.2). Sections of 3µm thickness were obtained using a microtome (RM2245, Leica Biosystems) (Figure 2.3) and then automatically stained with Hematoxylin-Eosin (H&E) in a multistainer (ST5020 Bath Array, Leica Biosystems) (Figure 2.4). Slides were examined at a light microscope (Nikon Eclipse 80i) (Figure 2.5).

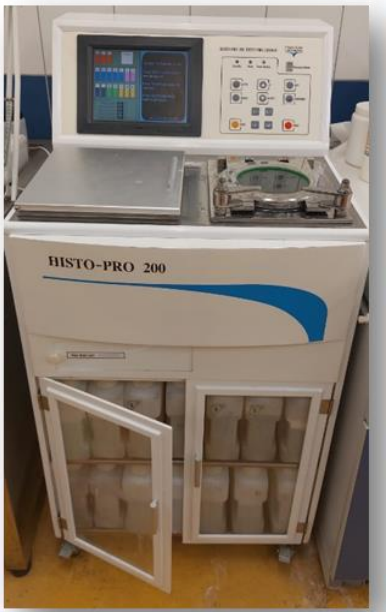


Figure 2.1. Automatic tissue processor (HISTO-PRO 200)

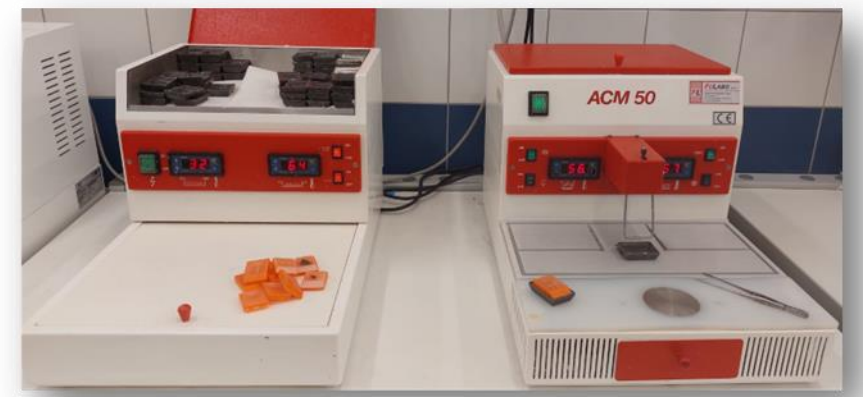


Figure 2.2. Paraffin- embedder (ACM 50)

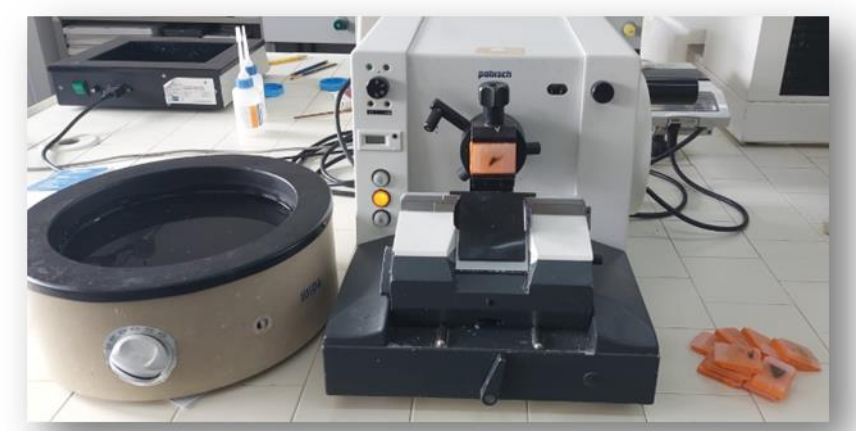


Figure 2.3. Microtome (RM2245, Leica Biosystems)

Figure 2.4. Multistainer (ST5020 Bath Array, Leica Biosystems)

Figure 2.5. Light microscope (Nikon Eclipse 80i)

Histopathological lesions were evaluated and classified by two pathologists, based on the morphological tissue changes, type, distribution, and severity of the inflammation. Slides were evaluated at a light microscope (Nikon Eclipse 80i) and photomicrographs of significant lesions were taken.

2.3.2. Classification of tissue lesions

Each organ was observed at the microscope and lesions were evaluated using the histopathological scoring as follows:

Table 2.1. Classification scheme of histopathological features in swine examined organs.

Intensity of inflammation	0	Normal
	1	Mild
	2	Moderate
	3	Severe
Type of inflammation	1	Neutrophilic
	2	Lymphoplasmacytic and macrophagic
	3	Eosinophilic
	4	Lymphoplasmacytic, macrophagic, and eosinophilic
Distribution of inflammation	1	Focal
	2	Multifocal
	3	Diffuse
Thickness of pleura, of lymph nodes and spleen capsule and heart	0	Normal
	1	Mild
	2	Moderate
	3	Severe
Lumen of bronchi and bronchioles	0	Normal
	1	Mild
	2	Moderate
	3	Severe
Epithelium hyperplasia of bronchi and bronchioli	0	Normal
	1	Mild
	2	Moderate
	3	Severe
Interstitium expansion (lungs and kidneys)	0	Normal
	1	Mild
	2	Moderate
	3	Severe
Renal tubular degeneration	0	Normal
	1	Mild
	2	Moderate
	3	Severe
Glomerular cellularity	0	Normal
	1	Mild
	2	Moderate
	3	Severe
Follicle lymph nodes hyperplasia	0	Normal
	1	Mild
	2	Moderate
	3	Severe
Hepatocyte degeneration	0	Normal
	1	Mild
	2	Moderate
	3	Severe
Villi morphology	0	Normal
	1	Mild
	2	Moderate
	3	Severe
White pulp/red pulp	Eosinophils/no eosinophils	

2.4. Nucleic acid extraction and Real-time PCR (RT-PCR)

2.4.1. DNA/RNA extraction

The 232 samples, submitted to the Istituto Zooprofilattico of Sardinia (IZS-SA), were prepared for DNA/RNA extraction. In labeled tubes, 0.5g of each tissue sample was suspended in 5 ml of Phosphate Buffered Saline (PBS) with magnetic bedding, homogenized (Bead Mill Homogenizer) (Figure 2.6) at 25°C, and centrifuged (BR4i Multifunction Centrifuge) for 6 minutes. DNA/RNA extraction was performed with MagMAX™ CORE Nucleic Acid Purification Kit and the MagMax96 extractor (Thermo Fisher) (Figure 2.7) according to manufacturer instructions [154]. The nucleic acids were stored at -80°C for further analysis.

Figure 2.6. Bead Mill Homogenizer

Figure 2.7. MagMax96 extractor (Thermo Fisher)

2.4.2. Real-time PCR (RT-PCR)

RT-PCR assays were done to detect the viral load of PRRSV, PCV2, PCV3, SVA, TTSuV, APPeV, LINDAV, PEAV, PDCoV, and PPVs (1 to 7) using different conditions, depending on the virus. RT-PCR positive controls for PCV2 and PCV3 have been provided by IZS of Sardinia, and the positive control for APPeV has been provided by IZS of Umbria and Marche. Due to the lack of biological material to be used as a positive control for SVA, LINDAV, PEAV, and PDCoV assays, synthetic positive controls encompassing the whole amplicon of each Real-time PCR were used.

PRRSV RT-PCR was performed on swine lungs, using the commercial kit LSI VetMax PRRSV EU/NA 2.0 lit (Life Technologies) according to the manufacturer's instructions.

PCV2 RT-PCR was performed on all samples, with cycling parameters of 10 minutes at 95°C; 40 cycles of 15 seconds at 95°C, and 60°C for 1 minute. The PCR reaction consisted of 12.5 µl of the TaqMan Fast Advanced Master Mix (Applied Biosystems), 0.2 µM of the detection probe, 0.9 µM of forward and reverse primers, and 5 µl of sample DNA in a final volume of 25 µl [202].

PCV3 RT-PCR was performed on all samples using the following conditions: 95°C for 10 minutes, 45 cycles of 95°C for 10 seconds, and 60°C for 30 seconds. The protocol of the PCR assay was as follows: 5 µl of the TaqMan Fast Advanced Master Mix, 2µl of the extracted DNA, 0.6 µM of the probe, 0.3 µM of primers, and sterile water added to reach a final volume of 10 µl [203].

TTSuV RT-PCR assay was done on all samples following the conditions: 95°C for 10 minutes; 50 cycles of 95°C for 15 seconds, and 60°C for 60 seconds. The PCR assay Master Mix was composed of 2 µl of extracted DNA and 5µl of TaqMan Fast Advanced Master Mix, 0.6 µM of forward and reverse primers, 0.3 µM of detection probe in a final volume of 10 µl [204].

PPV1, PPV2, PPV3, PPV4, PPV5, PPV6, and PPV7 RT-PCR was performed on all samples under the same conditions, as follows: 10 minutes at 95°C; 45 cycles of 10 seconds at 95°C,

and 60°C for 30 seconds. Three duplex RT-PCR assays were applied on PPV2 and PPV7, on PPV3 and PPV6, and on PPV4 and PPV5. Each of these 3 assays had a mix of 10 µl as the total volume composed of 2 µl of extracted DNA, 5 µl of the TaqMan Fast Advanced Master Mix, 0.6 µM of each primer, and 0.3 µM of detection probes [50]. Real-time for PPV1 was performed in singleplex with the same conditions described above except for primer/probe concentration (0.6/0.2 µM respectively).

Samples RNA was retrotrascribed to cDNA with LunaScript® RT Master Mix Kit (New England Biolabs), according to the manufacturer's instructions, and then the Real-time PCR for the detection of PEAV, PDCoV, SVA, APPeV, and LINDAV was performed.

SVA amplification was performed on all samples, using 5 µl of the TaqMan Fast Advanced Master Mix, 0.8 µM of each primer, 1.5 µM of detection probe, and 2 µl of cDNA in a final volume of 10 µl. PCR assay conditions were as follows: 95°C for 10 minutes, followed by 40 cycles of PCR, each cycle consisting of 95°C for 15 seconds, and 60°C for 60 seconds [72].

As for APPeV detection in lymph nodes, cycling parameters were as follows: 10 minutes at 95°C; followed by 45 cycles of 10 seconds at 95°C, and 60°C for 30 seconds, using 5 µl of TaqMan Fast Advanced Master Mix, 2 µl of sample cDNA, 0.3 µM of detection probe, and 0.6 µM of primers to reach a total final volume of 10 µl [145].

SYBR Green RT-PCR was used for the screening of LINDAV by mixing 0.3 µM of primers, 10 µl of SYBR PCR Master Mix 2x Qiagen, and 2µl of cDNA in a final volume of 20 µl. The amplification conditions of this assay were as follows: 15 minutes at 95°C, 40 cycles of 15 seconds at 94°C, 20 seconds at 60°C, and 20 seconds at 72°C; followed by dissociation curve analysis performed at 60°C for 15 seconds, and 95°C for 15 seconds [145].

The RT-PCR for PEAV and PDCoV was performed on intestine samples, following the same conditions: 94°C for 30 seconds, then 40 cycles of 94°C for 5 seconds, and 60°C for 30 seconds. 10 µl of TaqMan Fast Advanced Master Mix were combined with 0.8 µM of primers and 0.3 µM of detection probes and nuclease-free water to a final volume of 10 µl [205].

The different primers and probes used in each RT-PCR along with the relative references are listed in Table 2.2.

Table 2.2. Primers and probes used for the detection of swine viruses.

Virus	Primers and probes	Sequence	Reference
PCV2	P1570F P1642R P1591 probe	5'-TGGCCCGCAGTATTCTGATT-3' 5'-CAGCTGGGACAGCAGTTGAG-3' 5'-CCAGCAATCAGACCCCGTTGGAATG- 3'	Opriessnig *et al.* 2003 [202]
PCV3	PCV3 _353F PCV3_465R PCV3-_418probe	5'-TGACGGAGACGTCGGGAAAT-3' 5'-CGGTTTACCCAACCCCATCA-3' 5'-GGGCGGGGTTTGCGTGATTT- 3'	Franzo *et al.* 2018 [203]
TTSuV	QCOMF QCOMR QCOM probe	5'-CGAATGGYWGAGTTTWYGCCGC- 3' 5'-GCCCGAATTGCCCCTWGACTKCG-3' 5'- CTCCGGCACCCGCCCAG-3'	Brassard *et al.* 2009 [204]
PPV1	PPVF PPVR PPV probe	5'-CAGAATCAGCAACCTCACCA-3' 5'-GCTGCTGGTGTGTATGGAAG-3' 5'–TGCAAGCTTAATGGTCGCACTAGACA–3'	Opriessnig *et al.* 2011 [208]
PPV2	PPV2DF PPV2DR PPV2D probe	5'-TACTGAGCCCTAAGACTGACTACAAGC-3'' 5'-GTTTGTCTCGTTGTTCGTCTGATG-3' 5'-AACTGCTACATGAACCACTTTACCCCSTC-3'	Xiao *et al.* 2013 [209]
PPV3	PPV3F PPV3R PPV3 probe	5'-CAYGAYGAACGGTACGATGAAAT-3' 5'-GCGGTAAAACCTGTGAWAWTTGAAC-3' 5'-TAGGTTGATGAATAAGGAGATAGAGAGGGCGG-3'	Xiao *et al.* 2013 [209]
PPV4	ORF3 PPV4 F ORF3 PPV4 R ORF3 PPV4 probe	5'-TTTGCCAATAGTGCACAAGG-3' 5'-AGGCATCCATGGGTCTATCA-3' 5'-CAGAAAGCAAACTGAGATGTCC-3'	Gava *et al.*, 2015 [207]
PPV5	PPV5F PPV5R PPV5 probe	5'- GCATTGGTGTGTGTCTGTGTCC-3' 5'-GTGGCACATTTGTACATGGGAG-3' 5'-ACTTTGGTGTTGAGGGACTTAGCTTTTTTGTAC -3'	Xiao *et al.* 2013 [209]
PPV6	PPV6F PPV6R PPV6 probe	5'-GGCTTCATAATCCCTCCAAAACCT-3' 5'-GCTCATCTTCCTCTTGTTTCTCCTG-3' 5'-CCTCCTCCTCCTCCCTCTCCAATTCCT-3'	Cui *et al.* 2017 [210]
PPV7	PPV7F PPV7R PPV7 probe	5'- AGC AGA GAC AAA CAC AGA CG-3' 5'- CCA GTT TGC ATT GTT CCC ATC-3' 5'- CAG GCA GTG GTA GTG AAG GAT CCC -3'	Palinski 2016 [149]
SVV	SVV3DF SVV3DR SS3D-PR1	5'-AGAATTTGGAAGCCATGCTCT-3' 5'-GAGCCAACATAGARACAGATTGC-3' 5' -TTCAAACCAGGAACACTACTCGAGA- 3'	Fowler *et al.* 2017 [72]
APPeV	F primer R primer Probe (MGB)	5'-GGGCAGACGTCACYGAGTAGTACA-3' 5'-TCCGCCGGCACTCTATCA-3' 5'-TGTAGGGTCTACTGAGGCT-3'	Kaufmann *et al.* 2019 [145]
LINDAV	F primer R primer	5'-ACCCACTGGCGATGCCT-3' 5'-TCCGCCGGCATCCTATC-3'	Kaufmann *et al.* 2019 [145]
PDCoV	F primer R primer Probe	5'- ATTTGGACCGCAGTTGACA-3' 5'- GCCCAGGATATAAAGGTCAG-3' 5'- TAAGAAGGACGCAGTTTTCATTGTG- 3'	Huang *et al.* 2019 [205]
PEAV	F primer R primer Probe	5'- TCTCGGCTTACTCTAAACCC-3' 5'- CATCCACCATCTCAACCTC-3' 5'- AAGACCTAAATGCTGATGCCCCA- 3'	Huang *et al.* 2019 [205]

2.4.3. Sequencing

Sanger sequencing was performed to confirm the positivity for PPV2-7, APPeV, and TTSuV. The following samples were analyzed: 459 and 397 (PPV2), 397, 393 and 399 (PPV3), 361 (PPV4), 397 (PPV5), 397 (PPV6), 422 and 397 (PPV7); 350 (APPeV).

Sequencing was performed in both senses at the IZS using the primers reported in the table below and a DNA sequencing kit (dRhodamine Terminator Cycle Sequencing Ready Reaction; Applied Biosystems) on an ABI-PRISM 3500 Genetic Analyzer (Applied Biosystems, Waltham, MA, USA). BioEdit software version 7.0.0 was used to edit sequences [206] and Blastn (https://blast.ncbi.nlm.nih.gov) for their identification. Primer sequences are reported in Table 2.3.

Table 2.3. Primers used for the sequencing of emerging viruses.

Virus	Primer sequence
PPV2	PPV2-F6 GCTTTCTAGTCGGACCGGAAGT PPV2-R871 GCTCGGCCTTTCACGGTGGGC
PPV3	PPV3-F49 ACACCTACCTCGCCTATAAGAATCAG PPV3-R743 CTTCTCYGCTCCCTCCSGT
PPV4	PPV4F GCATTGGTGTGTGTCTGTG PPV4R GTGGCACATTTGTACATGG
PPV5	PPV5-F711 GGACGGGAACTCAAGTCCTA PPV5-R1591 TGTCCACCAACCACTGTACA
PPV6	PPV6-F203 CGAAAGCCTCTGTATTATGTCT PPV6-R1291 TTGCGTTATTCCAGTTAACAC
PPV7	PPV7-F370 GCAGCCGCTTCCTGGTGAG PPV7-2R CCAGRTCGGGYGCGTTTC
TTSuV	TTSuV1-fw CGGACCTGATTGAAGACTGAAAACCGTT TTSuV1-rew GGCAGCAAACGTGGTGCGAGCGAAG CGA TTSuVk2-fw TCGAGCTCCTGAGAGCGGAGTCAAGGGGCCTA
APPeV	APPV_5030-FW CCCAGGCAATACCTCACAAC APPV_5835-REV TTCCTCTGGCCCTGTTCTTC

2.5. Data collection and statistical analysis

Age and categories of swine (adults, post-weaning, and piglets), type of tissue, histopathological, and PCR results were registered and submitted to statistical analysis.

Microscopical results and RT-PCR data were analyzed using Stata 11.2 and Minitab 15 software (StataCorp LP, College Station, TX, USA; LEAD Technologies, Inc). In particular, histological data were submitted to the non-parametric Kruskal–Wallis test with Dunn's post

hoc comparison considering the intensity x distribution of the inflammation for each evaluated organ, while, after checking the normality, RT-PCR results were evaluated using the one-way ANOVA with Bonferroni post hoc comparison. Furthermore, categorical and ordinal variables were compared using the Pearson (r) correlation coefficient. A value of r approximately equal to 1 indicates a good correlation, a value near 0 indicates a poor correlation and a negative value indicates an inverse correlation. A p-value < 0.05 was considered significant.

Chapter 3

Results

3. Results

3.1. Gross and histopathology

3.1.1. Samples of adult pigs

3.1.1.1. Gross examination results

Macroscopic evaluation of different organs in adult pigs collected from the North Sardinian slaughterhouse is reported in Figure 3.1.

Grossly, all the examined lungs showed multifocal hyperemic areas associated with an increase in the volume of the parenchyma (Figure 3.1. A). The main bronchi showed abundant whitish-foamy fluid in the lumen (Figure 3.1.B). The morphological diagnosis of mild multifocal chronic interstitial pneumonia and bronchoalveolar edema was made. The kidney revealed mild to moderate multifocal chronic glomerulonephritis. The mediastinal lymph nodes appeared hyperemic, mildly enlarged, and moderately hyperplastic (Figure 3.1.C). The morphological diagnosis of mild multifocal lymph node hyperplasia was made. The intestine was characterized by a diffuse and catarrhal exudate, indicative of chronic, diffuse, mild to severe catarrhal enteritis.

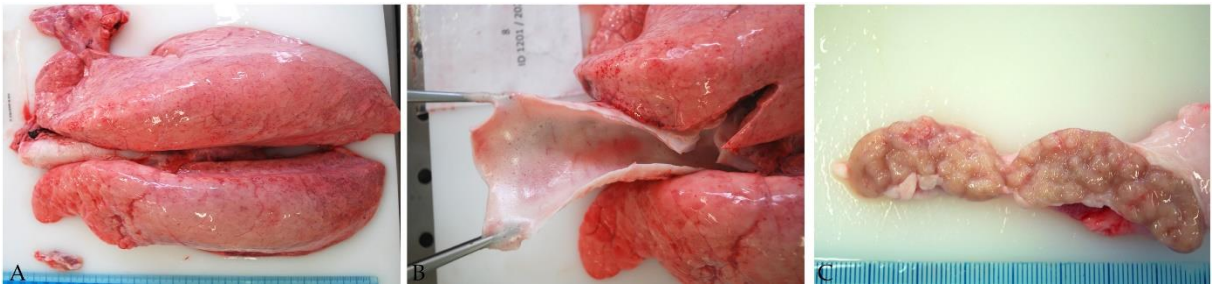


Figure 3.1. (A-B) Lungs of adult pigs. (A) Increase in the volume of the lung parenchyma and diffuse emphysema. (B) Abundant amount of whitish foamy fluid within tracheal lumen and alveoli (bronchoalveolar edema). (C) Mediastinal lymph nodes of adult pigs: moderately hyperplastic lymph nodes.

3.1.1.2. Histopathological results (Table 3.1.)

Lungs

Microscopic evaluation of lungs in adult pigs collected from the North Sardinian slaughterhouse is reported in Figure 3.2.

In the lamina propria of the bronchi, 7/10 pigs (70%) presented chronic mild and diffuse lymphoplasmacytic and eosinophilic inflammatory infiltrate. In particular, in 4/7 pigs the infiltrate was characterized by a scarce number of lymphocytes, plasma cells, and macrophages and was classified as chronic lymphoplasmacytic and macrophagic bronchitis, and in 3/7 pigs the infiltrate was mainly composed of eosinophils. Moderate and diffuse hyperplasia of the bronchus-associated lymphoid tissues (BALT) was detected in 2/10 pigs, showing lymphoplasmacytic and macrophagic bronchitis (Figure 3.2. A).

In 9/10 pigs (90%) a chronic mild and diffuse inflammatory infiltrate was present in the lamina propria of bronchioles. Six out of 9 pigs showed lymphoplasmacytic and macrophagic bronchiolitis characterized by a scarce number of lymphocytes, plasma cells, and macrophages, whereas, in 3/10 pigs, the infiltrate was mainly consisting of eosinophils (Figure 3.2.B). Mild to moderate BALT hyperplasia was detected in 3/10 pigs.

A multifocal to diffuse chronic interstitial pneumonia was observed in 6/10 pigs (60%), characterized by a mild (3/10) to moderate (3/10) inflammatory infiltrate of lymphocytes, macrophages, and plasma cells expanding the interstitium (Figure 3.2.D). Furthermore, in 2/6 pigs the alveoli showed mild destruction of alveolar septa with enlargement of alveolar spaces (emphysema) (Figure 3.2.C). Only 1/6 of the pigs presented a moderate diffuse alveolar red blood cell extravasation.

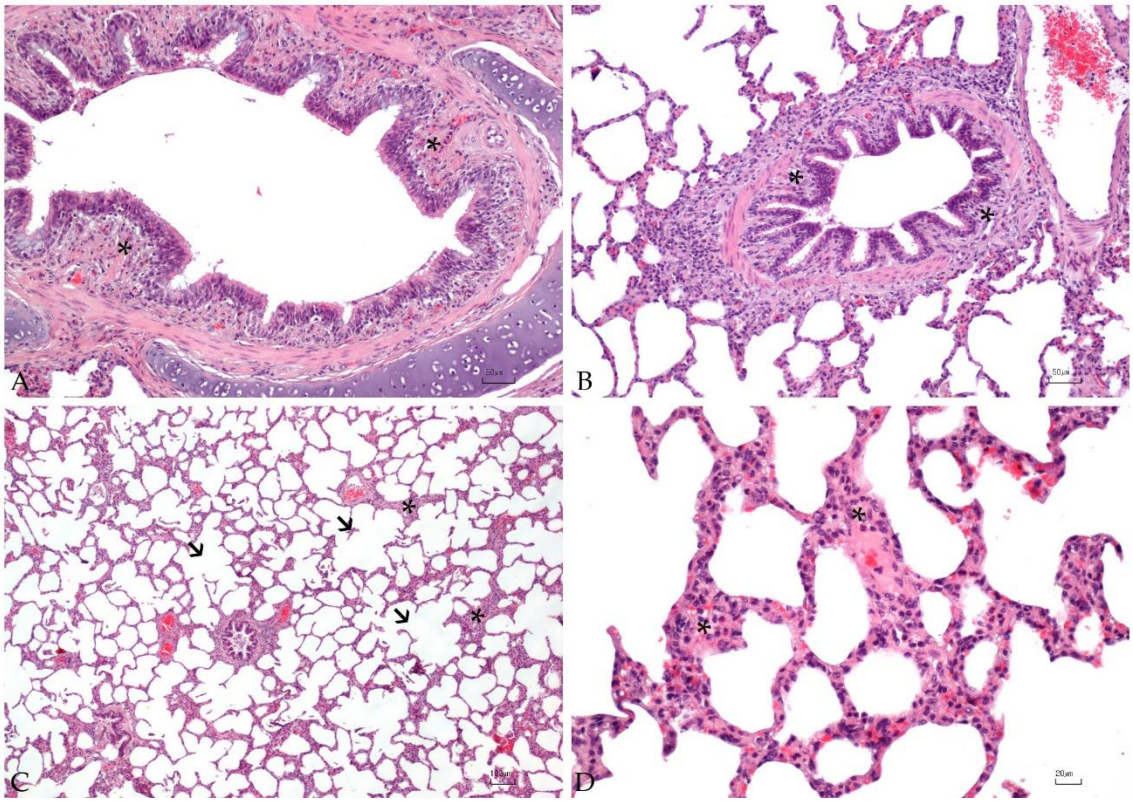


Figure 3.2. A-D. Adult pigs' lungs. Mild diffuse chronic bronchitis (A) and bronchiolitis (B) characterized by lymphoplasmacytic and macrophagic infiltrate in the lamina propria (asterisks) (HE, bar, 50µm). Mild diffuse chronic interstitial pneumonia (asterisks) with mild destruction of alveolar septa (emphysema) (arrows) (C) (HE, bar, 100µm). High magnification of interstitial pneumonia with mild infiltrate of lymphocytes, plasma cells, and macrophages within the interstitium (asterisk) (D) (HE, bar, 20µm).

The main histopathological findings of organs are reported in Figure 3.3.

Liver

The liver showed mild multifocal chronic lymphoplasmacytic and macrophagic hepatitis in 4/10 pigs (40%), characterized by a scarce number of lymphocytes, plasma cells, and macrophages, mainly placed in the periportal tracts (Figure 3.3.A). Eosinophilic hepatitis was detected in 1/10 pigs.

Intestine

The intestine showed a diffuse chronic lymphoplasmacytic, macrophagic, and eosinophilic enteritis in 4/10 pigs (40%), characterized by a mild (2/10) to moderate (2/10) infiltration of

lymphocytes, plasma cells, eosinophils, and macrophages in the lamina propria. In the remaining 6/10 pigs was observed eosinophilic enteritis, ranging from a moderate (6/6) diffuse infiltration of eosinophils in the lamina propria (Figure 3.3.B).

Kidney

An increase in glomerular cellularity was observed in 6/10 pigs (60%), which ranged from mild (5/10) to moderate (1/10). At the level of the interstitium, 5/10 pigs (50%) showed a focal to multifocal chronic lymphoplasmacytic and macrophagic nephritis, characterized by a mild (3/5) to moderate (2/5) infiltration of lymphocytes, plasma cells, and macrophages in the interstitium (Figure 3.3.C).

Heart

The heart didn't show any histopathological change in all examined adult pigs (Figure 3.3.D).

Spleen

Histopathological changes of the spleen were mild in the adult pigs' group and were mainly attributable to an increase of eosinophils in the white pulp in 8/10 pigs (80%) and a mild increase in the capsule thickness in 1/10 pigs (10%) (Figure 3.3.E).

Mediastinal lymph nodes

Mediastinal lymph nodes showed a mild increase population of lymphoid cells in 90% of adult pigs (9/10) (Figure 3.3.F). Five out of ten adult pigs showed diffuse eosinophilic lymphadenitis ranging from mild (3/5) to moderate (2/5).

Mesenteric lymph nodes

An increase in the population of lymphoid cells was also observed in the follicles of mesenteric lymph nodes in 9/10 pigs (90%), characterized by the presence of mild (8/10) to moderate (1/10) diffuse eosinophilic infiltration.

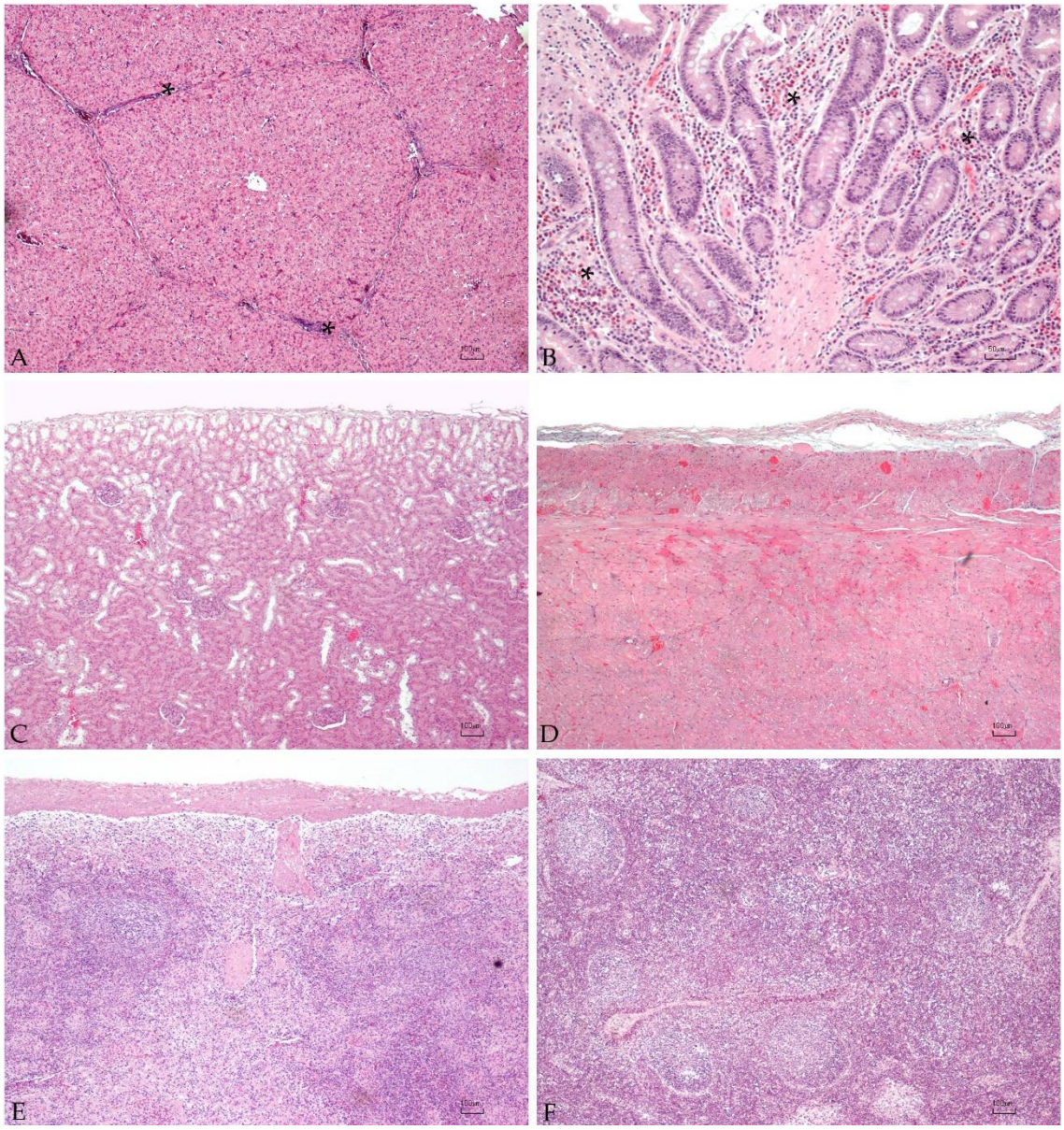


Figure 3.3. A-F. Adult pigs' organs. Mild chronic multifocal lymphoplasmacytic periportal hepatitis (asterisks) (A) (HE, bar, 100µm). Mild chronic multifocal lymphoplasmacytic and eosinophilic enteritis (asterisks) (B) (HE, bar, 50µm). Focal to multifocal mild chronic nephritis, characterized by an infiltrate of lymphocytes, plasma cells, and macrophages in the interstitium (C) (HE, bar, 100µm). Heart and spleen with no relevant histopathological changes (D, E) (HE, bar, 100µm). Mediastinal lymph node showing mild diffuse lymphoid hyperplasia (F) (HE, bar, 100µm).

3.1.2. Samples of post-weaning pigs

3.1.2.1. Gross examination results

Macroscopic evaluation of the different organs in post-weaning pigs collected from a North Sardinian slaughterhouse is reported in Figure 3.4.

All examined lungs showed a diffusely increased volume and consistency with evidence of rib impression (Figure 3.4.A). Abundant whitish foamy fluid was observed in the trachea and the main bronchi (Figure 3.4.B). The mediastinal lymph nodes appeared hyperemic, slightly enlarged, and moderately hyperplastic (Figure 3.4.C). The intestine was characterized by the presence of a diffuse and mild catarrhal exudate indicative of mild to severe acute catarrhal enteritis.

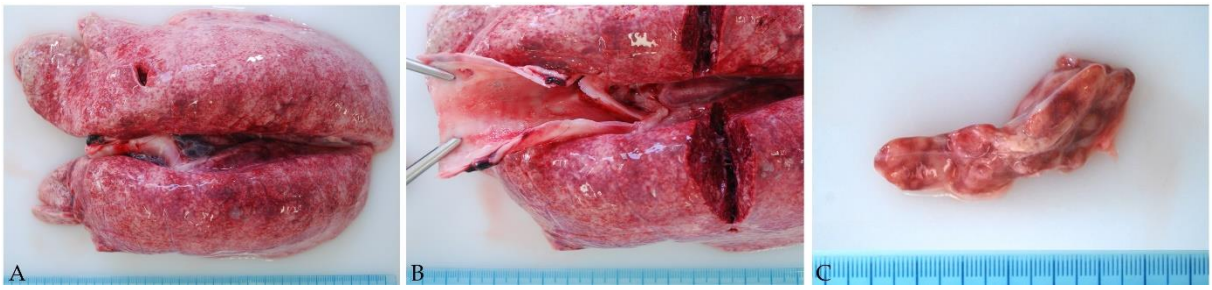


Figure 3.4. (A-B) Lung of post-weaning pigs. (A) Increase in the volume of the lung parenchyma and rib impression suggestive of a moderate, diffuse interstitial pneumonia and moderate multifocal hyperemia. (B) Moderate amount of whitish foamy fluid within tracheal lumen and alveoli (bronchoalveolar edema). (C) Mediastinal lymph nodes of post-weaning pigs, with moderate hyperemia and moderately hyperplastic lymph node.

3.1.2.2. Histopathological results (Table 3.1.)

Lungs

Microscopic evaluation of lungs in post-weaning pigs collected from the North Sardinian slaughterhouse is reported in Figure 3.5.

A mild chronic lymphoplasmacytic and macrophagic bronchitis was detected in 4/10 post-weaning pigs (40%), characterized by a focal to multifocal inflammatory infiltrate of lymphocytes, plasma cells, and macrophages in the lamina propria of the bronchi (Figure 3.5.A).

Bronchioles in post-weaning pigs were only mildly affected, presenting mild multifocal necrosis of the epithelium in 3/10 pigs (30%) (Figure 3.5.B). The lumen of the bronchioles showed a moderate number of macrophages and scarce mucus in 7/10 pigs (70%),

accompanied by the presence of scarce erythrocytes in one pig. BALT hyperplasia was detected in 5/10 pigs (50%). Histopathological changes in the alveolar septa, consisting of loss of type I alveolar cells and hypertrophy of type II alveolar cells, were detected in 3/10 pigs (30%). A scarce number of macrophages was detected in the lumen of the alveoli in 4/10 pigs (40%), whereas a moderate number of erythrocytes was found in the alveolar space of 1/10 pigs (10%). Mild to moderate multifocal emphysema was observed in 3/10 pigs (30%). Noteworthy, the main histopathological finding was the presence of a chronic multifocal to diffuse interstitial pneumonia in 10/10 post-weaning pigs (100%), mostly characterized by moderate (7/10) to severe (3/10) inflammatory infiltrate of lymphocytes, macrophages, and plasma cells expanding the interstitium (Figure 3.5.C-D).

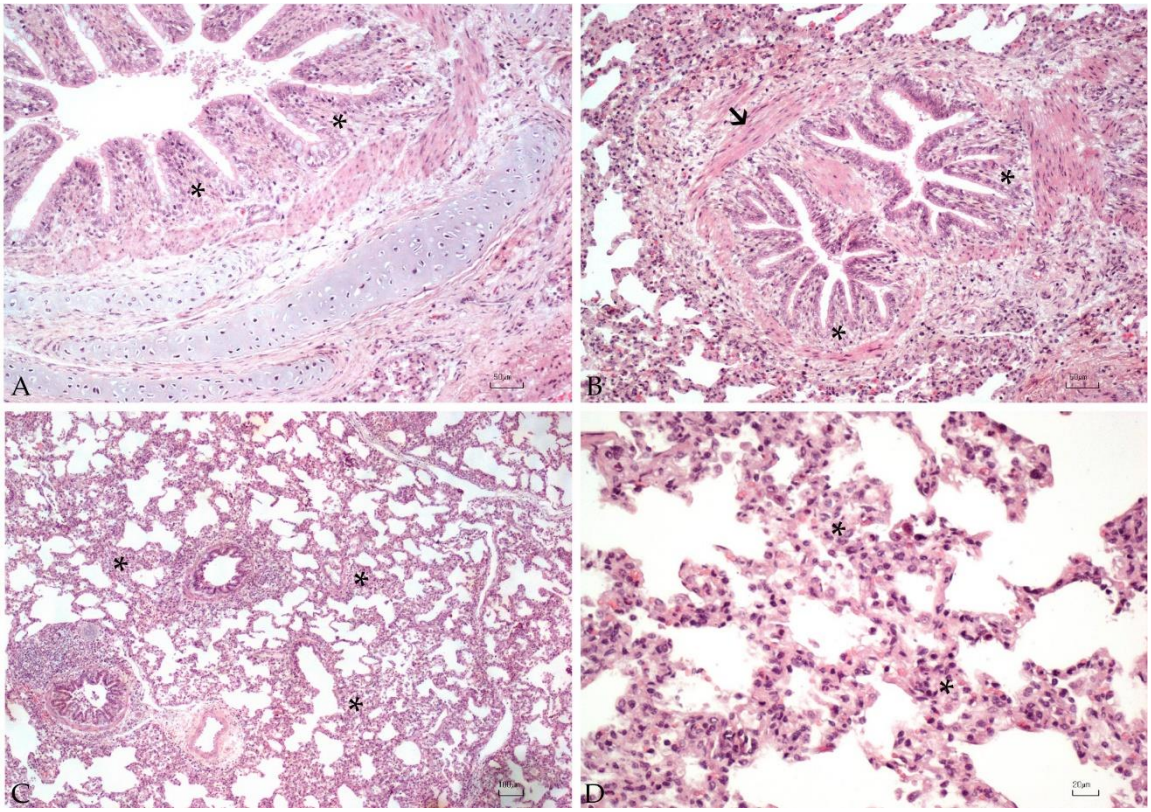


Figure 3.5. A-D. Post-weaning pigs' lungs. Mild diffuse chronic bronchitis (A) and bronchiolitis (B) characterized by lymphoplasmacytic and macrophagic infiltrate in the lamina propria (asterisks) (HE, bar, 50µm). Notice peribronchiolar hyperplasia of the smooth muscle layer (arrow) (HE, bar, 50µm). Moderate diffuse chronic interstitial pneumonia (asterisks) (C) (HE, bar, 100µm). High magnification of interstitial pneumonia with a moderate number of lymphocytes and macrophages expanding the interstitium (asterisks) (D) (HE, bar, 20µm).

The main histopathological findings of organs are reported in Figure 3.6.

Liver

A mild chronic lymphoplasmacytic and macrophagic periportal hepatitis was observed in 5/10 pigs (50%), characterized by a focal (3/10) to multifocal (2/10) infiltration of lymphocytes, plasma cells, and macrophages (Figure 3.6.A).

Intestine

A diffuse chronic lymphoplasmacytic and macrophagic enteritis was observed in 10/10 pigs (100%) ranging from mild (1/10) to moderate (8/10) and severe (1/10), with an inflammatory infiltrate characterized by lymphocytes, plasma cells, and macrophages and eosinophils at the level of the lamina propria. The Peyer's patches hyperplasia was detected in 1/10 pigs (Figure 3.6.B).

Kidney

A mild focal lymphoplasmacytic and macrophagic glomerulonephritis was detected in 2/10 pigs (20%) (Figure 3.6.C)

Heart

The main pathological findings were detected in the epicardium, which showed mild lymphoplasmacytic and macrophagic epicarditis in 5/10 pigs (50%), characterized by a focal to diffuse inflammatory infiltrate of lymphocytes, plasma cells, and macrophages, accompanied by a mild increase in thickness of the epicardium 4/5 pigs (Figure 3.6.D).

Spleen

No pathological findings were detected in the spleen of post-weaning pigs, except a mild to moderate hyperplasia of the white pulp in 7/10 pigs (70%) and mild edema in 2/10 pigs (20%) (Figure 3.6.E).

Mediastinal lymph node

An increase in the population of lymphoid cells of the follicles was detected in 10/10 pigs (100%). In 4/10 pigs (40%) the presence of extravasate red blood cells was detected in the follicles (Figure 3.6.F).

Mesenteric lymph node

An increase in the population of lymphoid cells of the follicles was detected in 10/10 pigs (100%), ranging from mild (8/10) to moderate (2/10). An increase in the number of eosinophils was observed in 3/10 pigs (30%), whereas 1/10 pigs (10%) showed moderate multifocal neutrophilic lymphadenitis.

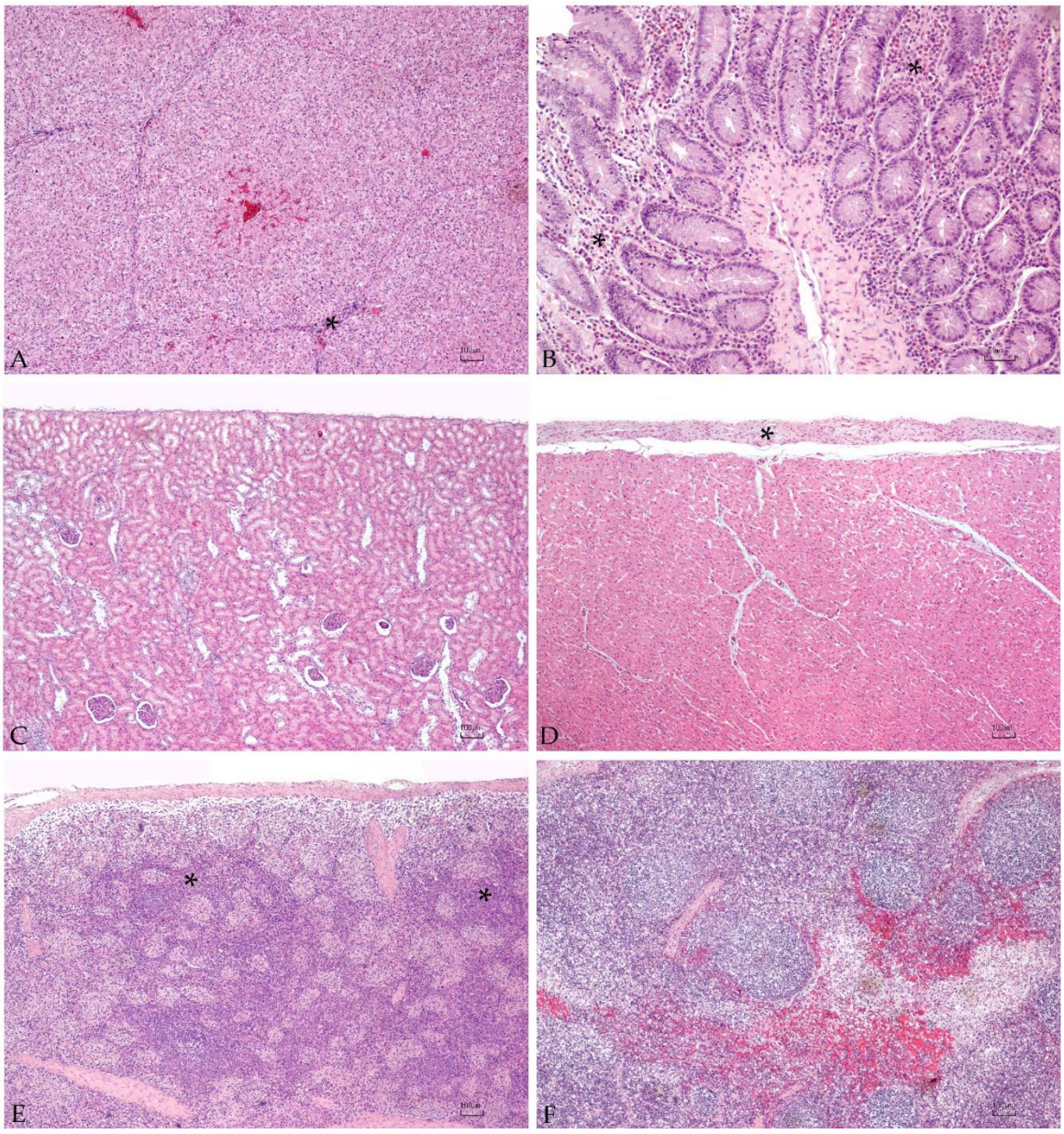


Figure 3.6. A-F. Post-weaning pigs' organs. Mild chronic multifocal lymphoplasmacytic periportal hepatitis and moderate multifocal congestion (asterisk) (A) (HE, bar, 100µm). Mild chronic multifocal lymphoplasmacytic and eosinophilic enteritis (asterisks) (B) HE, bar, 50µm). Focal to multifocal mild chronic nephritis, characterized by an infiltrate of lymphocytes, plasma cells, and macrophages in the interstitium (C) (HE, bar, 100µm). Heart showing mild chronic lymphoplasmacytic epicarditis, with an increase in thickness of epicardium (asterisk) (D) (HE, bar, 100µm). Spleen showing moderate hyperplasia of the white pulp (asterisks) (E) (HE, bar, 100µm). Mediastinal lymph node showing diffuse lymphoid hyperplasia and congestion (F) (HE, bar, 100µm).

3.1.3. Samples of piglets

3.1.3.1. Gross examination results

Macroscopic evaluation of the different organs in piglets collected from a North Sardinian slaughterhouse is reported in Figure 3.7.

All evaluated lungs showed a diffusely increased volume and consistency (Figure 3.7.A). A moderate, locally extensive chronic fibrinous and suppurative bronchopneumonia of the cranial ventral margins was detected in 1/9 pigs (11%) (Figure 3.7.A-B). Abundant whitish foamy fluid was observed in the lumen of the trachea and the main bronchi. The intestine was characterized by the presence of a diffuse and mild to moderate amount of exudate indicative of acute catarrhal enteritis. All the mediastinal lymph nodes appeared slightly enlarged, diffusely and moderately hyperplastic, and focally hyperemic (Figure 3.7.C).

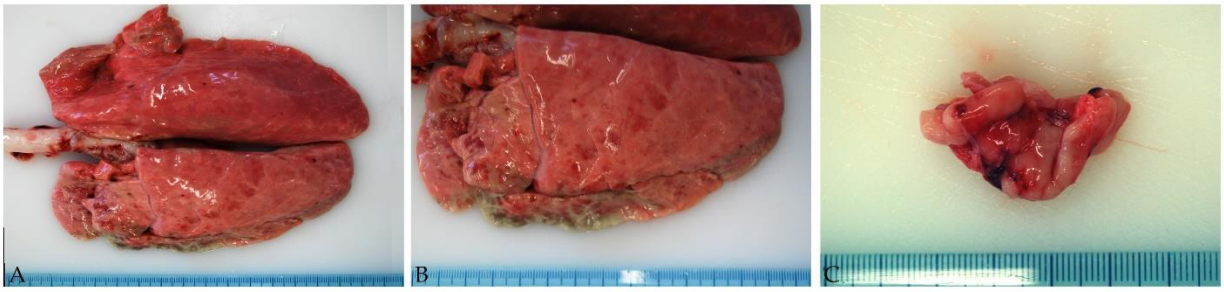


Figure 3.7. (A-B) Lung of piglets. (A) moderate multifocal hyperemia associated with cranio-ventral moderate fibrino-suppurative bronchopneumonia. (B) High magnification of cranio-ventral, moderate, fibrino-suppurative bronchopneumonia. (C) Mediastinal lymph nodes of post-weaning, with focal hyperemia and mild hyperplastic lymph node.

3.1.3.2. Histopathological results (*Table 3.1.)*

Lungs

Microscopic evaluation of lungs in piglets collected from the North Sardinian slaughterhouse is reported in Figure 3.8.

The pleura showed a mild increase in thickness in 2/9 piglets (22%), 1 of which was associated with mild lymphoplasmacytic and macrophagic pleuritis, characterized by inflammatory infiltrates of lymphocytes, macrophages, and plasma cells.

A mild chronic lymphoplasmacytic and macrophagic bronchitis was detected in 8/9 piglets (89 %), characterized by a multifocal to diffuse infiltration of lymphocytes, plasma cells, and macrophages in the lamina propria of bronchi (Figure 3.8.A). In the bronchi, a mild to severe detachment epithelial of epithelial cells was also detected in 3/9 piglets (33%). The lumen of the bronchi was characterized in 9/9 piglets (100%) by the presence of a mild (2/9) to moderate (4/9) and abundant (3/9) amount of mucus intermingled with a variable number of macrophages. In addition, in 1/9 piglets with abundant mucus in the lumen of the bronchi was observed the presence of bacterial colonies of cocci and bacilli.

At the level of the lamina propria of bronchioles a multifocal to diffuse chronic lymphoplasmacytic and macrophagic bronchiolitis was detected in 8/9 piglets (89%), characterized by a mild (7/8) to moderate (1/8) infiltrate of lymphocytes, plasma cells, and macrophages (Figure 3.8.B). In 9/9 piglets (100%) a moderate amount of mucus intermingled with scarce macrophages was detectable in the lumen of bronchioles.

Alveoli were affected in 6/9 piglets (67%) with mucus and macrophages in the lumen, and the remaining piglets presented a moderate multifocal to diffuse alveolar inflation or emphysema.

The main histopathological finding in piglets was chronic diffuse interstitial lymphoplasmacytic and macrophagic pneumonia in 9/9 piglets, characterized by moderate (2/9) to severe (7/9) inflammatory infiltrates of lymphocytes, plasma cells, and macrophages that expanded the interstitium, often associated with the presence of extravasated erythrocytes (8/9) (Figure 3.8.C-D).

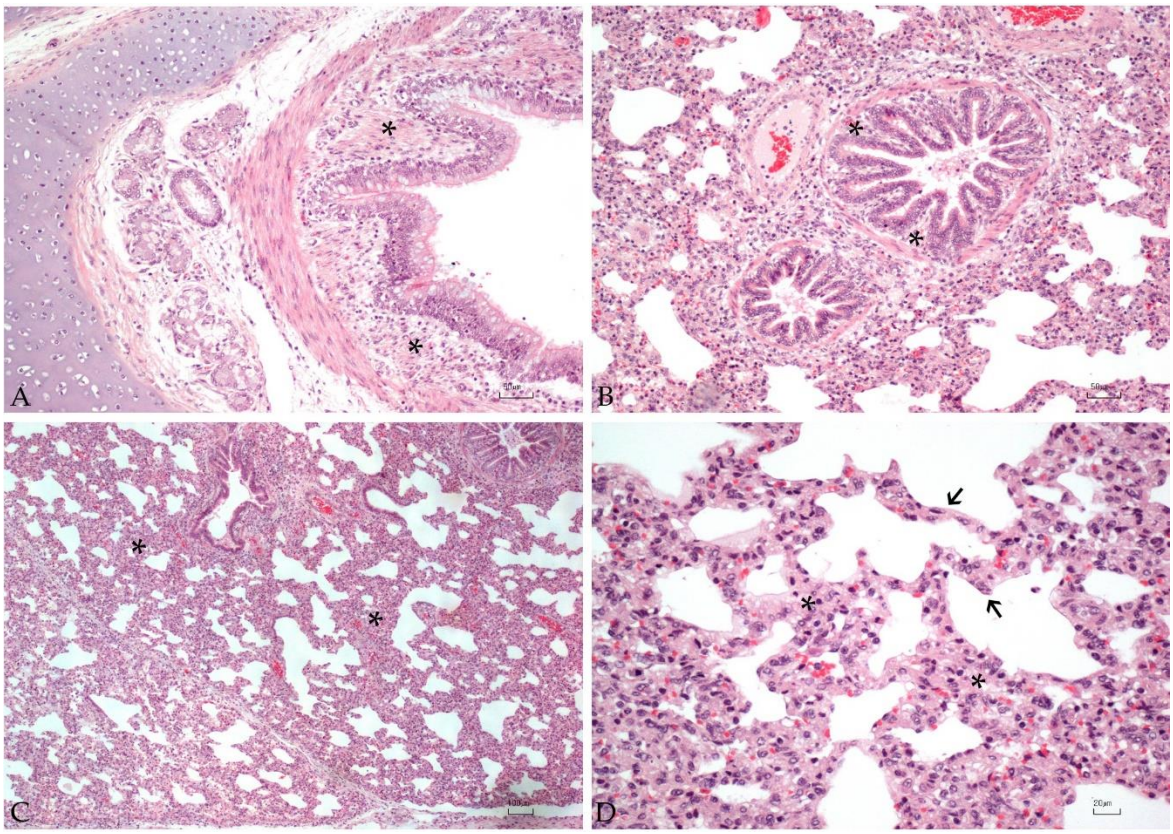


Figure 3.8. A-D. Piglets' lungs. Mild diffuse chronic bronchitis (A) and bronchiolitis (B) characterized by lymphoplasmacytic and macrophagic infiltrate in the lamina propria (asterisks) (HE, bar, 50µm). Severe diffuse chronic interstitial pneumonia (asterisks) (C) (HE, bar, 100µm). High magnification of interstitial pneumonia with a high number of lymphocytes and macrophages expanding the interstitium (asterisks) Notice type II pneumocyte hyperplasia (arrows) (D) (HE, bar, 20µm).

The main histopathological findings of organs are reported in Figure 3.9.

Liver

Mild chronic lymphoplasmacytic and macrophagic periportal hepatitis was observed in 3/9 piglets (33%) that presented a multifocal lymphoplasmacytic and macrophages inflammation of the periportal tracts (2/3) (Figure 3.9.A) with one piglet showing severe focal nodular lymphoplasmacytic and macrophagic inflammation in the liver capsule. Moreover, moderate multifocal congestion was detected in 4/ 9 piglets (44%).

Intestine

Morphological changes in the intestinal villi were detected in 7/9 piglets (78%), showing mild (6/7) to moderate (1/7) fusion of the villi apex. Mild to moderate, diffuse, chronic enteritis

was observed in 9/9 piglets (100%), characterized by lymphocytes, plasma cells, and macrophages and showing eosinophils, expanding the lamina propria. Hyperplasia of Peyer's patches was detected in 2/9 piglets (22%) (Figure 3.9.B).

Kidney

The kidney was affected by a mild to moderate chronic lymphoplasmacytic and macrophagic nephritis, characterized by a focal (1/3) to multifocal (2/3) inflammatory infiltrate of lymphocytes, plasma cells, and macrophages that expanded the interstitium (Figure 3.9.C). In 2/3 piglets a reduction of Bowman's spaces was detected.

Heart

In the epicardium of 3/9 piglets (33%) was observed an increase in thickness associated with mild to severe chronic lymphoplasmacytic and macrophagic epicarditis, characterized by a focal to diffuse infiltrate of lymphocytes, plasma cells, and macrophages (Figure 3.9.D).

Spleen

Spleen in 5/9 piglets (56%) had moderate hyperplasia of the white pulp (Figure 3.9.E), whereas in 3/9 (33%) of the remaining piglets a moderate diffuse edema was observed. The splenic capsule presented a moderate increase in thickness in 1/9 piglets (11%).

Mediastinal lymph node

Follicles showed an increase in the population of lymphoid cells in 9/9 piglets (100%) (Figure 3.9.F), with moderate severity in most cases (6/9). Mild multifocal edema was observed in 6/9 piglets (67%), whereas an increase in hemosiderin-laden macrophages and erythrocytes was detected in 4/9 piglets (44%).

Mesenteric lymph node

Follicle increase in the population of lymphoid cells was detected also in mesenteric lymph nodes of 8/9 piglets (89%), mostly of moderate degree (7/8). A mild multifocal increase in eosinophils number was also observed in 9/9 piglets (100%), whereas mild multifocal edema was identified in 4/9 piglets (44%).

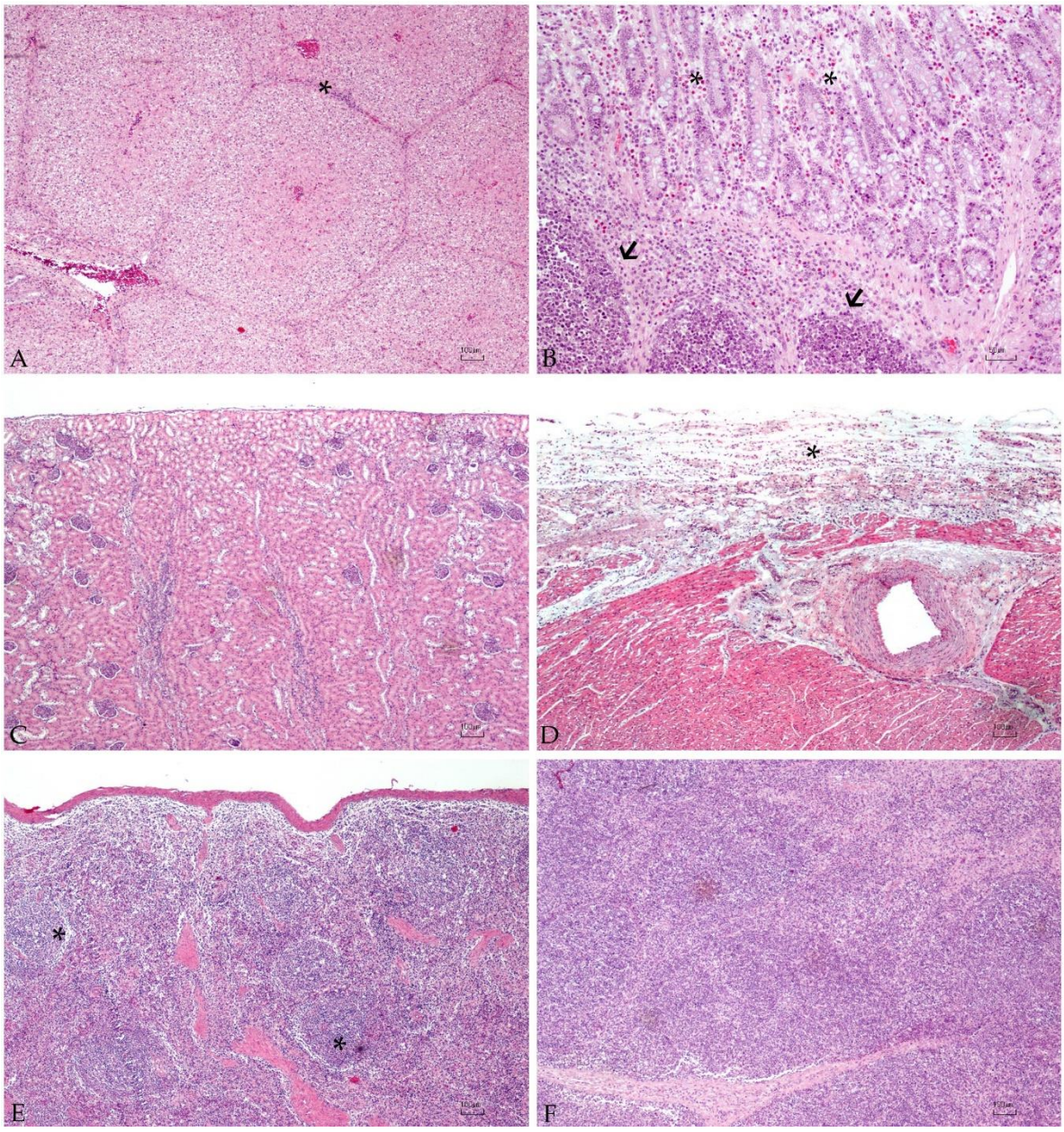


Figure 3.9. A-F. Piglets' organs. Mild chronic multifocal lymphoplasmacytic periportal hepatitis (asterisk) and moderate multifocal congestion (A) (HE, bar, 100µm). Mild to moderate chronic multifocal lymphoplasmacytic and eosinophilic enteritis (asterisks) (HE, bar, 100µm) and hyperplasia of Peyer's patches (arrows) (B) (HE, bar, 50µm). Multifocal moderate chronic nephritis characterized by an infiltrate of lymphocytes, plasma cells, and macrophages in the interstitium (C) (HE, bar, 100µm). Heart showing severe multifocal chronic lymphoplasmacytic epicarditis, with an increase in thickness of epicardium (asterisk) (D) (HE, bar, 100µm). Spleen showing moderate hyperplasia of the white pulp (asterisks) (E) (HE, bar, 100µm). Mediastinal lymph node showing moderate diffuse lymphoid hyperplasia (F) (HE, bar, 100µm).

Table 3.1. Classification of the histopathological lesions in organs of swine categories.

Organs		Adult pigs			Post-weaning pigs			Piglets		
		Mi	M	S	Mi	M	S	Mi	M	S
Lung										
Pleura	Increase in thickness	-	-	-	-	-	-	2/9	-	-
	Inflammation LPM	-	-	-	-	-	-	1/9	-	-
Bronchi	Lumen material	-	-	-	4/10	-	-	2/9	4/9	3/9
	Lamina propria inflammation LPM	7/10	-	-	4/10	-	-	8/9	-	-
	Epithelium detachment	-	-	-	-	-	-	2/9	-	1/9
Bronchioles	Lumen material	-	-	-	3/10	4/10	-	8/9	1/9	-
	Lamina propria inflammation LPM	6/10	1/10	-	1/10	-	-	7/9	1/9	-
	Epithelium detachment	-	-	-	2/10	-	-	-	-	-
Alveoli	Lumen material	1/10	-	-	4/10	1/10	1/10	-	-	6/9
	Epithelium detachment	-	-	-	1/10	3/10	-	-	-	-
Interstitium	Inflammation LPM	3/10	2/10	1/10	1/10	6/10	3/10	-	2/9	7/9
Heart										
Epicardium	Increased thickness	-	-	-	4/10	-	-	1/9	1/9	1/9
	Inflammation LPM	-	-	-	5/10	-	-	2/9		1/9
Mediastinal lymph node										
Capsule	Increased thickness	1/10	-	-	-	-	-	-	-	-
Follicles	Increased cellularity	9/10	-	1/10	1/10	9/10	-	1/9	6/9	2/9
	Inflammation E	2/10	1/10	-	-	-	-	-	-	-
Mesenteric lymph node										
Capsule	Increased in thickness	-	-	-	-	-	-	-	-	-
Follicles	Increased in cellularity	8/10	-	-	2/10	8/10	-	1/9	7/9	-
	Inflammation E	-	1/10	-	-	1/10	-	-	-	-
Intestine										
Villi	Morphological changes	-	-	-	-	-	-	6/9	1/9	-
Lamina propria	Inflammation E	-	5/10	1/10	-	-	-	-	-	-
	Inflammation LPM	-			1/10	6/10	1/10	1/9	3/9	-
	Inflammation LPME	-	2/10	2/10	-	2/10	-	2/9	3/9	-

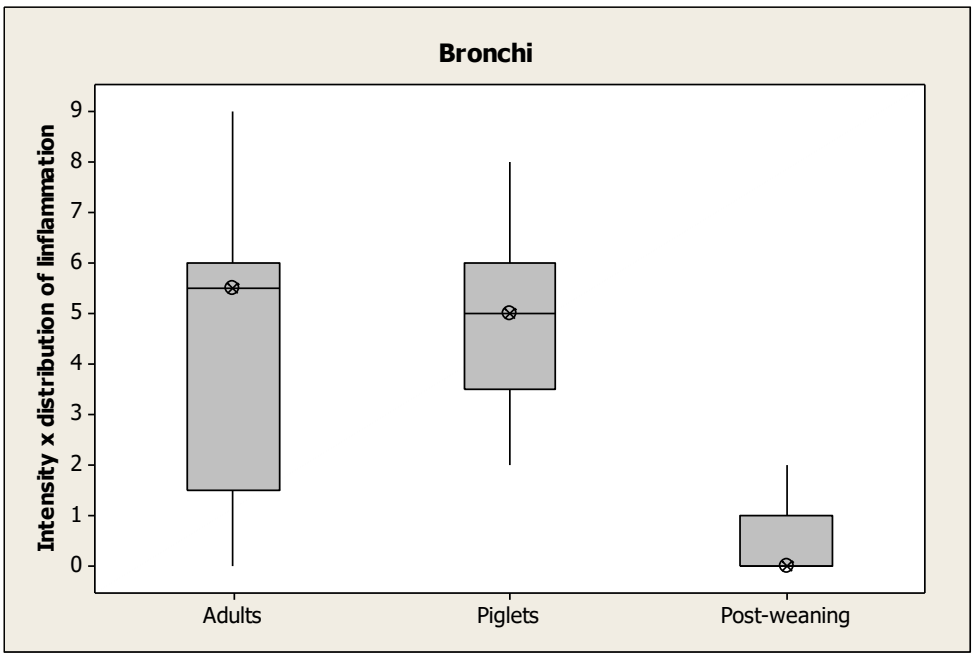
Kidney										
Glomeruli	Increased in cellularity	5/10	1/10	-	-	-	-	1/9	-	1/9
Tubules	Degeneration	-	-	-	-	-	-	-	-	-
Interstitium	Inflammation LPM	3/10	2/10	-	2/10	-	-	3/9	-	-
Liver										
Hepatocytes	Degeneration	-	-	-	-	-	-	-	-	-
	Inflammation LPM	4/10	-	-	5/10	-	-	3/9	-	-
Spleen										
Capsule	Increase in thickness	1/10	-	-	-	-	-	-	1/9	-
White pulp	Eosinophils increase		8/10		-	-	-	-	-	-
	Hyperplasia	5/10	2/10	-	-	-	-	4/9	1/9	-
	Inflammation acute neutrophilic	-	-	-	-	-	-	-	1/9	-

Mi: mild, M: moderate, S: severe. LPM: Lymphoplasmacytic and macrophagic inflammation; LPME Lymphoplasmacytic, eosinophilic, and macrophagic inflammation, E: eosinophilic inflammation

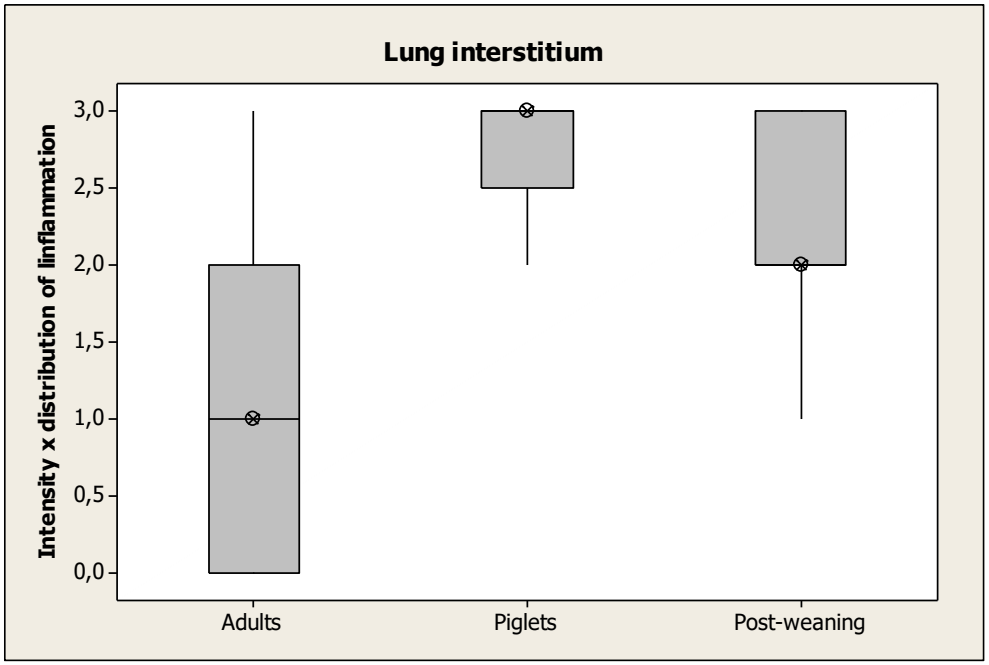
3.2. Statistical analysis of histopathology results

Histological results were compared using the non-parametric Kruskal–Wallis test with Dunn's post hoc comparison considering the intensity x distribution of the inflammation for each evaluated organ. The histological evaluations highlight the presence of statistically significant differences between the severity of the lesions in the different classes of swine. In particular, as reported in graph 3.1, adults and piglets were characterized by more severe bronchiolar lesions than post-weaning pigs (Kruskal-Wallis Test, $\chi 2$ chi-square 13.771; $p < 0.05$), while there were no statistically significant differences between adult pigs and piglets ($p > 0.05$).

Considering graph 3.2, post-weaning pigs were characterized by more severe lesions in the lung interstitium than adults (Kruskal-Wallis Test, $\chi 2$ 12.304; $p < 0.05$), while there were no statistically significant differences between adult pigs and piglets ($p > 0.05$).



Graph 3.1. Boxplot of bronchi lesions in adult, post-weaning, and piglets. Boxplot represents the median (line), the mean (cross within a circle), 25th to 75th percentile (box), and outside (black cross) values.



Graph 3.2. Boxplot of lung interstitium lesions in adult, post-weaning, and piglets. Boxplot represents the median (line), the mean (cross within a circle), 25th to 75th percentile (box), and outside (black cross) values.

No statistically significant differences were observed in the 3 age categories regarding the histological lesions in the heart, the mediastinal lymph node, the intestine, the kidney, the liver, the mesenteric lymph node, and the spleen ($p > 0.05$).

3.3. Statistical analysis of Real-time PCR results

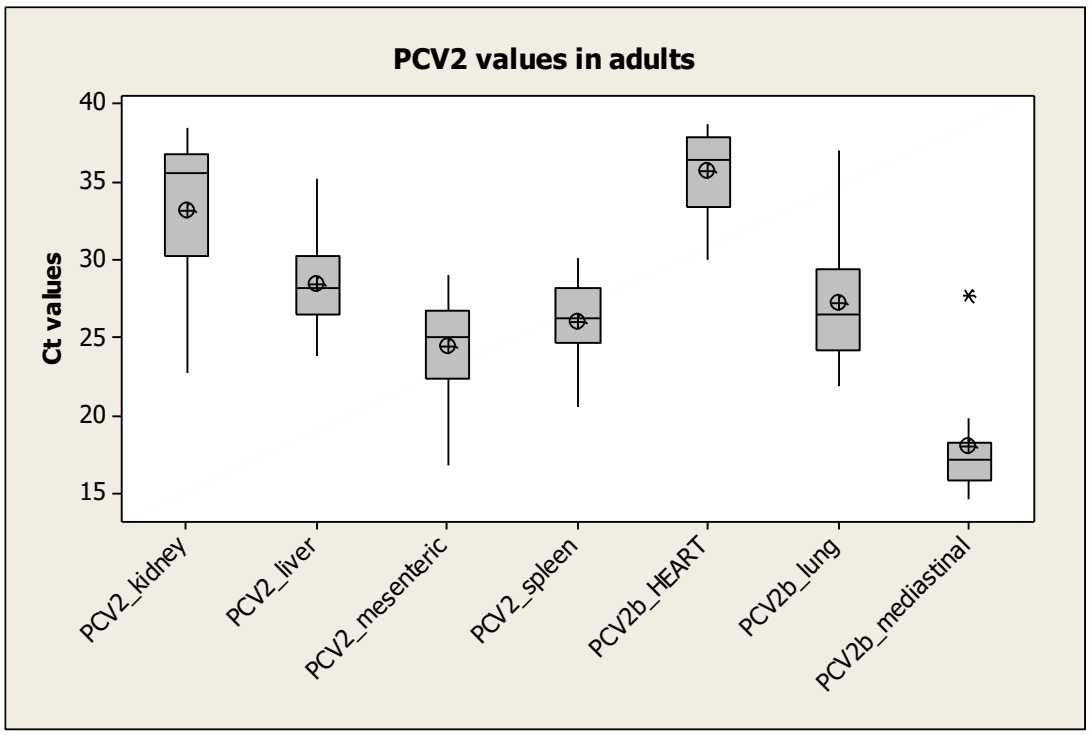
3.3.1. Non-emerging viruses

3.3.1.1 Samples of adult pigs

Regarding the non-emerging viruses, we tested three of the most frequent viruses in intensive pig farms PRRS, PCV2, and PPV1 by Real-time PCR.

PRRS: RT-PCR assay which was done on lungs belonging to adult pigs resulted in 20% positivity (2 pigs out of 10) for the PRRS-EU strain with a mean Ct value of 36.59±0.41, whereas no positivity was found for the PRRS-NA strain.

PCV2: RT-PCR assay which was done on all tissues belonging to adult pigs showed 100% positivity at the level of the lung, livers, mediastinal lymph nodes, and spleens, where all these tissues were positive with the following mean Ct values: 27.22±4.43 for the lungs, 28.46 ±3.10 for the livers, 18.06±3.65 for the mediastinal lymph nodes, and 26.05±26.04 for the spleens. At the level of the heart, 60% (6 pigs out of 10) positivity was recorded with a mean Ct value of 35.60±3.09. Ninety percent (9 pigs out of 10) of each of the mesenteric lymph nodes and the kidneys were positive with a mean Ct value of 24.39±3.53 for mesenteric lymph nodes and 33.14±4.90 for kidneys. PCV2 was also detected in 70% (7 pigs out of 10) of tested intestines that were positive with a mean Ct value of 27.92±4.94. In particular, as shown in graph 3.3, mean Ct values were statistically higher in the kidney and heart compared to other organs, while lower values were observed in the mediastinal lymph node (Anova $\chi 2$ (6) = 3.7246 Prob> $\chi 2$ = 0.714, F=19.75, $p < 0.05$) (Graph 3.3)



Graph 3.3. Graph of PCV2 Ct values in the different organs of adult pigs. Boxplot represents the median (line), the mean (cross within a circle), 25th to 75th percentile (box), and outside (black cross) Ct values.

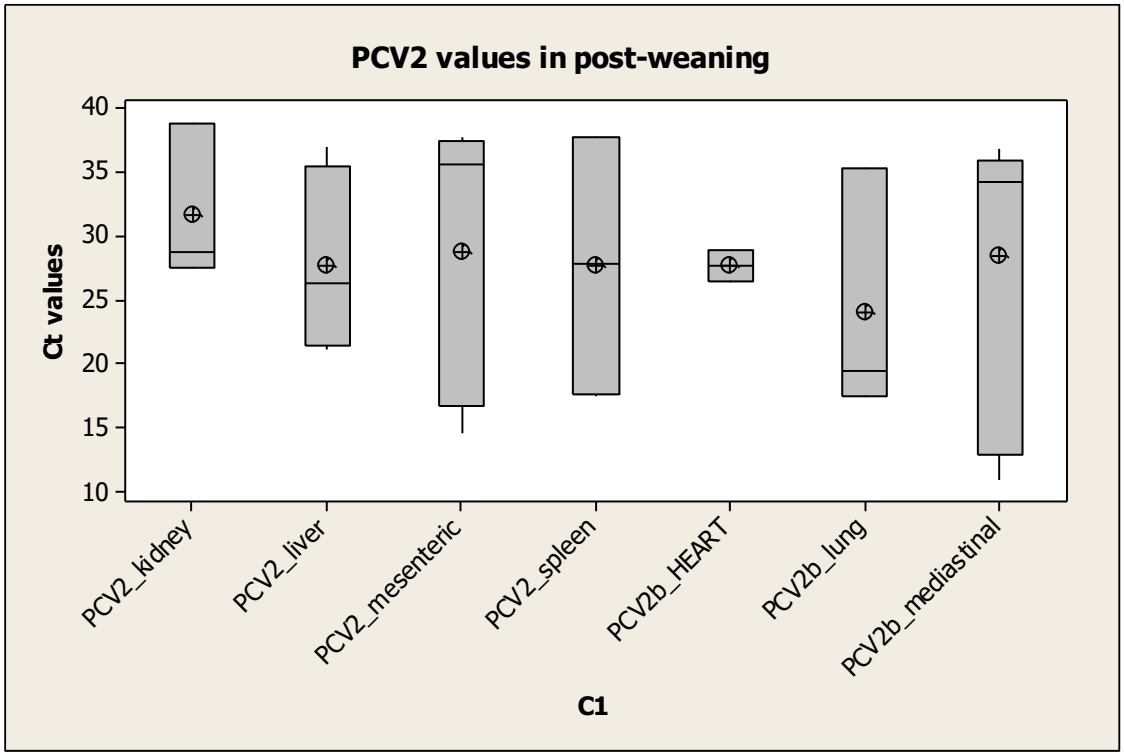
PPV1: PPV1 was detected in only 1 spleen of 1 pig out of 9 (11.11%) with a Ct value of 37.73 and in 1 kidney of 1 pig out of 10 (10%) with a Ct value of 40.53.

3.3.1.2 Samples of post-weaning pigs

PRRS: RT-PCR assay which was done on lungs belonging to post-weaning pigs showed 20% positivity (2 pigs out of 10) for the PRRS-EU strain with a mean Ct value of 34.30±4.35, whereas no positivity was found for the PRRS-NA strain.

PCV2: RT-PCR assay which was done on all tissues belonging to post-weaning pigs showed 70% positivity (7 pigs out of 10) in mediastinal lymph nodes having a mean Ct value of 28.46±11.40. At the level of the liver, spleen, and intestine, positivity was 40% where PCV2 was detected in 4 pigs out of 10 with mean Ct values: 27.66±7.52 for livers, 27.65±11.36 for spleens, and 29.97±7.08 for intestines. Fifty percent (5 pigs out of 10) of mesenteric lymph nodes were found positive and had a mean Ct value of 28.73±11.06. At the level of the lungs and the kidneys, 30% (3 pigs out of 10) were found positive for the virus with a mean Ct value

of 24.05±9.7 at the level of the lungs and 31.64±6.17 for the kidneys. As for the tested hearts, 20% (2 pigs out of 10) were found positive for PCV2 with a mean Ct value of 27.58±1.75. No statistically significant differences were observed between the mean Ct value (Anova $\chi 2$ (6) = 3.4520 Prob> $\chi 2$ = 0.750, F=0.15, p > 0.05) (Graph 3.4).



Graph 3.4. Graph of PCV2 Ct values in the different organs of post-weaning pigs. Boxplot represents the median (line), the mean (cross within a circle), 25th to 75th percentile (box), and outside (black cross) Ct values.

PPV1: PPV1 was detected in 2 lungs of 2 pigs out of 10 (20%) with a mean Ct value of 38.44±0.32, in the mediastinal lymph node of 1 pig out of 10 (10%) with a Ct value of 39.77, and in 1 intestine sample out of 10 (10%) with a Ct value of 37.63. (Graph 3.4)

RT-PCR assay which was done on lungs belonging to post-weaning pigs showed 20% positivity (2 pigs out of 10) for the PRRS-EU strain with a mean Ct value of 34.30±4.35, whereas no positivity was found for the PRRS-NA strain.

3.3.1.3. Samples of piglets

PRRS: RT-PCR assay which was done on lungs belonging to piglets presented 33% positivity (3 piglets out of 9) for the PRRS-EU strain with a mean Ct value of 37.38±2.13, whereas, for the strain PRRS-Na, 11% (1 piglet out of 9) showed positivity with a Ct value of 36.94.

PCV2: RT-PCR assay which was done on all tissues belonging to piglets showed 22% positivity (2 piglets out of 9) for the lungs, livers, and intestines, with mean Ct values of 29.67±8.84 for the lungs, 28.21±3.14 for the livers, and 36.93±0.33 for the intestines, respectively. At the level of the mediastinal lymph nodes, 44% (4 piglets out of 9) positivity was recorded with a mean Ct value of 30.51±10.51. As for the remaining tissues, 11.11% positivity (1 piglet out of 9) was recorded for the hearts, spleens, mesenteric lymph nodes, and kidneys with the following mean Ct values: 35.49 for the heart, 22.59 for the spleen, 27.43 for the mesenteric lymph node, and 31.23 for the kidney. No statistically significant differences were observed between the mean Ct values (Anova $\chi2$ (2) = 1.0204 Prob> $\chi22$ = 0.600, F= 0.20; $p > 0.05$).

PPV1: PPV1 was detected in 2 lungs of 2 piglets out of 9 (22.22%) with a mean Ct value of 40± 0.25.

3.3.2. Emerging viruses

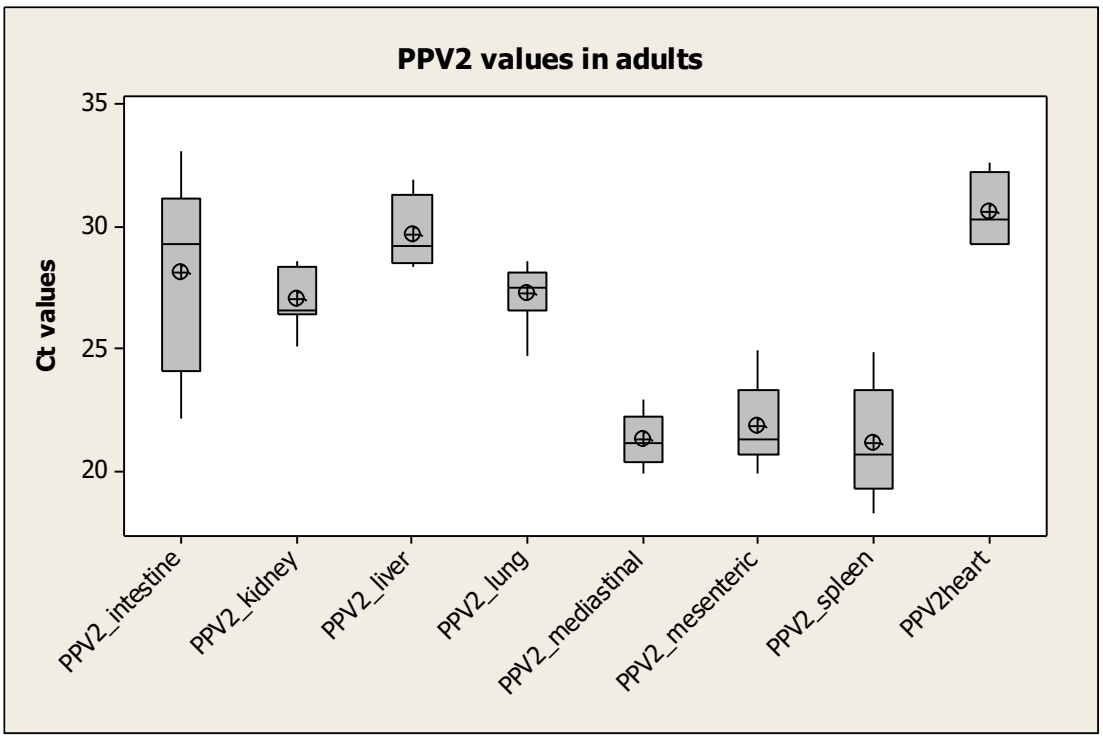
3.3.2.1 Samples of adult pigs

PCV3*:* RT-PCR assay applied on tissues belonging to the adult pigs showed a 10% positivity (1 pig out of 10) for several tissues, as follows: the lungs with a mean Ct value of 19.91, the livers with a mean Ct value of 22.27, the hearts with a mean Ct value of 32.11, the spleen with a mean Ct value of 21.97, the mesenteric lymph nodes with a mean Ct value of 26.40, and the kidneys with a mean Ct value of 33.14. As for the mediastinal lymph nodes and the intestines, 20% (2 pigs out of 10) were positive for PCV3 with mean Ct values, of 21.03±5.02 and 35.53±5.8, respectively. No statistically significant differences were observed between the mean Ct values (Anova $\chi2$ (1) = 0.0134 Prob> $\chi2$ = 0.908; F= 1.69, $p > 0.05$).

PPV 2, 3, 4, 5, 6, 7: PPV2 was detected in all the tested samples. Lungs, mediastinal lymph nodes, and spleens of all pigs (100%) were found positive with mean Ct values of 27.29±1.12, 21.29±1.09, and 21.14 ±2.36, respectively. Four livers of 4 pigs out of 10 (40%) were positive for PPV2 with a mean Ct value of 29.69±1.55. The same percentage of positivity was for heart samples where 4 pigs out of 10 (40%) were positive with a mean Ct value of 30.64±1.6. Regarding mesenteric lymph nodes and intestine samples, PPV2 was detected in 9 pigs out of 10 (90%) for each of these tissues with mean Ct values of 21.83±1.66 and 28.12±3.87, respectively. As for the kidneys, 8 pigs out of 10 (80%) were found positive for PPV2 with a mean Ct value of 27.08±1.25.

Statistically significant differences were observed between mean Ct value (Anova $\chi 2$ (7) = 22.4992 Prob> $\chi 2$ = 0.002; F= 25.13, $p < 0.05$). In particular, Ct values of mediastinal, mesenteric lymph node and spleen were significantly lower compared to other examined organs (Graph 3.5).

RT-PCR assay applied on tissues belonging to the adult pigs showed a 10% positivity (1 pig out of 10) for several tissues, as follows: the lungs with a mean Ct value of 19.91, the livers with a mean Ct value of 22.27, the hearts with a mean Ct value of 32.11, the spleens with a mean Ct value of 21.97, the mesenteric lymph nodes with a mean Ct value of 26.40, and the kidneys with a mean Ct value of 33.14. As for the mediastinal lymph nodes and the intestines, 20% (2 pigs out of 10) were positive for PCV3 where the virus was detected with mean Ct values, respectively, 21.03±5.02 and 35.53±5.8. No statistically significant differences were observed between the mean Ct values (Anova chi2(1) = 0.0134 Prob>chi2 = 0.908; F= 1.69, $p > 0.05$)



Graph 3.5. Graph of PPV2 Ct values in the different organs of adult pigs. Boxplot represents the median (line), the mean (cross within a circle), 25th to 75th percentile (box), and outside (black cross) Ct values.

RT-PCR assay for the detection of PPV3 resulted in the positivity of 1 liver of 1 pig out of 10 (10%) with a Ct value of 23.40, 1 mediastinal lymph node of 1 pig out of 10 (10%) with a Ct value of 23.17, 1 spleen out of 9 (11.11%) with a Ct value of 24.13, 1 mesenteric lymph node (10%) with a Ct value of 23.45, 1 intestine sample out of 10 (10%) with a Ct value of 28.01, and 2 kidneys out of 10 (20%) with a mean Ct value of 29.77±4.44. No statistically significant differences were observed between the mean Ct values ($F= 0.58$, $p > 0.05$)

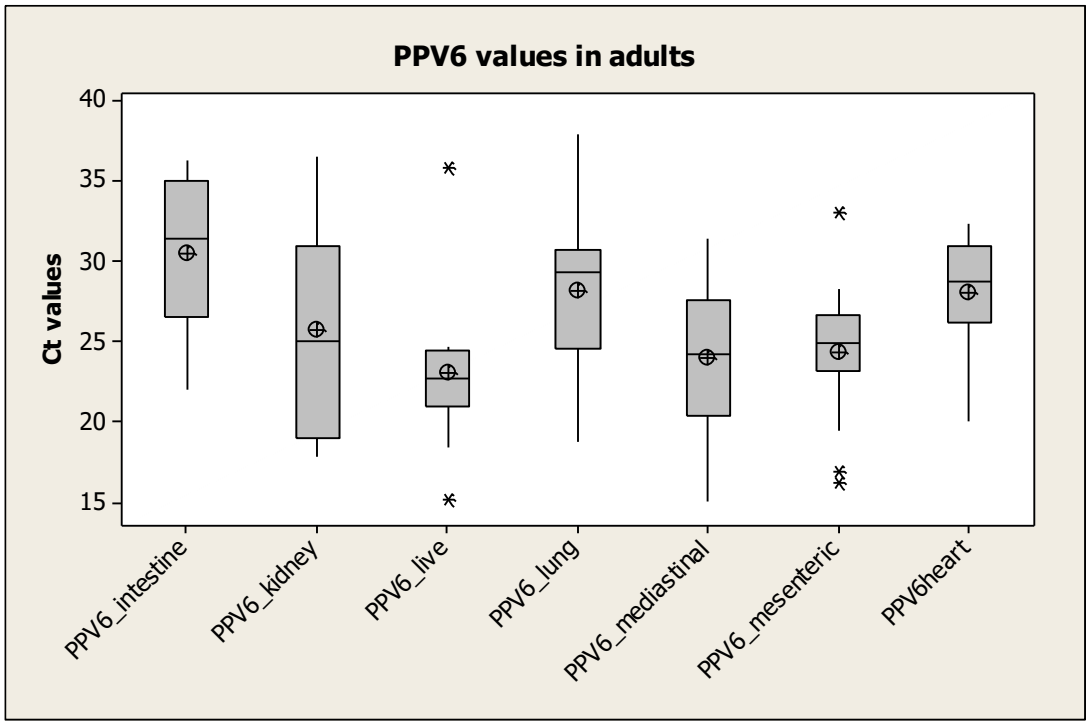
PPV4 was only found in 1 intestine (10%) with a Ct value of 31.76, and in 3 kidneys (30%) with a mean Ct value of 31.76 ±6.16. No statistically significant differences were observed between mean Ct values ($p > 0.05$).

PPV5 was detected in 3 lungs out of 10 (30%) with a mean Ct value of 36.84±2.1, 4 mediastinal lymph nodes of 4 pigs out of 10 (40%) with a mean Ct value of 30.86±1.22, 7 hearts (70%) with a mean Ct value of 27.88±10.96, 7 spleens out of 9 (78%) with a mean Ct

value of 27.31±4.21, 9 mesenteric lymph nodes (10%) with a mean Ct value of 26.59±8.59, 2 intestine samples (20%) and 5 kidneys (50%) with mean Ct values 35.99±2.52 and 34.65±1.97, respectively.

No statistically significant differences were observed between mean Ct value (Anova $\chi 2$ (6) = 21.5226 Prob> $\chi 2$ = 0.001; F= 1.75, $p > 0.05$).

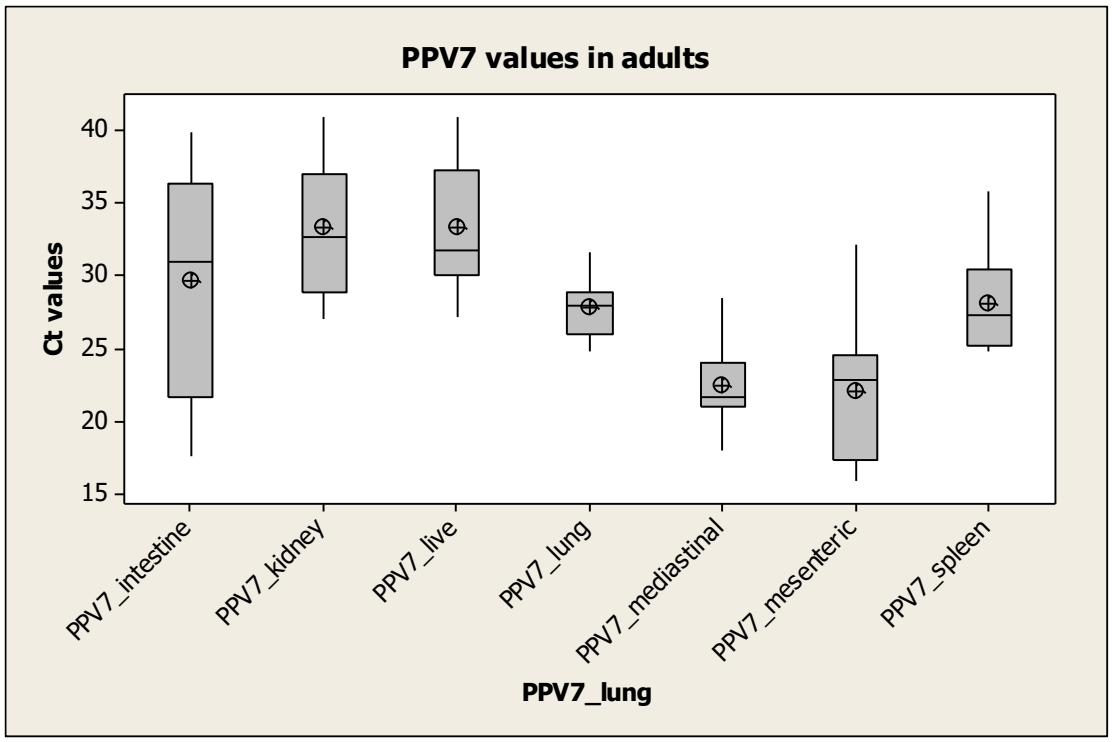
PPV6 was detected in all tissue samples of all pigs (100%) with mean Ct values as follows: 28.14±1.96 for lungs, 23.07±5.31 for livers, 28.03±3.7 for hearts, 23.15±2.98 for spleens, 25.44±4.76 for mesenteric lymph nodes, 30.49±4.72 for intestines, and 25.67±6.37 for kidneys. As for the mediastinal lymph nodes, 9 pigs out of 10 (90%) were found positive for PPV6 with a mean Ct value of 23.96±5.06. Statistically significant differences were observed between the mean Ct values (Anova $\chi 2$ (6) = 3.8286 Prob> $\chi 2$ = 0.700; F= 3.24, $p < 0.05$). In particular, the intestine has a higher value compared to the liver ($p < 0.05$) (Graph 3.6).



Graph 3.6. Graph of PPV6 Ct values in the different organs of adult pigs. Boxplot represents the median (line), the mean (cross within a circle), 25th to 75th percentile (box), and outside (black cross) Ct values.

RT-PCR assay which was done for the detection of PPV7 resulted in the positivity of all lungs, mediastinal lymph nodes, and spleens of all pigs with mean Ct values 27.84±1.96, 22.48±2.78, and 28.05±3.61, respectively. As for the remaining tissues, the heart resulted negative, and the liver, mesenteric lymph nodes, intestines, and kidneys resulted with a positivity of 90 % for each tissue with mean Ct values 33.33±4.49, 22.03±5.04, 29.56±7.73, and 33.28±4.62, respectively.

Statistically significant differences were observed between mean Ct value (Anova $\chi 2$ (6) = 17.7336 Prob> $\chi 2$ = 0.007; F= 9.11, $p < 0.05$). In particular, the intestine, kidney, and liver have a higher value compared to the mesenteric and mediastinal lymph nodes ($p < 0.05$) (Graph 3.7).

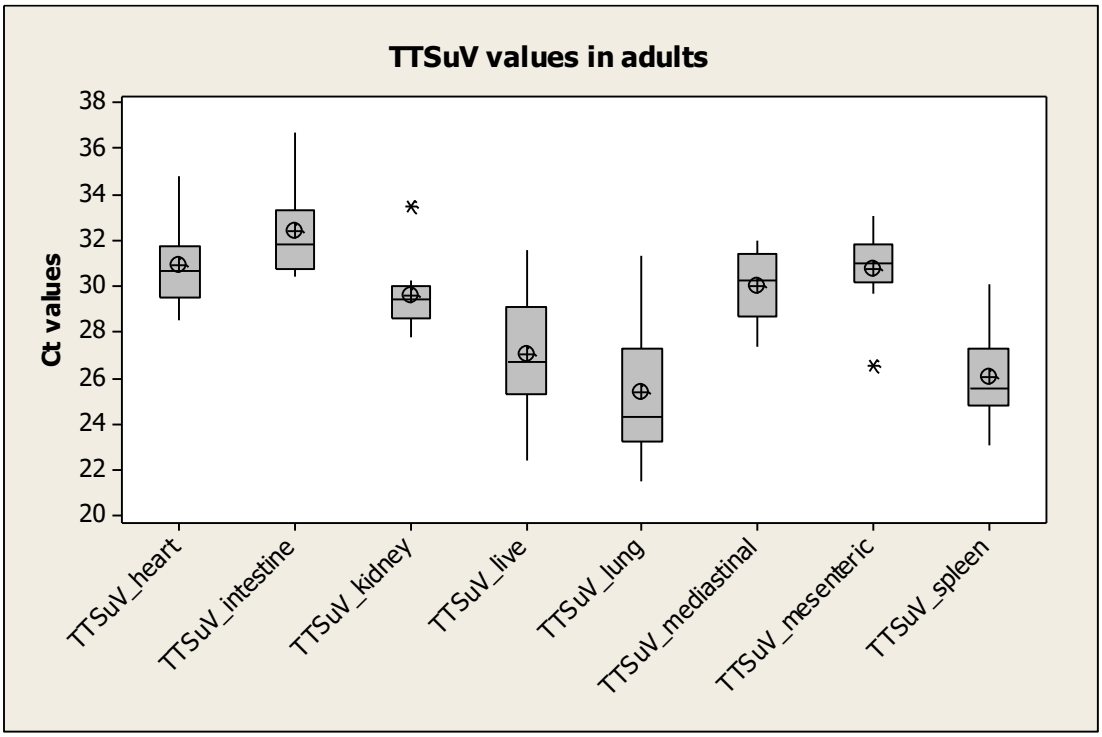


Graph 3.7. Graph of PPV7 Ct values in the different organs of adult pigs. Boxplot represents the median (line), the mean (cross within a circle), 25th to 75th percentile (box), and outside (black cross) Ct values.

TTSuV: all tissues of all pigs (100%) resulted positive for TTSuV with mean Ct values as follows: 25.38±3.10 for lungs, 27.02±2.78 for livers, 30.05±1.63 for mediastinal lymph nodes,

30.92±1.96 for hearts, 26.06±2.04 for spleens, 30.75±1.79 for mesenteric lymph nodes, 32.44±2.05 for intestines, and 29.63±1.56 for kidneys.

Statistically significant differences were observed between mean Ct value (Anova $\chi 2$ (7) = 7.6004 Prob>$\chi 2$ = 0.369; F= 13.50, $p < 0.05$). In particular, the heart and intestine have a higher value compared to the liver, lungs and spleen, the kidney has a higher value compared to the lungs and spleen, while the mediastinal and mesenteric nodes have higher values compared to the liver, lung, and spleen ($p < 0.05$). (Graph 3.8)



Graph 3.8. Graph of TTSuV Ct values in the different organs of adult pigs. Boxplot represents the median (line), the mean (cross within a circle), 25th to 75th percentile (box), and outside (black cross) Ct values.

***APPeV**:* RT-PCR assay done on mediastinal lymph node samples was positive for APPeV of 5 pigs out of 10 (50%) with a mean Ct value of 34.70±5.92 and 2 pigs out of 10 (20%) were positive for the virus in mesenteric lymph node samples with a mean Ct value of 35.47±3.04. All RT-PCR assays for SVA, LINDAV, PEAV, and PDCoV applied on all tissues belonging to adult pigs resulted in negative.

3.3.2.2 Samples of post-weaning pigs

PCV3: RT-PCR assay applied on the tissues belonging to post-weaning pigs showed a 20% positivity (2 pigs out of 10) for the lungs with a mean Ct value of 29.07±12.15, for the livers with a mean Ct value of 31.01±9.72, for the hearts with a mean Ct value of 31.23±31.23. Fifty percent of tested intestines (5 pigs out of 10) were found positive for PCV2 with a mean Ct value of 34.62±5.40. As for the mediastinal lymph nodes, spleens, mesenteric lymph nodes, and kidneys, PCV2 was detected in only 10% (1 pig out of 10) with mean Ct values, respectively: 20.62, 19.25, 23.28, and 35.09.

No statistically significant differences were observed between mean Ct values (Anova $\chi 2$ (3) = 1.3371 Prob> $\chi 2$ = 0.720; F= 0.79, $p > 0.05$).

PPV 2, 3, 4, 5, 6, 7: PPV2 was detected in 5 lungs of 5 pigs out of 10 (50%), 5 livers (50%), 5 hearts (50%), 5 spleens (50%), 5 mesenteric lymph nodes (50%), and 5 intestine samples (50%) with mean Ct values of 21.21±5.47, 25.34±6.11, 24.49±3.78, 17.83±3.50, 18.23±2.09, and 22.69±6.89, respectively. Six mediastinal lymph nodes of 6 pigs out of 10 (60%) were positive with a mean Ct value of 19.33±6.37. Regarding kidney samples, PPV2 was detected in 7 pigs out of 10 (70%) with mean Ct values of 21.44±4.10.

No statistically significant differences were observed between mean Ct value (Anova $\chi 2$ (7) = 6.9516 Prob> $\chi 2$ = 0.434; F= 1.54, $p > 0.05$).

RT-PCR assay for the detection of PPV3 resulted in the positivity of only 1 liver of 1 pig out of 10 (10%) with a Ct value of 37.91.

PPV4 was found in 3 lungs (30%) with a mean Ct value of 37.42±1.08, in 2 mediastinal lymph nodes (20%) with a mean Ct value of 39.15±1.23, and in 1 intestine sample out of 10 (10%) with a Ct value of 37.79. No statistically significant differences were observed between mean Ct value (F= 2.82, $p > 0.05$).

PPV5 was detected in 4 lungs out of 10 (40%) with a mean Ct value of 29.03±7.36, 3 livers (30%) with a mean Ct value of 30.64±4.89, 3 mediastinal lymph nodes of 3 pigs out of 10

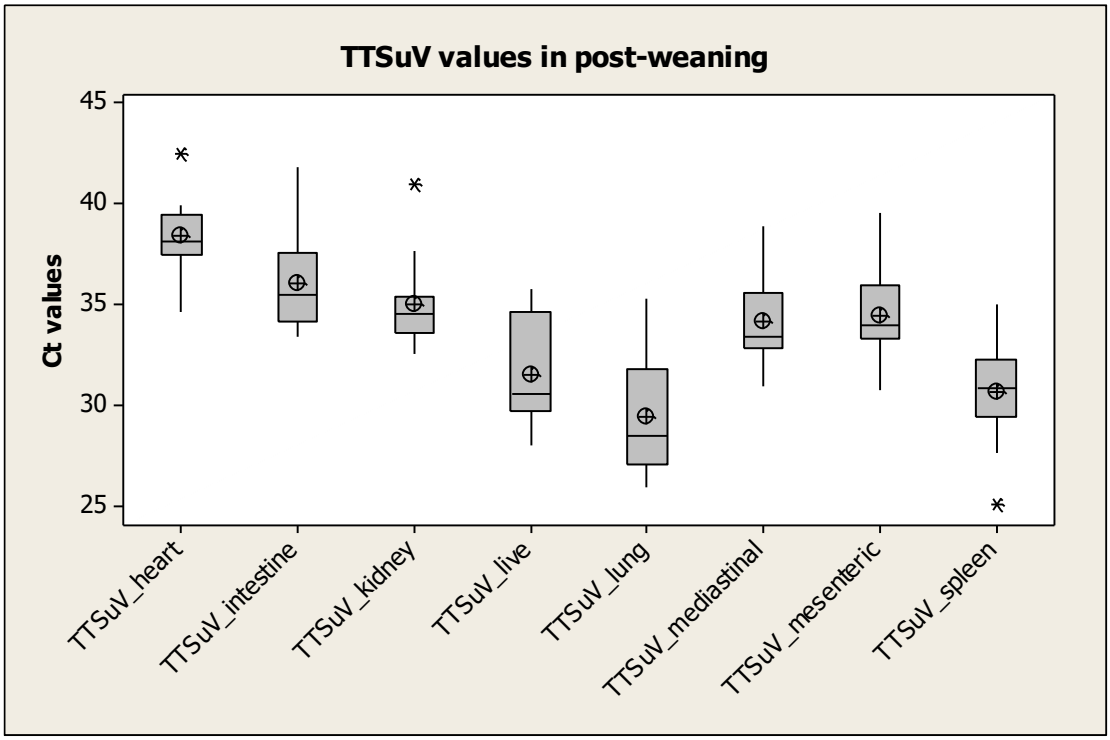
(30%) with a mean Ct value of 27.51±6.38, 10 hearts (100%) with a mean Ct value of 31.64±10.30, 8 spleens out of 9 (88.89%) with a mean Ct value of 24.66±9.21, 7 mesenteric lymph nodes (70%) with a mean Ct value of 27.54±7.52, 2 intestine samples (20%) and 3 kidneys (30%) with mean Ct values of respectively 33.42±1.63 and 27.17±5.68. No statistically significant differences were observed between mean Ct value (Anova $\chi 2$ (7) = 4.4315 Prob> $\chi 2$ = 0.729; F= 0.60, $p > 0.05$).

PPV6 was detected in 2 livers of 2 pigs out of 10 (20%) with a mean Ct value of 35.08±3.90, and in 1 heart out of 10 (10%) with a Ct value of 39.27. No statistically significant differences were observed between mean Ct value (F= 0.77, $p > 0.05$).

RT-PCR assay, which was done for the detection of PPV7, resulted in the positivity of 4 lungs out of 10 (40%), 2 livers (20%), 5 mediastinal lymph nodes (50%), 3 spleens (30%), 3 mesenteric lymph nodes (30%), and 3 intestine samples (30%) with mean Ct values of 30.50±2.1, 33.58±1.54,34.53±6.17, 31.87±4.05, 30.35±3.34, and 38.05±3.1, respectively. No statistically significant differences were observed between mean Ct value (Anova $\chi 2$ (5) = 4.2378 Prob> $\chi 2$ = 0.516; F= 1.60, $p > 0.05$).

TTSuV: all tissues of all pigs (100%) resulted positive for TTSuV with mean Ct values as follows: 29.39±2.98 for lungs, 31.56±2.69 for livers, 34.14± 2.27 for mediastinal lymph nodes, 38.44±2.06 for hearts, 30.67±4.05 for spleens, 34.46±2.40 for mesenteric lymph nodes, 36.03±2.53 for intestines, and 35.05±2.46 for kidneys.

Statistically significant differences were observed between mean Ct value (Anova $\chi 2$ (7) = 1.6003 Prob> $\chi 2$ = 0.979; F= 13.95, $p < 0.05$). In particular, the heart has a higher value compared to the other examined organs, the intestine has a higher value compared to the other examined organs except for the kidney, the kidney has a higher value compared to the other examined organs except for the mediastinal and mesenteric lymph nodes, while the mediastinal and the mesenteric lymph nodes have higher values compared to the lung and the spleen ($p < 0.05$) (Graph 3.9).



Graph 3.9. Graph of TTSuV Ct values in the different organs of post-weaning pigs. Boxplot represents the median (line), the mean (cross within a circle), 25th to 75th percentile (box), and outside (black cross) Ct values.

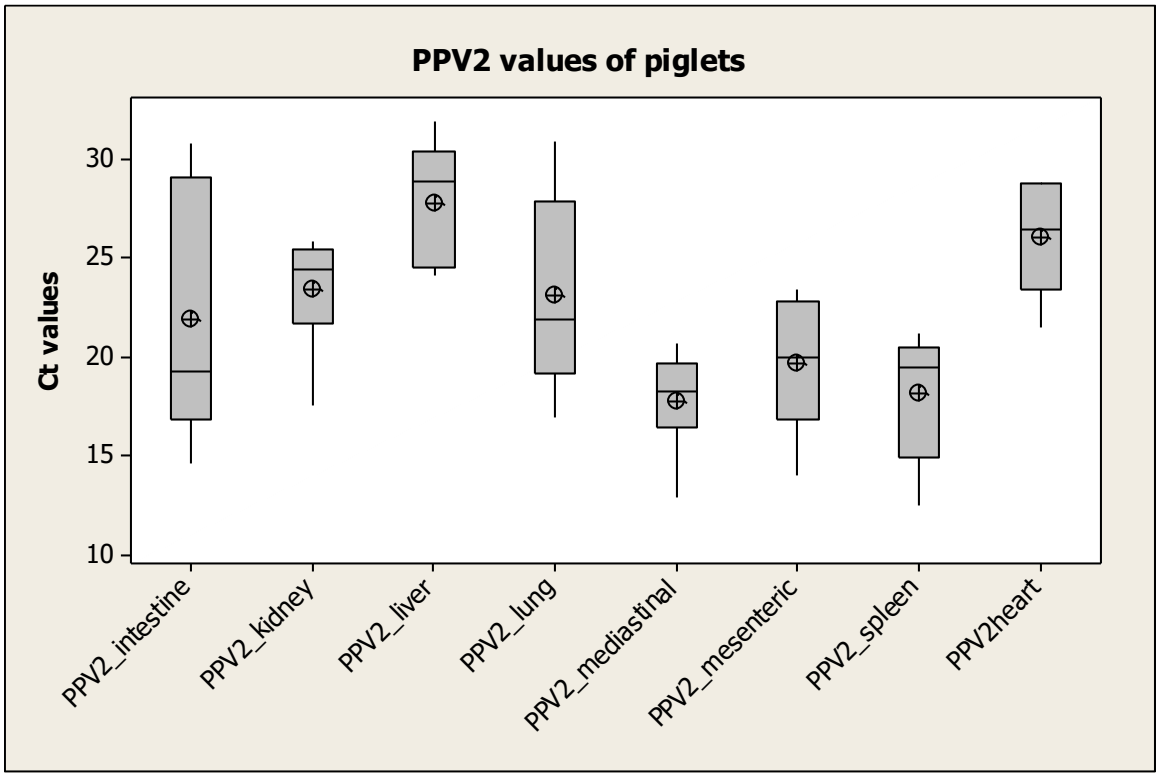
All RT-PCR assays for SVA, APPeV, LINDAV, PEAV, and PDCoV applied on all tissues belonging to post-weaning pigs resulted in negative.

3.3.2.3. Samples of piglets

PCV3: RT-PCR assay applied on tissues belonging to piglets revealed a 44% positivity (4 piglets out of 9) at the level of the lungs with a mean Ct value of 35.59±4.88, a 22% positivity (2 piglets out of 9) at the level of the liver, the spleen, and the intestine with mean Ct values 36.68±1.14, 31.26±3.23, and 35.95±0.45, a 33% positivity (3 piglets out of 9) at the level of the kidney with a mean Ct value of 35.39±4.40, and 11% positivity (1 piglet out of 9) for the mediastinal lymph node, heart, and mesenteric lymph node with respective mean Ct values as follows: 32.33, 37.64, and 33.27. No statistically significant differences were observed between mean Ct value (Anova $\chi 2$ (4) = 4.1021 Prob> $\chi 2$ = 0.392; F= 0.51, p > 0.05).

PPV 2, 3, 4, 5, 6, 7: PPV2 was detected in 8 lungs of 8 piglets out of 9 (89%), 5 livers (56%), 8 mediastinal lymph nodes (89%), 6 hearts (67%), 8 spleens (89%), 7 mesenteric lymph nodes (78%), 8 intestine samples (89%), and 8 kidneys (89%) with mean Ct values of 23.06±4.83, 27.73±3.19, 17.71±2.44, 25.97±2.97, 18.12±3.23, 19.68±3.53, 21.84±6.36, and 23.35±2.86, respectively.

Statistically significant differences were observed between mean Ct value (Anova $\chi 2$ (7) =9.7024 Prob> $\chi 2$ = 0.206 F= 5.37, $p < 0.05$). In particular, the Ct values of the liver were significantly higher compared to mediastinal, and mesenteric lymph nodes and spleen, while the Ct values of the heart were significantly higher than mediastinal nodes and the spleen (Graph 3.10).



Graph 3.10. Graph of PPV2 Ct values in the different organs of piglets. Boxplot represents the median (line), the mean (cross within a circle), 25th to 75th percentile (box), and outside (black cross) Ct values.

RT-PCR assays for the detection of PPV3 resulted in the positivity of 1 liver of 1 piglet out of 9 (11%) with a Ct value of 32.93 and 1 heart of 1 piglet out of 9 (11%) with a Ct value of 6.11.

No positivity was found for PPV4 in any of the tested samples of all piglets. No statistically significant differences were observed between mean Ct value (F= 1.27, p > 0.05).

PPV5 was detected in 7 lungs out of 9 (78%) with a mean Ct value of 30.18±9.40, 4 livers (44%) with a mean Ct value of 30.64±5.23, 5 mediastinal lymph nodes of piglets out of 9 (56%) with a mean Ct value of 26.31±4.51, 7 hearts (78%) with a mean Ct value of 28.48±7.49, 6 spleens out of 9 (67%) with a mean Ct value of 19.44±8.76, 10 mesenteric lymph nodes (100%) with a mean Ct value of 26.61±7.94, 4 intestine samples (44%) and 4 kidneys (44%) with mean Ct values of respectively 28.73±7.09 and 26.13±4.12. No statistically significant differences were observed between mean Ct value (Anova $\chi 2$ (7) = 4.3014 Prob> $\chi 2$ = 0.744; F= 1.28, p > 0.05).

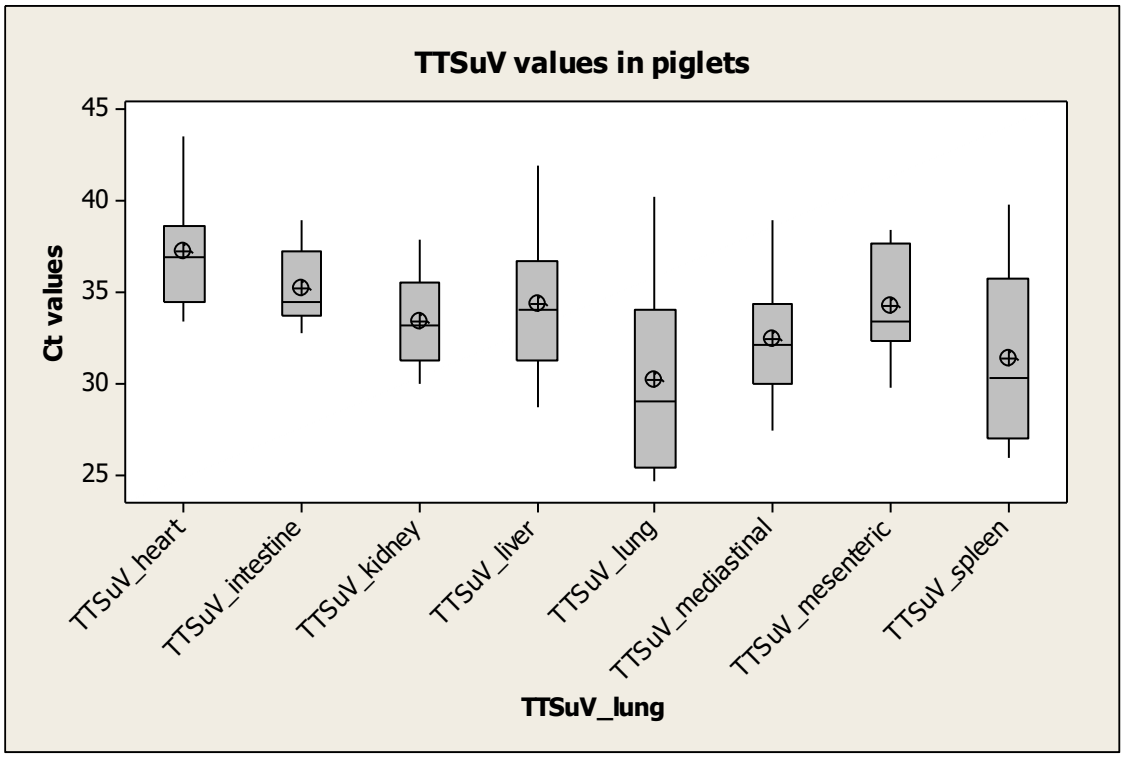
PPV6 was detected in 1 lung of 1 piglet out of 9 (11.11%) with a Ct value of 36.75, and in 5 livers of 5 piglets out of 9 (56%) with a mean Ct value of 33.31±2.44.

RT-PCR assay which was done for the detection of PPV7 resulted in the positivity of 3 lungs of 3 piglets out of 9 (33%), 2 livers (22%), 3 mediastinal lymph nodes (33%), 1 heart (11%), 2 spleens (22%), 3 mesenteric lymph nodes of 3 piglets out of 9 (33%), 2 intestine samples (22%), and 1 kidney of 1 piglet out of 9 (11%) with mean Ct values 26.98±10.62, 27.69±3.06, 24.23±4.68, 32.04, 24.77±1.61, 17.73±9.94, 23.57±3.20, and 29.14, respectively. No statistically significant differences were observed between mean Ct value (Anova $\chi 2$ (5) = 4.3506 Prob> $\chi 2$ = 0.500; F= 0.67, p > 0.05).

TTSuV: all the tested lungs and spleens of all piglets (100%) were found positive with mean Ct values respectively 30.13±5.32 and 31.32±5.08. As for the remaining organs, positivity was 8 out of 9 (89%) piglets for each of the liver, mediastinal lymph node, heart, mesenteric lymph node, intestine, and kidney with mean Ct values as follows: 34.29±4.29, 32.40±3.45, 37.15±3.16, 34.23±3.07, 35.16±2.15, and 33.32±2.61.

Statistically significant differences were observed between mean Ct value (Anova $\chi 2$ (7) = 9.5042 Prob> $\chi 2$ = 0.218; F= 2.80, P < 0.05). In particular, the Ct values of the heart were

significantly higher compared to the lung and spleen, while the Ct values of the heart were significantly higher than the mediastinal lymph nodes and the spleen ($P < 0.05$) (Graph 3.11).



Graph 3.11. Graph of TTSuV Ct values in the different organs of piglets. Boxplot represents the median (line), the mean (cross within a circle), 25th to 75th percentile (box), and outside (black cross) Ct values.

All RT-PCR assays for SVA, APPeV, LINDAV, PEAV, and PDCoV applied on all tissues belonging to piglets resulted in negative.

Table 3.2. Ct values - Mean (±SD)- of non-emerging viruses in several organs of the different swine categories.

Virus		Lu	He	Li	Sp	In	Ki	Md LN	Ms LN
PRRS	**A**	2/10	Nt	Nt	Nt	Nt	Nt	Nt	Nt
	Mean Ct	36.59± 0.41	-	-	-	-	-	-	-
	PW	2/10	Nt	Nt	Nt	Nt	Nt	Nt	Nt
	Mean Ct	34.30± 4.35	-	-	-	-	-	-	-
	P	3/9	Nt	Nt	Nt	Nt	Nt	Nt	Nt
	Mean Ct	37.38± 2.13	-	-	-	-	-	-	-
PCV2	**A**	10/10	6/10	10/10	9/9	7/10	9/10	10/10	9/10
	Mean Ct	27.22± 4.43	35.60±3.09	28.46±3.10	26.05 ±26.04	27.92 ±4.94	33.14 ±4.90	18.06 ±3.65	24.39 ±3.53
	PW	3/10	2/10	4/10	4/10	4/10	3/10	7/10	5/10
	Mean Ct	24.05± 9.7	27.58±1.75	27.66±7.52	27.65 ±11.36	29.97 ±7.08	31.64 ±6.17	28.46 ±11.40	28.73 ±11.06
	P	2/9	1/9	2/9	1/10	2/9	1/9	4/9	1/9
	Mean Ct	29.67± 8.84	35.49	28.21±3.14	22.59	36.93 ±0.33	31.23	30.51 ±10.51	27.43
PPV1	**A**	N	N	N	1/9	N	1/10	N	N
	Mean Ct	-	-	-	37.73	-	40.53	-	-
	PW	2/10	N	N	N	1/10	N	1/10	N
	Mean Ct	38.44± 0.32	-	-	-	39.77	-	39.77	-
	P	2/9	N	N	N	N	N	N	N
	Mean Ct	40.00± 0.25	-	-	-	-	-	-	-

A: adult pigs, PW: post-weaning pigs, P: piglets, Lu: lung, He: heart, Li; liver, Sp: spleen, In: intestine, Ki: kidney, Md Ln: mediastinal lymph node, Ms Ln: mesenteric lymph node, Nt: not tested, N: negative

Table 3.3. Ct values - Mean (±SD)- of emerging viruses in several organs of the different swine categories.

Virus		Lu	He	Li	Sp	In	Ki	Md LN	Ms LN
PCV3	**A**	1/10	1/10	1/10	1/9	2/10	1/10	2/10	1/10
	Mean Ct	19.91	32.11	22.27	21.97	27.92 ±5.8	33.14	21.03 ±5.02	26.40
	PW	2/10	2/10	2/10	1/10	5/10	1/10	1/10	1/10
	Mean Ct	29.07 ±12.15	31.23 ±31.23	31.01 ±9.72	19.25	29.97	35.09	20.62	23.28
	P	4/9	1/9	2/9	2/9	2/9	3/9	1/9	1/9
	Mean Ct	35.59 ±4.88	37.64	36.68 ±1.14	31.26 ±3.23	36.93 ±0.45	35.39 ±4.40	32.33	33.27
TTSuV	**A**	10/10	10/10	10/10	9/9	10/10	10/10	10/10	10/10
	Mean Ct	25.38 ±3.10	30.92 ±1.96	27.02 ±2.78	26.06 ±2.04	32.44 ±2.05	29.63 ±1.56	30.05 ±1.63	30.75 ±1.79
	PW	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10
	Mean Ct	29.39 ±2.98	38.44 ±2.06	31.56 ±2.69	30.67 ±4.05	36.03 ±2.53	35.05 ±2.46	34.14	34.46 ±2.40
	P	9/9	8/9	8/9	9/9	8/9	8/9	8/9	8/9
	Mean Ct	30.13 ±5.32	37.15 ±3.16	34.29 ±4.29	31.32 ±5.08	35.16 ±2.15	33.32 ±2.61	32.40 ±3.45	34.23 ±3.07
PPV2	**A**	10/10	4/10	4/10	9/9	9/10	8/10	10/10	9/10
	Mean Ct	27.29 ±1.12	30.64 ±1.6	29.69 ±1.55	21.14 ±2.36	28.12 ±3.87	27.08 ±1.25	21.29 ±1.09	21.83 ±1.66
	PW	5/0	5/10	5/10	5/10	5/10	7/10	6/10	5/10
	Mean Ct	21.21 ±5.47	24.49±3 .78	25.34 ±6.11	17.83 ±3.50	22.69 ±6.89	21.44 ±4.10	19.33 ±6.37	18.23 ±2.09
	P	8/9	6/9	5/9	8/9	8/9	8/9	8/9	7/9
	Mean Ct	23.06 ±4.83	25.97±2 .97	27.73 ±3.19	18.12 ±3.23	21.84 ±6.36	23.35 ±2.86	17.71 ±2.44	19.68 ±3.53
PPV3	**A**	N	N	1/10	1/9	1/10	2/10	1/10	1/10
	Mean Ct	-	-	23.40	24.13	28.01	29.77 ±4.44	23.17	23.45
	PW	N	N	1/10	N	N	N	N	N
	Mean Ct	-	-	37.91	-	-	-	-	-
	P	N	1/9	1/9	N	N	N	N	N
	Mean Ct	-	6.11	32.93	-	-	-	-	-
PPV4	**A**	N	N	N	N	1/10	3/10	N	N
	Mean Ct	-	-	-	-	31.76 ±6.16	31.76	-	-
	PW	3/10	N	N	N	1/10	N	2/10	N
	Mean Ct	37.42 ±1.08	-	-	-	37.79	-	39.15 ±1.23	-
	P	N	N	N	N	N	N	N	N
	Mean Ct	-	-	-	-	-	-	-	-
PPV5	**A**	3/10	7/10	N	7/9	2/10	5/10	4/10	9/10
	Mean Ct	36.84 ±2.1	27.88±1 0.96	-	27.31 ±4.21	35.99 ±2.52	34.65 ±1.97	30.86 ±1.22	26.59 ±8.59
	PW	4/10	10/10	3/10	8/10	2/10	3/10	3/10	7/10
	Mean Ct	29.03 ±7.36	31.64±1 0.30	30.64 ±4.89	24.66 ±9.21	33.42 ±1.63	27.17 ±5.68	27.51 ±6.38	27.54 ±7.52
	P	7/9	7/9	4/9	6/9	4/9	4/9	5/9	9/9
	Mean Ct	30.18 ±9.40	28.48±7 .49	30.64 ±5.23	19.44 ±8.76	28.73 ±7.09	26.13 ±4.12	26.31 ±4.51	26.61 ±7.94
PPV6	**A**	10/10	10/10	10/10	9/9	10/10	10/10	9/10	10/10
	Mean Ct	28.14 ±1.96	28.03±3 .7	23.07 ±5.31	23.15 ±2.98	30.49 ±4.72	25.67 ±6.37	23.96 ±5.06	25.44 ±4.76
	PW	N	1/10	2/10	N	N	N	N	N

	Mean Ct	-	39.27	35.08 ±3.90	-	-	-	-	-
	P	1/9	N	5/9	N	N	N	N	N
	Mean Ct	36.75	-	33.31 ±2.44	-	-	-	-	-
PPV7	**A**	10/10	N	9/10	9/9	9/10	9/10	10/10	9/10
	Mean Ct	27.84 ±1.96	-	33.33 ±4.49	28.05 ±3.61	29.56 ±7.73	33.28 ±4.62	22.48 ±2.78	22.03 ±5.04
	PW	4/10	N	2/10	3/10	3/10	N	5/10	3/10
	Mean Ct	30.50 ±2.1	-	33.58 ±1.54	31.87 ±4.05	38.05 ±3.1	-	34.53 ±6.17	30.35 ±3.34
	P	3/9	1/9	2/9	2/9	2/9	1/9	3/9	3/9
	Mean Ct	26.98 ±10.62	32.04	27.69 ±3.06	24.77 ±1.61	23.57 ±3.20	29.14	24.23 ±4.68	17.73 ±9.94
APPeV	**A**	Nt	Nt	Nt	Nt	Nt	Nt	5/10	2/10
	Mean Ct	-	-	-	-	-	-	34.70	35.47
	PW	Nt	Nt	Nt	Nt	Nt	Nt	Nt	Nt
	Mean Ct	-	-	-	-	-	-	-	-
	P	Nt	Nt	Nt	Nt	Nt	Nt	Nt	Nt
	Mean Ct	-	-	-	-	-	-	-	-

A: adult pigs, PW: post-weaning pigs, P: piglets, Lu: lung, He: heart, Li; liver, Sp: spleen, In: intestine, Ki: kidney, Md Ln: mediastinal lymph node, Ms Ln: mesenteric lymph node, Nt: not tested, N: negative

3.4. Sequencing results

The sequencing performed on several samples confirmed the positivity for PPV2-7, APPeV, and for both *TTSuV1* and *TTSuV2k* genus. One adult pig confirmed to be coinfected with PPV2, PPV3, PPV5, PPV6, PPV7, TTSuV1 and TTSuV2k, along with PPV1 and PCV2.

The Blast analysis showed the highest similarity (97%) of PPV2 Sardinian sequences with two Chinese strains collected in 2009 (GenBank accession number GU938299) and 2017 (MK378208).

The PPV3 and PPV4 sequences obtained in this study had a similarity of 99% and 95% with strains from Germany collected in 2010 (KC296751) and the USA (collection data 2011, JQ236639), respectively.

PPV5 sequences showed an identity of 99% with several Chinese strains collected in 1999 and 2017.

PPV6 Sardinian sequences revealed the highest similarity (99%) with a group of strains from Spanish (year 2012, MH558679), Poland (year 2012, KX384813), China (year 2017, MN326256), and USA (year 2014, KR709268).

PPV7 Sardinian sequences showed 95% identity with a Chinese strain collected in 2011 (MN326256).

APPeV Sardinian sequence had the highest similarity (95%) with strains collected in Spain (LT855204, LT631728) and Germany (year 2005, KU041639).

Finally, Sanger sequencing from TTSuV-positive samples revealed the presence in the samples of both TTSuV1 and TTSuV2k. TTSuV1 sequences had the highest similarity (98%) with strains from Brazil (KX833781) and South Korea (JF451485). Instead, TTSuV2k sequences had 98% identity with strains from China collected in 2015 (MK263732) and 2016 (MK263716).

Chapter 4

Discussion and Conclusions

4.1. Discussion

Pork consumption increased with population growth and the pork meat demand has consecutively intensified production to satisfy the market. An increase in farms, housing high pig densities, contributes to rapid pathogens transmission increasing swine risk exposure [2,26]. Thus, once a virus is introduced to intensive farms its spread could be severe due to the increased susceptibility of the herd to immunosuppression [26].

Swine disease is believed to be mostly related to viruses as a primary insult that could induce an infection by secondary agents [211]. Swine emerging viruses are ubiquitous worldwide and have acquired great relevance as common viral agents that could negatively affect the swine industry as reported for PRRS, ASF, FMD, and PCV2 [27-29]. Emerging viruses may be subclinical in swine herds or may cause mild to severe clinical signs and lesions of various degrees of severity leading to pig death in more severe cases.

Unfortunately, it is difficult to differentiate pathological aspects caused by emerging viruses and other more common viruses, and recurrent lesions in pig organs, such as interstitial pneumonia, lymphoid depletion, enteritis, vasculitis, and glomerulonephritis, are similar when emerging and common viruses are involved making it difficult to associate lesions to viruses. [117,140,141,149]. In addition, co-infection is scarcely reported in the swine population, although the common coexistence of several viruses in intensive systems may alter the severity of the disease in swine [26, 212]. Regrettably, swine diseases consecutive to infection by emerging viruses are understudied leading to limited knowledge of their biology, transmission, and pathogenic potential. [32,36,40]. Although some authors reported that macroscopical and histopathology examinations of lesions are important tools to assign a prominent role to the causative agents and despite several diagnostic techniques (PCR, Real-time PCR, histopathology, ISH, IHC) have been used to detect swine emerging viruses, diagnosis and clinical assessment of viral diseases are still difficult to achieve [211].

In Sardinia, intensive farms are extremely rare. Most pork-producing farms are for self-consumption and a only few intensive pig farms are present in Oristano and Cagliari provinces [195,197]. Unfortunately, the swine industry has suffered since 1978, after the spread of ASF in both domestic and wild pig populations [195]. Very little to null information is known about the possible presence of swine emerging viruses in Sardinian pig farms, and even less about the presence of co-infections in these herds.

A multidisciplinary approach was used to evaluate tissue lesions in several organs of different swine age categories by histopathology and to detect the presence of some emerging and most common swine viruses by Real-time PCR. We considered only statistically significant results even though several lesions were reported in many other organs. Histopathology showed chronic mild diffuse lymphoplasmacytic and macrophagic bronchitis and bronchiolitis (70% to 90% respectively) associated with mild to moderate multifocal to diffuse chronic interstitial lymphoplasmacytic and macrophagic pneumonia in 60% of adult pigs. Post-weaning pigs showed chronic multifocal to diffuse, moderate to severe interstitial pneumonia (100%), whereas mild chronic lymphoplasmacytic and macrophagic bronchitis was present in 40% of the subject. The main histopathological finding in piglets was a chronic moderate to severe and diffuse interstitial lymphoplasmacytic and macrophagic pneumonia (100%) associated with mild chronic lymphoplasmacytic and macrophagic bronchitis and bronchiolitis in 89% of the piglets. Data analyses highlighted that adult pigs and piglets were characterized by more severe bronchiolar lesions than post-weaning pigs (Kruskal-Wallis Test, $p < 0.05$), whereas post-weaning pigs were characterized by more severe lesions in the lung interstitium than adults (Kruskal-Wallis Test, $p < 0.05$). As reported by Ruggeri *et al.,* 2020 lung lesions are one of the most frequented lesions affecting piggeries worldwide [213]. Moreover, our data slightly differ from what has been reported regarding the association between the different types of lesions (i.e: catarrhal bronchopneumonia, purulent bronchopneumonia, interstitial pneumonia, etc) and the swine categories, being interstitial pneumonia more frequently recorded in

growing/fattening pigs than post-weaning. Furthermore, this difference could be attributable to the different adopted protocols based on only macroscopical evaluation or macroscopical and histological description [213]. Samples were also tested by RT-PCR for emerging swine viruses such as PCV3, PPV2 to PPV7, TTSuV, APPeV, SVA, LINDAV, PDCoV, SeACoV, and two of the most common viruses in swine (PCV2 and PRRS).

PRRS was detected in 33% of lungs in piglets and 20% in each of the adult and post-weaning pig categories. Furthermore, the high Ct values detected were indicative of a low viral load and suggested a low presence of PRRSV in examined samples. Concerning PCV2 it was detected in 100% of adult pig's mediastinal lymph nodes, lungs, liver, and spleen. Statistically significant differences were noticed between mediastinal lymph nodes (lowest mean Ct value) compared with other organs ($p < 0.05$). In post-weaning pigs and piglets, PCV2 was detected also in all organs with lower percentages than in adults. However, both post-weaning pigs and piglets did not show statistically significant differences between tested organs, with lower Ct values observed in the lung and the spleen, respectively. PCV2 causes PCVAD [31,35] with several organs involved including the lung, spleen, lymph nodes, kidney, and heart. In addition, PCV2 targets the thymus and lymphoid cells leading to lymphoid depletion and then immunosuppression, thus causing the pigs more susceptible to being infected with other opportunistic viruses and bacteria [36]. A study on PCV2 reported that the lung lesions caused by PCV2 consist of granulomatous interstitial pneumonia associated or not with bronchiolitis and bronchiolar fibrosis, granulomatous lymphadenitis, and lymphoid depletion with, sometimes classical intracytoplasmic botryoid inclusion bodies [214]. Another recent review reported that PCV2 infection causes interstitial to granulomatous pneumonia characterized by the expansion of peribronchial space and alveolar septa by macrophages and lymphocytes. However interstitial pneumonia in PCV2 and PRRSV infections share similarities with often overlapping morphology of lesions, but a granulomatous pattern of inflammation associated with multinucleated macrophages in lymphoid organs is more typical of PCV2 infection than

PRRSV. Based on our results, it seems plausible that the observed lung lesions characterized by interstitial pneumonia are more related to PCV2 infections (100%) than PRRSV (33%) positivity in lungs by RT-PCR. Adding to this, lower PCV2 Ct values than PRRSV in all the examined categories were observed, suggesting a great involvement of this pathogen. Furthermore, as reported by Sarli *et al.,* 2021 the simultaneous presence of different viruses causing pulmonary involvements is frequent in swine herds, and localizing causative agents by alternative methods such as IHC or ISH with the lesions is a key point to understanding the etiopathogenesis of the disease [211]. Moving on to the emerging viruses, PCV3 was detected in all swine categories, mainly in the lungs of piglets (4/9) and the intestine of 5 out of 10 post-weaning pigs with a mean detection rate of 38% (11 swine out 29) and lower Ct values in the lung, spleen and mediastinal lymph node. Interestingly, as reported by Klaumann *et al.,* 2018 the PCV3 genome has been detected in all age categories with a similar frequency. Furthermore, similarly to our results, the highest prevalence of PCV3 PCR positivity has been detected in animals after weaning [160 and references therein]. No statistically significant differences were observed between the mean Ct value in the examined organs ($p > 0.05$) suggesting a broad tropism of PCV3 in the swine tissue as mentioned by Chen *et al*., 2021 [162].

Interestingly, compared to our results, Dei Giudici and coauthors in 2020 reported a lower (17.64%) detection rate among domestic pigs in Sardinia. This apparent dissimilarity is probably related to the different samples analyzed including, among others, also blood, aborted fetus, placenta, and brain. Furthermore, similarly to our study, Dei Giudici and coauthors as well as Klauman *et al.,* in 2018 reported a frequent coexistence of PCV3 with other viruses (i.e PCV2, PRRSV, PEDV, PPV, TTSuV1 and 2, PDCoV) [154, 160 and references therein].

Of interest, Dei Giudici hypothesizes that PCV3 impacts the pig immune system similarly to PCV2 causing immunosuppression as already reported [154]. Furthermore, it is still unclear if PCV3 could have a role as a secondary agent in immunomodulated or immunosuppressed pigs, or if the co-infection frequency isn't related to the pig's immune system condition [160 and

references therein, 163]. Regarding PCV3 pathogenicity, it has been demonstrated in experimental infection that the virus could infect inoculated swine, although, they didn't always show lesions [163]. Accordingly, further and deep studies involving a great number of samples, as well as ISH to detect viral genome and PCV3-specific monoclonal antibody for IHC, are necessary to demonstrate the real incidence and risk of this virus for swine herds in Sardinia and its role in the pathogenicity of swine co-infection viruses. A very important and noticeable result was achieved regarding TTSuV being detected in 100% of the three age swine categories. This data is in agreement with several worldwide reports and could be attributable to the capacity of Anellovirus to spread horizontally, mainly via the fecal–oral route but also vertical and transplacental/intra-uterine. Adding to this, TTSuV was found in the boars' semen, indicating transmission via sexual route [215 and references therein]. In adult and post-weaning pigs TTSuV detection was 100% in all organs tested, whereas 100% of lungs and spleen were affected in piglets and 89% in the remaining organs suggesting a broad spectrum of tissue tropism of this virus. Furthermore, statistically significant differences were noticed in piglets, adult and post-weaning pigs between lung and spleen with the lowest means Ct values compared with other organs ($p < 0.05$).

Interestingly, also Li *et al*., 2019 reported a high prevalence of TTSuV1 and TTSuV2, during co-infection with PCVs, in particular in the lung and spleen. Similarly, in our cases, Sanger sequencing from TTSuV-positive samples revealed the presence of both TTSuV1 and TTSuV2k. TTSuV1 sequences had the highest similarity with strains from Brazil and South Korea while TTSuV2k sequences had 98% identity with strains from China suggesting a global and widespread diffusion of those viruses [91]. Of note, it is not clear whether TTSuV infection could be a primary agent of the disease, as it is detected in both healthy and affected pigs [39,215]. Accordingly, studies aimed to investigate the macroscopical and microscopical aspects of TTSuV infection are scarce, and the correlation between the virus and lesions is still not fully proven [215 and references therein]. Previous studies reported the presence of TTSuV

DNA in the heart, liver, lung, lymph nodes, kidney, and spleen [89,105,109], and some authors such as Krakowka and Ellis in 2008, and Mei *et al.,* 2011 in experimentally infected pigs with TTSuV [108,118–120] detected, among other lesions, progressive and severe interstitial pneumonia [119]. In our study, a multifocal to diffuse chronic macrophagic and lymphoplasmacellular interstitial pneumonia was observed in 60% of adult pigs, in 100% of post-weaning pigs, and in 100% of piglets suggesting a possible involvement of TTSuV in lung lesions. Furthermore, TTSuV has been found in combination with PCV2, PRRSV, and PPV, influencing the development of PDNS and PMWS [92,106,113,116], leading to difficulties in distinguishing pathological aspects. Notably, the probability of co-infection by TTSuV and PCV2 within pig herds is very high, but it is reported that it is sometimes subclinical, and the underlying disease is related to the viral load of each implicated virus. TTSuV may play a role in generating immune system damage and affecting the disease dynamics if present [92]. Adding to this, and despite the limited data on gross and histopathological lesions caused by TTSuV and PCV3, these two viruses are related to interstitial pneumonia, glomerulonephritis, and lymphoid depletion [120,149,150,158]. These lesions make it difficult to distinguish between these two viruses and differentiate them from PCV2.

Overall, based on our data it seems plausible that TTSuV may play a role in lung swine disease, but further studies are needed to clarify if the Anellovirus may enhance the development of disease caused by other swine pathogenic agents. Regarding Parvoviruses (PPV2 to PPV7), the most frequent strain found in samples was PPV2. PPV2 was detected in most of the examined tissue (lung, spleen lymph nodes, intestine, and kidney) of 100% and 90% of adults and piglets, and to less extent in post-weaning. Variable prevalence of PPV2 has been reported by several epidemiological studies. A high prevalence ranging from 8.8% to 83% of PPV2 was detected from serum, tonsils, and hearts in clinically healthy pigs, while PPV2 was found within alveolar macrophages, in 28% of lung samples presenting interstitial pneumonia, suggesting that the virus is associated, but may not be the sole causative agent, of the porcine respiratory disease

complex [216 and references therein]. In our study, molecular results of PPV2 were statistically significant for both adults and piglets where Ct values of mediastinal lymph node, mesenteric lymph node, and spleen were significantly lower compared to other examined organs ($p < 0.05$) similar to what has been recently reported by Lagan and coauthors in 2020 suggesting lymphoid tissue as possible target [169]. However, limited knowledge of viral pathogenesis is due to difficulties in the culture of single-stranded DNA viruses. In addition, the presence of PPV6 and PPV7 was also statistically significant in adult pigs ($p < 0.05$), while no statistically significant differences were observed between mean Ct values for all PPV2 in post-weaning pigs ($p > 0.05$). No statistically significant differences were observed between mean Ct values for the remaining PPVs. Overall, novel PPVs 2 to 7 viruses have been recently reported in the literature, but their viral pathogenicity is still to be determined and clarify being detected in both clinically healthy and diseased animals. A retrospective histopathological study demonstrated that PPV2 was significantly associated with interstitial and bronchointerstitial pneumonia [169], while other authors considered unclear the significance of PPV2 infection in porcine respiratory disease complex as well its association with other co-infections [216 and references therein]. Moreover, since interstitial pneumonia is the most representative lesion in organs, and they are 100% positive also for PPV2 we can propose that this virus could be an important contributor to the development of lung lesions.

Globally, to the best of our knowledge, this is the first study to report the presence of PPVs from 2 to 7 in Italy and Sardinia, while only a few reports have been reported in Europe [217] Additional deep studies are necessary to clarify the clinical significance of this novel emerging virus. Furthermore, PCR regarding APPeV showed positivity of 50% in mediastinal lymph nodes and 20% in mesenteric lymph nodes of adult pigs, like in previous studies, where APPeV was detected in the lymphoid organs presenting the highest viral load [126,140,141]. To the best of the authors' knowledge, APPeV is reported for the first time in a Sardinian intensive farm.

Regarding co-infections, they are increasingly being reported. However, little is known about their impact on other co-infecting pathogens. The most common consequence of co-infection is viral interference, leading to suppressing replication, enhancing viral replication, or coexisting with other viruses. Co-infections generally induce a negative effect on health, altering pathological lesions and severity. Lesions are usually associated with one pathogen that is causing the disease, but multiple agents could be implicated [212]. Reports of coexisting agents in the respiratory system and the effect of this co-infection on the host are recently increasing but there is limited knowledge about the influence of these agents [211,212]. A recent paper reported the detection of more than one pig's viruses showing respiratory symptoms in endemic farms [212,213,218]. Notably, our PCR results, showed the presence of more than one virus in the three pig categories, meaning there is a co-infection in various combinations in a high percentage of the lung samples of more than one emerging and non-emerging swine virus. All adult pigs showed a percentage of 100% of lung and mediastinal lymph nodes co-infection with PCV2 TTSuV and PPV2,6,7. In addition, one adult pig was confirmed to be coinfected with PPV2, PPV3, PPV5, PPV6, and PPV7 along with PPV1, PCV2, and TTSuV.

Piglets showed a percentage of 100% of lung and mediastinal lymph nodes co-infection with TTSuV and PPV2. Post-weaning pigs showed a percentage of 50% of lung and mediastinal lymph node co-infection with TTSuV and PPV2.

Porcine respiratory disease complex (PRDC), in which the main contributory pathogens are PRRSV, PCV2, and Influenza A virus (IAV), is a multifactorial syndrome responsible for sanitary problems in growing pigs and losses in pig farms and is considered the outcome of several co-infections between viruses and/or bacteria [216]. Mei *et al.,* 2011 reported that TTSuV causes lung lesions in pigs and it was considered to have a high prevalence in the lungs of pigs affected by PRDC [120,216]. Recent work reported that there can be a significant

association between interstitial pneumonia and PPV2, in the absence of other clinically significant swine viruses and bacteria [169,216].

By our results, both TTSuV and PPV2 seem to be involved in lung swine pathology and we suggest including these two emerging viruses in the routine diagnostic investigation for PRDC. Swine pathology presents a high prevalence in mixed infections, posing an important threat to herd health when compared with infection by a single pathogen. Nevertheless, it is critical to know the implicated pathogens in PRDC for the execution of control measures and prevention. Further investigations, such as immunohistochemistry and in situ hybridization, are needed to localize emerging viruses in the organs of coinfected pigs, to better clarify the role of each emerging pathogen and its real impact on swine health. However, our work presents some limitations, due to the missing information about important bacterial agents implicated in PRDC, although, it is crucial to have a better knowledge and understanding of each involved pathogen since the combinations of viral with other infectious agents constantly change over time, with the likelihood of the emergence of new viruses complicating disease severity.

4.2. Conclusions

Intensive pig farms are stress-inducing systems that negatively impact animal welfare and lead to an increased susceptibility to viral diseases. Our work aimed to highlight the viral circulation in an intensively reared Sardinian pig herd and its impact on swine health status. This goal was accomplished by the evaluation and classification of the macroscopic and histopathological lesions in tissue samples of pigs from different age categories. Histology highlighted the presence of chronic, moderate to severe, lymphoplasmacytic, and macrophagic interstitial pneumonia in all pig categories, with more severe bronchiolar lesions in adult pigs and piglets, and a high severity of interstitial lesions in post-weaning pigs. Emerging viruses are seldom reported in the literature and their role in the pathogenesis of lesions is sometimes still unclear. This study offers the first report of several newly emerging viruses, detected by PCR assays, which haven't been previously reported in the Sardinian domestic pig population. In addition, the sequencing results confirmed the coexistence of some of these viruses (PPV2-7, TTSuV) in tissue samples of several pigs, regardless of age categories. The infection of the host by multiple viruses could favor the creation of recombinant viruses. In addition, viral recombination poses a crucial threat to the host, since it may also alter the dynamics of the disease, the efficacy of the predicted treatment, and the evolution of the virus, as well as changes in the severity of the disease. This work detected PPV2-7, APPeV, and TTSuV in Sardinia in intensively reared pigs and described the histopathological lesions in affected subjects. However, our work presents some limitations, due to the missing data on the mixed infection of viruses and bacteria within swine tissues. Further investigations, such as immunohistochemistry and in situ hybridization, are needed to localize specific viruses in the organs of coinfected pigs, to better clarify the role of each pathogen and its impact on swine health. This work may also be improved by screening a bigger scale of the pig population in Sardinia, testing a larger number of samples, and expanding to more intensive farms. Also, using samples from extensive pig herds and wild boars could give information about the circulation of emerging viruses in pig populations that

are not reared under stressful intensive conditions, to investigate the epidemiology and behavior of these viruses in different host systems.

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